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Pest Management with Natural Products

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Pest Management with Natural Products

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Foreword

The ACS Symposium Series was first published in 1974 to provide a mechanism for publishing symposia quickly in book form. The purpose of the series is to publish timely, comprehensive books developed from the ACS sponsored symposia based on current scientific research. Occasionally, books are developed from symposia sponsored by other organizations when the topic is of keen interest to the chemistry audience.

Before agreeing to publish a book, the proposed table of contents is reviewed for appropriate and comprehensive coverage and for interest to the audience. Some papers may be excluded to better focus the book; others may be added to provide comprehensiveness. When appropriate, overview or introductory chapters are added. Drafts of chapters are peer-reviewed prior to final acceptance or rejection, and manuscripts are prepared in camera-ready format.

As a rule, only original research papers and original review papers are included in the volumes. Verbatim reproductions of previous published papers are not accepted.

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Preface

This volume and the contributed chapters therein are a result of the Natural Products for Pest Management symposium held at the 244th ACS National Meeting in Philadelphia, PA August 19–23, 2012 to honor the late Horace (Hank) Cutler and his contributions in the field of natural products chemistry. The symposium introduced recent discoveries and applications of natural products from insect, terrestrial plant, microbial, and synthetic sources for the management of insects, weeds, plant pathogenic microbes, and nematodes. The symposium brought together scientists from academic, government, and private research laboratories around the world. Discussed were natural products with insect repellent and attractant, insecticide, nematicide, herbicide, and fungicide activities, and their current and potential future roles in pest management. Also highlighted was the emission of volatile natural products from trees as a method to detect early stages of pathogen infection. In addition to recent advances, the symposium included reviews of important natural products that have proven successful as commercial products as well as the significance of responsible product stewardship.

The symposium was dedicated to the late Dr. Horace Cutler, whose 50-year career in natural products chemistry focused on the discovery of compounds for pest management. Chapter 1 of this volume summarizes some of his research. Several presentations discussed the synthesis of compounds based on natural products, as well as recent progress in research concerning the modes of action of natural product pesticides. These areas of research are important topics that offer solutions to the ever-increasing resistance of agricultural pests to herbicides, insecticides, and fungicides. This compilation of current investigations, significant past discoveries, and potential directions provides researchers — chemists, entomologists, ecologists, plant pathologists, weed scientists, nematologists, physiologists, and biochemists — with practical approaches for the use of natural products for the management of agricultural and urban pests. The purpose of this book is to further disseminate the wealth of knowledge presented at the 2012 ACS Natural Products for Pest Management Symposium to a more extensive audience. Pest management issues cover a broad range of scientific disciplines. Reflective of this were the talented and multidisciplinary group of scientists that presented their findings.

The editors offer their sincere appreciation to the chapter authors for their valuable and enlightening contributions. Additionally, we wish to extend our gratitude to the chapter reviewers for their valuable time and input. Finally, we thank Laura Koivunen and Morgan Weidinger for the cover art.

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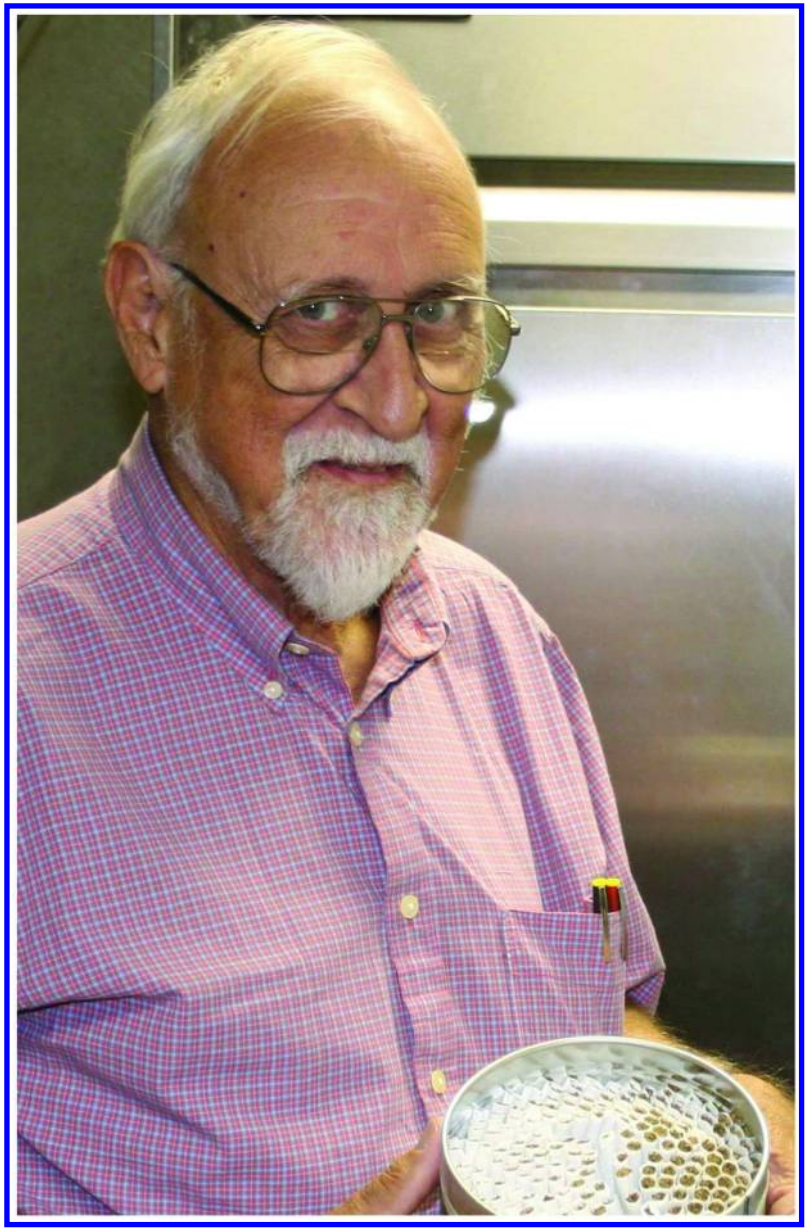

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Editors' Biographies

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Joel R. Coats (Ph.D., University of Illinois) is Charles F. Curtiss Distinguished Professor of Entomology in the Department of Entomology at Iowa State University in Ames, Iowa. His research focuses on natural products as insecticides and repellents, as well as the environmental fate and effects of agrochemicals. He has mentored a total of 44 graduate students who received advanced degrees in his laboratory. He has published 140 refereed journal articles, 39 book chapters, 7 review articles, and 10 books; he also holds 9 patents. He is a Fellow of the AAAS, the ESA, and the AGRO Division of ACS.

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Stephen O. Duke (Ph.D., Duke University) is Research Leader of the Natural Products Utilization Research Unit of the U.S. Department of Agriculture in Oxford, Mississippi. His research is on discovery and development of natural product-based pest management products. He is Chair-Elect of the Agrochemicals Division (AGRO) of ACS and has authored or co-authored almost 400 journal articles and book chapters, co-edited 6 books, and co-authored one book. His honors include Fellow of AAAS, AGRO, and the Weed Science Society of America and an Honorary Doctorate from the University of the Basque Country. He is Editor-in-Chief of *Pest Management Science*.

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Marja E. Koivunen (Ph.D., University of California, Davis) is a Research Director with Eurofins Agroscience Services in Sanger, CA. She conducts contract research for agrochemical industry clients with an emphasis in testing biopesticide candidates to control weeds, insects, plant diseases, and nematodes. Her previous experience includes discovery and development of new biopesticides in an industry R&D setting. She has authored or co-authored over 20 refereed journal articles and a book chapter. She is an active member of ACS AGRO, co-organizer of the AGRO student poster competition, and reviewer for scientific journals including the *Journal of Agricultural and Food Chemistry.*

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Chapter 1

Pest Management with Natural Products

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Natural products for pest control is not a new concept – products from nature have been used to control pests since the early beginning of agriculture circa 8000 B.C. to repel or kill biting arthropods. Throughout the years, natural products have played a direct role in controlling weeds, insects, plant pathogens and nematodes in the field, or indirectly as leads for development of modern pesticides through chemical syntheses. In addition to classic natural products chemistries for pest management there has been increased interest in the use of volatile natural products for pest management in agriculture. Hence, the two-day symposium of Natural Products for Pest Management honoring the work and contributions of the late Dr. Horace (Hank) Cutler as part of the Agrochemicals Division (AGRO) program at the ACS national meeting in Philadelphia in 2012, was very timely and provided an excellent opportunity to learn about past and present projects of natural products chemistry laboratories in academia, government, and industry. This introduction provides a brief and informative look at the varying topics discussed at the AGRO division symposium on natural

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products. The compilations range from current research on hot topics and glimpses into past discoveries, to in-depth reviews on important topics in natural products. More importantly, this ACS symposium series book provides researchers of all disciplines with a practical approach for the management of pests, urban or agricultural, using natural products.

Introduction

Natural products for pest control is not a new concept – products from nature have been used to control pests since the early beginning of agriculture circa 8000 B.C. to repel or kill biting arthropods. Throughout the years, natural products have played an important role either directly by controlling weeds, insects, plant pathogens and nematodes in the field, or indirectly as leads that have been used to create modern pesticides through chemical synthesis. Due to the continuous use of products with identical or similar modes of action there has been a rapid increase in pesticide resistance among targeted pests. With their complex and diverse chemistries natural products have generated new interest in the development of commercial pesticides with novel modes of action. At the same time, public awareness of potential short- and long-term health effects connected to pesticides has guided pesticide manufacturers to develop so-called green chemistries with less potential for residues and harmful effects on non-target organisms.

Since the turn of the millennium, increased interest in natural products as pest control agents for both agricultural and urban pests has opened the market to biopesticides, and the modern tools provided by natural products chemistry and biotechnology have facilitated fast development of new pesticide products entering the competitive market place. Hence, the two-day symposium of Natural Products for Pest Management honoring the work and contributions of the late Dr. Horace (Hank) Cutler as part of the Agrochemicals Division (AGRO) program at the ACS national meeting in Philadelphia in 2012, was very timely and gave us an excellent opportunity to learn about past and present projects of natural products chemistry laboratories in academia, government, and industry. Chapter 2 in this book authored by Dr. Stephen Cutler highlights the legacy and achievements of his father, Dr. Hank Cutler, that helped lay the foundation for natural products chemistry as a resource for agrochemical product development.

Biopesticides are quickly advancing to the forefront of crop protection. The U.S. Environmental Protection Agency (EPA) defines biopesticides as pesticides derived from natural materials such as animals, plants, microbes, and certain minerals. The importance of natural product pesticides is highlighted in the recent statistics that valued the global pesticide market at $49.9 billion in 2012. Total market value of pesticides is expected to reach nearly $67.5 billion in 2017 after increasing at a five-year compound annual growth rate of 6.2%. As a segment, biopesticides were expected to total $2.1 billion in 2012, and surpass $3.7 billion in 2017, with an annual growth rate of 12%. This suggests that natural product pesticides are slowly achieving mainstream status, demonstrated by a large number of licensing agreements and acquisitions in this sector. These topics,

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discussed in Chapter 3 by Asolkar *et al*., substantiate the growing interest in new chemistries derived from natural sources. Instead of using these chemistries as leads for synthesis, more emphasis is placed on understanding the function of individual compounds and the positive combination effects that occur in complex natural matrices. Natural products played an important role in pest control during the early years of agriculture and crop husbandry and may well become a major component of future pest management. The ACS-AGRO symposium presentations, captured here as outstanding and informative chapters, clearly indicate a renewed interest in natural products research and development, and emphasize the value of natural product chemistries in modern pest control.

An additional tool for management of insect pests in agricultural settings has been the use of volatile natural products, which are more commonly identified as either host plant volatiles (e.g., kairomones) or pheromones. These volatile chemical signals between plants and insects often play a significant role in communicating to the insect an appropriate food source, a safe ovipositional site, or the identity of a non-host plant. Researchers have utilized this form of communication between plants and insects and gone on to develop blends of volatiles that attract insect pests or to enhance the attractiveness of a pheromone by mixing kairomones with these pheromones. Several presentations at the 244th ACS-AGRO symposium highlighted the use of host plant volatiles or pheromones to control or monitor insect pests.

Chapters 4, 5, and 6 in Pest Management with Natural Products directly address the multiple uses of volatiles for manipulating insect behavior, monitoring an insect pest, or even diagnosing plant health. In Chapter 4 Mafra-Neto *et al.* provide a nice example of formulating semiochemicals into an emulsion for the controlled-release of semiochemicals for use in mating disruption, attract and kill, and repellent treatments. Chapter 5 provides an overview on the use of host plant volatiles and pheromones for monitoring or control of an agricultural insect pest. And finally, the use of volatiles in agriculture and their relation to the development of in-field instruments for the detection of pathogens, fungi, or other forms of plant distress is discussed at length; the review by Aksenov *et al*. in Chapter 6 provides an in-depth look at the collection, analysis, and data processing of plant volatiles.

In Chapter 7, Gross *et al.* report on a quantitative structure-activity relationship (QSAR) study that uses an insect octopamine receptor expressed in a yeast strain. It investigates the structural parameters of monoterpenoids that are optimal for their binding at that receptor. Research by Patt *et al*. reported in Chapter 8 focuses on natural and synthetic compounds that influence the feeding responses of the insidious Asian citrus psyllid; the work reveals that some synthetic ligands were capable of enhancing the effects of a natural feeding stimulant. Chapter 9 from Zou *et al*. addresses pheromone chemistry of mealybugs and scale insects. It provides valuable detailed pathways for synthesis of a series of irregular terpenoids that serve roles in chemical communication for those plant-sucking insect pests. Chapter 10 by Miresmailli presents work on characterizing the differential volatilization of individual compounds in a natural insect repellent after it is applied to human skin. A portable gas chromatograph was used to track the evaporation of multiple monoterpenoids in the air above the

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treated skin. Chapters 7, 8, 9, and 10 illustrate the importance of understanding the physical, chemical and biological properties of natural products, with emphasis on several terpenoids, which can manipulate the behavior of pests to our advantage or kill them.

One of the biggest pesticide needs is new herbicides with new modes of action, as there has been no new herbicide mode of action introduced in over 20 years. Chapter 11 by Evidente *et al.* outlines the work of these authors in discovery of microbial metabolites with novel structures that might be useful in fighting parasitic weeds, and Chapter 12 deals with efforts by Macias *et al*. to discover new herbicides, based on phytochemical phytotoxins. In both cases, the structures of the phytotoxins discovered are unlike any commercial herbicides, making a new mode of action likely. Using metabolomic methods, Pederson *et al*. in Chapter 13 provides evidence to support the view that clover is producing allelochemicals that have effects on other plants much like those of some commercial herbicides. These chapters illustrate the potential of natural sources for new herbicide discovery.

Natural product pesticides of today serve the same purpose as the products our ancestors used to control weeds, insects, plant pathogens or nematodes. However, thanks to the advancement in the field of natural product chemistry, we now know more about the detailed chemical composition of products that originate from plants and microbes. We appreciate nature's ability to adjust to ever-changing environmental conditions, and we no longer assume that everything that comes from nature is non-toxic or benign. Also, we have methods to identify and quantify active compounds in natural sources, and use the information as clues to develop pesticides with novel modes of action as discussed in Chapter 14 by Duke and Dayan. And finally, what would the science of natural product chemistry be without attempts to manipulate the metabolic pathways in plants and microbes capable of producing compounds that can be used to control agricultural and urban pests? Chapter 15 by Hahn discusses the art of using microbes as production plants for active pesticide chemistries.

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Chapter 2

From the Rainforest to Your Grocery Store and Medicine Cabinet: 50 Years of Natural Products Research

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Over the past 50 years, many biologically active natural products have been discovered, which have utility in the agriculture and pharmaceutical industries. It is interesting to find there is an indisputable delight that comes from migrating across scientific disciplines. In this transition it happens that chemical structures and their derivatives turn up like old friends. Perhaps another metaphor exists between the chemistry of pharmaceuticals and agrochemicals with the common thread being chemistry. Of course, modern civilization is built upon the pillars of agrochemicals and pharmaceuticals with the first giving rise to an abundance of food and the latter keeping the mind and body healthy. This paper will interweave examples of agrochemicals that possess pharmaceutical effects and, conversely, medicinal agents that have agrochemical properties. Included in these examples are biologically active natural products discovered by Horace "Hank" Cutler during his 50 years as a natural products chemist.

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Introduction

Growing up in a research environment had certain rewards and pleasures. I can recall, as if it were yesterday, when at the age of 5 years old, I was first exposed to my father's laboratory in Tifton, Georgia. At that point in his career he was working with the United States Department of Agriculture – Agriculture Research Service (USDA-ARS) and his primary research focus included the use of bioassays in the isolation and structural elucidation of biologically active secondary metabolites with potential utility as agrochemicals. There were three things that struck me that particular day: 1) the smell of the solvents used in his isolation work; 2) the tall columns (some of which were taller than a 5-year old boy) filled with chromatography media and dripping solvent and other "goodies" into an automatic fraction collector and the funny clicking sound it would make as it changed from one test tube to another; and 3) the delicious chocolate candy bars tucked in the freezer. It was the latter that would serve as the hook for my return visits and subsequently serve as the "catalyst" for my development as a scientist. However, this story is less about me and more about the 50-plus years of research conducted by Horace "Hank" G. Cutler as seen through my eyes.

Although his primary focus for roughly 35 years of research was in agrochemicals, he spent the last 15 years of his career investigating the biological utility of natural products as both agrochemicals and pharmaceuticals. As he began making the transition into the pharmaceutical arena, there was trepidation and anxiety on his part. As thrilling as it was to learn a new area of research and to migrate between disciplines, there is great suspicion that the knowledge bank might become depleted. Today it is extremely difficult to keep current in one discipline much less learning the concepts, terms, and reading the literature of a second area of research. Even so, he found comfort in the discipline of pharmacy with some "old friends" showing up and presenting themselves as chemical structures and templates that possessed biological effects in both agricultural and pharmaceutical uses. The first example includes the class of benzodiazepines, which he isolated as an agrochemical in 1984 and later learned belonged to an important therapeutic class used to manage anxiety in humans; the irony should not be lost on the reader that it was through this gateway that his trepidation with working in two disciples was abated. Over time, he became comfortable with the evaluation of natural products as potential pharmaceuticals and potential agrochemicals.

The reader might argue that some examples are not truly natural products for which as a penitent I plead *mea culpa*. Even so, the synthetic and semi-synthetic examples are provided in order to tell a story that includes optimization of a lead compound or how similar chemical classes of agents were independently being developed as agrochemicals and pharmaceuticals. Furthermore, for those readers interested in a more comprehensive story of natural product development as therapeutic agents or as agrochemicals they are directed to the work of Newman and Cragg (*1*) or Cantrell, Dayan, and Duke (*2*). Whenever possible this chapter will weave examples of natural products that potentially have both agrochemical and pharmaceutical utility.

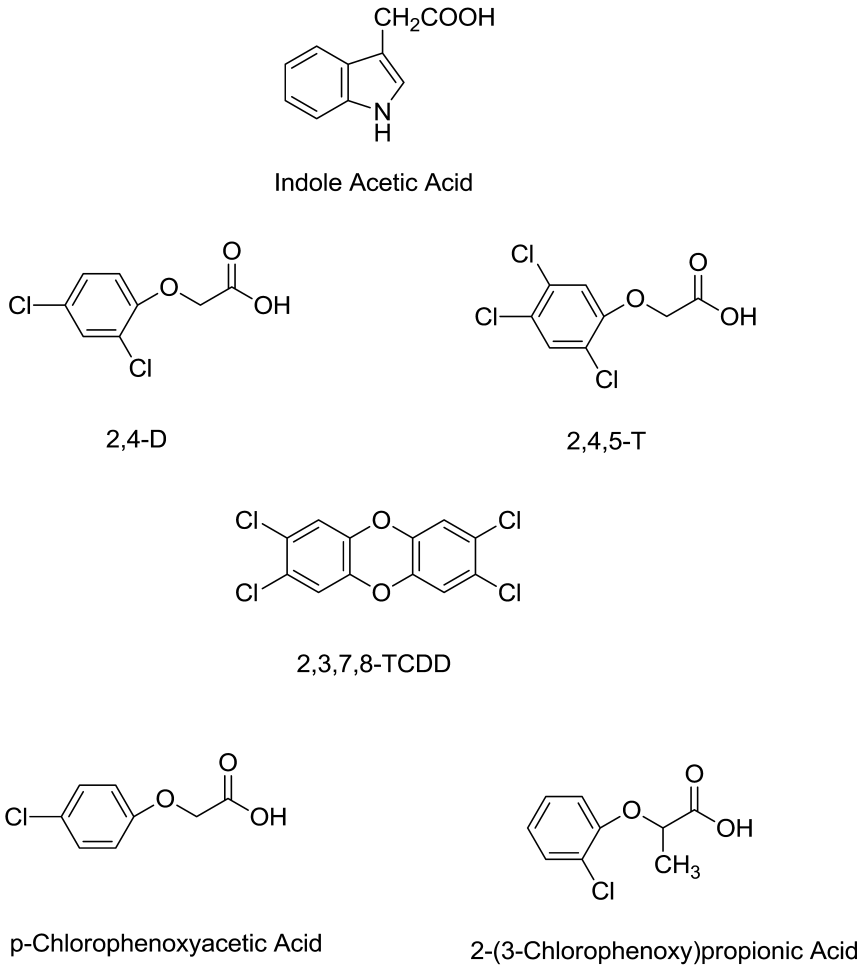


Figure 1. Plant growth regulators belonging to the aryl acetic acid and aryloxy acetic acid classes.

Phenoxy Derivatives

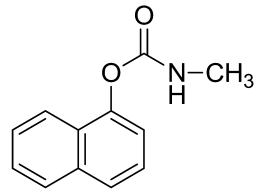
In 1954 Hank Cutler received a Union Carbide Fellowship to work with Lawrence J. King at the Boyce Thompson Institute for Plant Research in New York City. William Boyce Thompson, who acquired his wealth in copper mining, recognized the importance of ensuring a healthy supply of food. He established the institute in 1920 with a $10 million endowment and named it after his parents. Larry King was interested in expanding on the plant growth regulating properties of indole-3-acetic acid. Earlier, Zimmerman and Hitchcock, both of whom were also employed at Boyce Thompson, had recognized that the structure of IAA

might be a good template for synthesizing agriculturally useful compounds. In an exceptionally brief and succinct 27-line 1941 JACS publication, Pokorny reported the synthesis of 2,4-dicholorphenoxyacetic acid (2,4-D) and its congener 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) (Figure 1) (*3*). These two compounds were successfully used as herbicidal agents in World War II and the Vietnam War. The defoliant properties allowed Allies to be more successful in engaging the enemy while in the jungle theater, since the product deprived the enemy of vegetative cover. It was in the Vietnam War that the defoliate, which was a combination of 2,4-D and 2,4,5-T, was named Agent Orange (*4*). The name came from the color of the orange-striped 55 U.S. gallon barrels in which the herbicide was shipped. Ironically, it was the success of Agent Orange that also led to a major disaster; the demand of the product far exceeded the supply and in an attempt to produce more material, the reaction temperature was raised to increase the synthesis rate. This resulted in the production of a by-product, the potent carcinogen dioxin (2,3,7,8-tetrachlorodibenzo[b,e][1,4]dioxin) or TCDD (Figure 1) (*5*). Over time, other phenoxy derivatives were introduced into the agrochemical market including p-chlorophenoxyacetic acid and 2-(3-chloropenoxy)propionic acid, among others.

King recognized the significance of the phenoxy work and proposed that generating derivatives of naphthalene acetic acid with a carbamate might be beneficial as an herbicide or plant growth regulator. He asked his colleague Joseph Lambrech to make "six novelty carbamates" of which one was carbaryl (Sevin) (Figure 2) (*6*). Although this compound lacked herbicidal activity, it was discovered to possess significant insecticidal activities primarily due to its cholinesterase inhibitor properties. It is the carbamate that provides the inhibitory effects on the enzyme that is normally responsible for hydrolyzing acetylcholine. This leads to an excess of acetylcholine in the synaptic clef, which results in paralysis and death of the insect. As a side-note, there are agrochemicals possessing this mechanism that are currently used to manage Alzheimer's Disease, which, in some patients, respond to elevated levels of acetylcholine in the synaptic cleft.

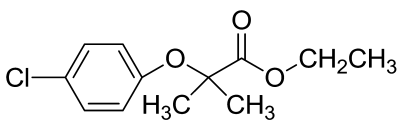
It is interesting when history provides separate, yet similar events, occurring independent of one other. Within the pharmaceutical industry there is a class of phenoxy acetic acids developed as lipid lowering agents shortly after the plant growth regulator development of this chemical class began. These hypolipidemics are structurally very similar to the plant growth regulators and are identified as clofibrate (Atromid), gemfibrozil (Lopid), and fenofibrate (Tricor and Lipophen) (Figure 3). They are useful in managing Type III hyperlipoproteinemia and to a lesser extent, in managing Type IIb and IV hyperlipoproteinemia. In these examples, there is an elevated level of lipoproteins including triglycerides, very-low-density lipoproteins (VLDL), and low-density lipoproteins (LDL). The lipid transport mechanism of the human body exists to shuttle lipids, such as cholesterol, in the aqueous environment of the blood. Normally, there is a balance between the production of these lipids, their storage, and degradation. However, when the balance is lost, there is an opportunity for the lipids to accumulate as deposits in the walls of the arteries, which can lead to atherosclerosis and other cardiovascular diseases. The mechanism of action for the phenoxy compounds to

lower triglyceride levels in the plasma is not completely understood. It is believed that these compounds do not interfere with the biosynthesis of lipids, but rather increase lipid excretion through the biliary tract, which in turn results in normal or near-normal homeostasis of blood lipoproteins (*7*).

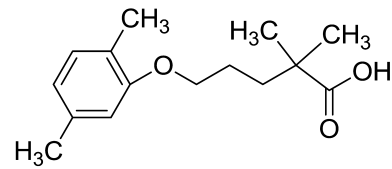


Carbaryl
Sevin

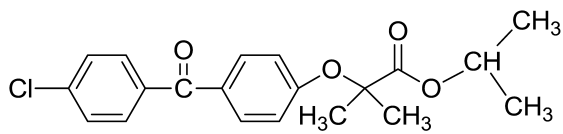
Figure 2. Structure of the insecticidal agent carbaryl (Sevin).



Clofibrate
Atromid



Gemfibrozil
Lopid



Fenofibrate
Tricor
Lipophen

Figure 3. Structures of phenoxy acids possessing lipid lowering effects.

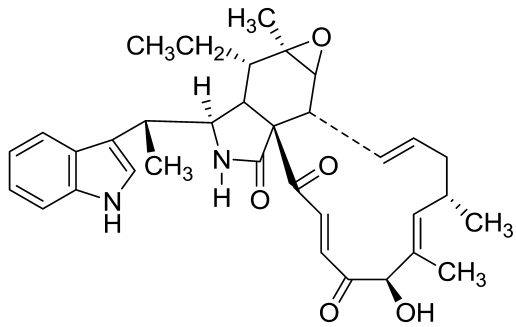
The structural similarities of agrochemicals and pharmaceuticals captured our interest and we embarked on a study that would bridge the gap between these two disciplines. It was surmised that the pharmaceuticals used to manage hyperlipidemia might possess agrochemical properties. With the use of the etiolated wheat coleoptile bioassay it was determined that clofibrate possessed plant growth regulating properties. Clofibrate significantly inhibited ($P < 0.01$)

the growth of etiolated wheat coleoptile 100% at both 10^{-3} and 10^{-4} M, relative to controls. It is of interest to note that in this assay 2,4-D inhibits the growth of wheat coleoptiles at 10^{-3} and 10^{-4} M, 100 and 50%, respectively (*8*). Ten days following 10^{-2} M clofibrate treatment on week-old beans (*Phaseolus vulgaris* L. cv. Black Valentine), the leaves became malformed with a leathery appearance. This same treatment concentration on week-old corn plants (*Zea Mays* L. cv. Norfolk Market White) induced chlorotic streaks on the leaves within 48 hours. After 10 days, bean and corn plants were inhibited by as much as 50% as compared to controls.

Although studies have yet to be reported on the lipoprotein effects of phenoxy plant growth regulators, one can take pleasure in anticipating that these congeners might influence mammalian disorders and have potential utility as pharmaceuticals.

Macrocyclics (Chaetoglobosin K)

In 1980, the structure of chaetoglobosin K (Figure 4), a secondary metabolite from *Diplodia macrospora*, was determined by single crystal X-ray analysis (*9*). This indolylcytochalasin possesses potent activity in the etiolated wheat coleoptile at concentrations as low as 10^{-7} M (*10*). The wheat sections gave an appearance that resembled bananas with tapered ends and were unlike the normal shapes observed in this assay in which the sections were straight. This morphologic effect is something that was noted with any cytochalasin tested in this assay (*11*). Overall, the structure of chaetoglobosin K is interesting in that a differing feature from the other cyctochalasins is that the former has an indole nucleus at the C-10 position rather than a phenyl ring. This is a feature that all natural product chemists find interesting, since there is a relationship to indole-3-acetic acid, serotonin, and the ergot alkaloid, lysergic acid diethylamide (LSD). One should anticipate that compounds possessing an indole feature will show interesting biological effects; the scientific question is in what biological system will the activity present itself.



Chaetoglobosin K

Figure 4. Structure for chaetoglobosin K.

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While evaluating secondary metabolites for their potential utility in influencing the gap junction mediated intercellular communication between rat-cultured glial cells it was determined that chaetoglobosin K was able to prevent two organochloride pesticides, dieldrin and endosulfan, from inhibiting this cellular communication (*12*, *13*). In a dose-dependent fashion (1–10μM), chaetoglobosin K prevents dieldrin or endosulfan induced inhibition of gap-junction communication. Evaluation of the gap-junction protein expressed by the glial cells showed that phosphorylation of connexin 43 was normal in those treated with chaetoglobosin K and either of the organochloride pesticides while the control group without the cytochalasin demonstrated that the organochlorides disrupted the normal phosphorylation of the connexin 43. This study suggests that chaetoglobosin K stabilizes the native phosphorylation state of connexin 43 and prevents the organochloride induced inhibition of gap junction cellular communication. This finding has led into the investigation of chaetoglobosin K as an anti-cancer agent (*14*). In recent years, this compound has been shown to promote apoptosis, inhibit the growth of *ras*-transfomed cells, prevent tumor-promoter disruption of cell communication by agents known to interfere with cell-to-cell gap-junction communication, and reduce *Akt* (protein kinase B) activation in neoplastic cells (*15*). The significance of this finding is that this is the first report in the scientific literature that demonstrates inhibition of two oncogenic pathways by a single compound. In the field of cancer treatment, most patients receive a cocktail of drugs that target different pathways and the utility of an agent that targets multiple pathways underscores its potential as an anti-neoplastic therapy.

Flavones

While a graduate student, my work focused on the synthesis and biological evaluation of natural products including flavones. Flavonoids have been shown to possess a broad range of biological activities including control of carcinomas, viruses, fungi, bacteria, etc. Within the National Cancer Institute (NCI) of the National Institutes of Health hundreds of flavones have been evaluated in tumor models. In the late 1980s, the flavone, flavone-8-acetic acid (Figure 5), was being investigated for its utility against solid tumors (e.g., colon, pancreatic, etc) versus soft tumors (e.g., leukemias) (*16*, *17*). This was a significant finding since many anticancer agents that came through the NCI screening program lacked significant effects on solid tumors. This finding helped shape how NCI currently conducts its primary screens. Prior to the recognition that compounds possessing anti-neoplastic properties had different mechanisms of action (particularly solid versus soft anticancer properties), the primary screen of NCI was only soft tumor lines such as L1210 and P388 leukemias. Since 1990, the NCI utilizes a screening panel of 60 tumor cell-lines, including both solid and soft tumor models.

In the late 1980s a series of flavone-8-carboxylic acid derivatives (Figure 5) were synthesized in an attempt to develop novel anticancer agents that were effective in managing solid tumors such as melanoma (*18*, *19*). It was at this

time that Hank's and my research began to merge. The broad spectrum of activity associated with flavones and the search for plant growth regulators from this class became a tantalizing project.

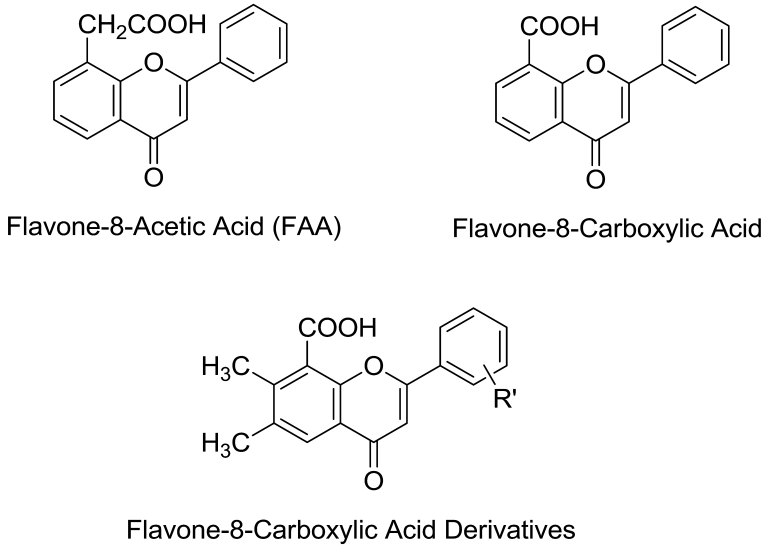

Figure 5. Structures of flavones possessing anticancer properties.

It is of interest to note that the initial evaluation of these derivatives in the NCI screen failed to show any significant biological effects against their leukemia cell lines. However, one derivative, flavone-8-carboxylic acid had significant anticancer properties. Additional studies in a B16 melanoma model demonstrated that this compound had utility as a lead candidate and modifications of the 2-aryl ring was observed to markedly influence activity in a dose-dependent manner. Within the series, the 3′-amino, 4′-methyl derivative of flavone-8-carboxylic acid had the best increase-in-lifespan for C57BL/6 mice exposed to B16 melanoma.

In the etiolated wheat coleoptile assay several of the flavone-6,7-dimethyl-8-carboxylic acid derivatives demonstrated significant growth inhibition at 10^{-3} M. The most interesting one is flavone-4′-methyl-8-carboyxlic acid with 56% inhibition.

Benzodiazepines

I can recall the day as if it were yesterday; I was visiting Hank at the Richard B. Russell building in Athens, Georgia. On his desk were several 8.5 x 11 print-outs of chemical structures he was going to photograph while preparing slides for a scientific presentation. Earlier, I had learned of the therapeutic class of agents known as the benzodiazepines, and two of his slides included this chemical class of agents. He had isolated two benzodiazepines (Figure 6) from an aberrant strain of *Penicillium cyclopium* growing on pecans (*Carya illinoinensis*)

(*20*). Although the initial report of these compounds was 20 years prior (*21*, *22*) to the 1984 publication, it was the first report including the biological effects as both a plant growth regulator and as a lead pharmaceutical. The scientific talk he was preparing was to share his discovery of the agrochemical properties of the benzodiazepines. However, before I move into that aspect of the story I must admit that it came as a real surprise that there were no boundaries between my discipline (pharmaceuticals) and his (agrochemicals), and it was this class of compounds that opened our collaboration that would comprise almost 25 years of research.

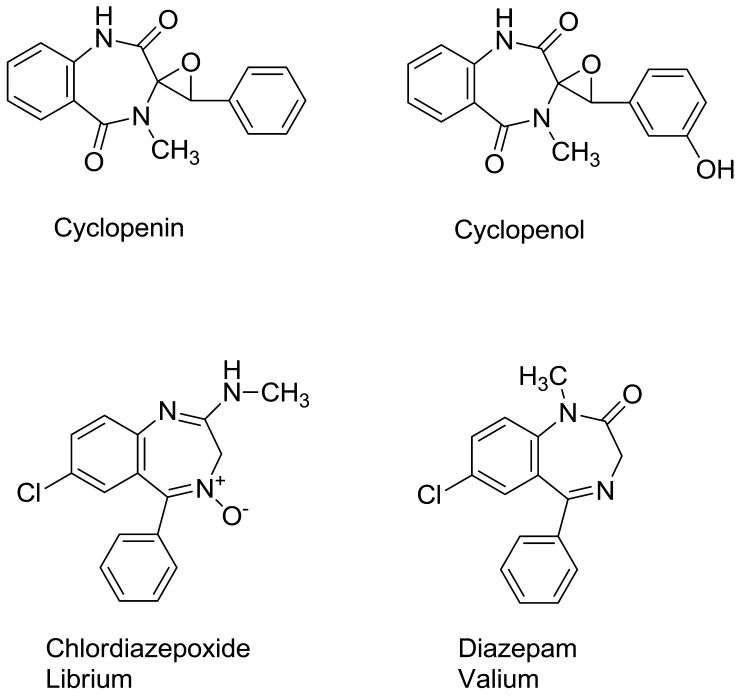

Figure 6. Structures of natural and synthetic benzodiazepines.

Within the pharmaceutical arena the benzodiazepines were first introduced serendipitously in 1955 (*23*). Leo Sternbach who had fled northern Europe during a very unsettling period when Hitler was rattling his saber, landed a job at Roche Pharmaceuticals. There he continued his work on the synthesis of dyestuff. By accident, he synthesized chlordiazepoxide (Librium) and diazepam (Valium), which are two tranquilizers that possess antianxiety, skeletal muscle relaxant, and anticonvulsant properties, among other effects. He was asked to clean up the lab and as he so eloquently states, the laboratory had reached a critical stage. One can readily visualize the clutter of the lab with no space to place anything; coffee mugs filled with test tubes, etc., an all too familiar story for most scientists. One of the samples that his pharmacology friend tested had significant effects and was patented as Librium only 2 years after its pharmacologic effects were discovered.

This is a remarkable story but compounded when one considers that it now typically takes 12-17 years from the discovery process to commercialization of a New Chemical Entity (NCE) or drug. Even more fascinating is that in the eleventh hour the chemistry was sorted out and the correct structure was included in the patent application. In retrospect, it is difficult to envision Sternbach, missing the chemical structure of Librium as there were reports in the scientific literature of naturally occurring benzodiazepines (*21*, *22*). However, it underscores how little the agrochemical and pharmaceutical scientists were paying attention to one another in scientific literature and one can only assume each was operating in a vacuum.

In the etiolated wheat coleoptile bioassay, cyclopenin inhibited coleoptile growth by 100%, while cyclopenol inhibited this growth 20%. It is of interest to note that at 0.025% cyclopenol was highly active against late blight (*Phytophthora infestans*) of potato (*Solanum tuberosum*) while at the same concentration cyclopenin was moderately active. As most historians know this fungus was responsible for the 1846 and 1847 Great Famines in Ireland and resulted in mass emigration from Ireland to various parts of the world including the United States.

With respect to the pharmacological properties of these natural benzodiazepines in day-old chicks dosed at 250 mg/kg (via intubation) cyclopenin induced drowsiness within 2 hours. At higher doses (500 mg/kg) it showed marked sedation and ataxia. In these studies cyclopenol didn't appear to possess any pharmaceutical effects (*20*). These findings, along with the structure-activity-relationship of the therapeutic benzodiazepines offer a nice project for a young graduate student interested in crossing scientific disciplines.

Conclusion

Over the past quarter of a century, Hank and I have enjoyed collaborating on various research projects that allowed us to cross the scientific disciplines of pharmaceuticals and agrochemicals. In recent years, our research has focused on the evaluation of his New Zealand collection of fungi. This work has included the investigation of secondary metabolites that have binding affinity for cannabinoid and opioid receptors. The potential utility of agents possessing affinity for these receptors include managing wasting-syndrome in AIDS patients, appetite, anxiety, nausea and vomiting (in patients receiving anticancer chemotherapy), pain, inflammatory diseases, and other disorders. Since this work has yet to yield compounds with both pharmaceutical and agrochemical utility the reader is directed to other publications in the scientific literature (*24*, *25*).

When Hank was a boy and was learning to swim, he experienced his first big challenge, which included him diving into a pool of water. He asked friends what it was like and they mumbled incoherent drivel. When the big day arrived he was nervous and stepping to the edge of the pool he sensed an increase in his breathing and heart rate. This anxiety was supported with thoughts of water forced up his nostril, hitting the bottom of the pool, and being able to surface; this was long before he learned about Boyles and Charles laws of partial pressure relative to the air in his lungs. The big moment came and he leaped into the water, surfaced, and

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breathed in fresh air. It was the same exhilaration he would feel almost 50 years later when he undertook the challenge of learning a new discipline by jumping into another pool of scientific knowledge. Exploration, after all, is the very essence of which the human spirit is built upon and I have to thank my mentor, my friend, and my father for providing me the insight to this fundamental human need. Not only did he supply me with the genetic architecture for human existence but also the innate substance that drives the search for novel discoveries. His genetic footprint on the field of natural products research is immeasurable and there are many scientists throughout the world that have benefitted from their interactions with him as both a scientist and a friend.

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Chapter 3

Discovery and Development of Natural Products for Pest Management

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Natural products from microbial sources have been successfully used for the development of new biopesticides for pest management. Interesting chemical structures and novel modes of action make these secondary metabolites very attractive due to improved efficacy, environmental and non-target safety, and mitigation of resistance development. In the search for new biopesticide leads, microbes isolated from soil and plants are screened for activity against pests such as insects, plant pathogens, nematodes and weeds. An example of promising molecules obtained through bioassay-guided research into a microbial hit selected for development will be discussed in detail. The discovery, development and use of biopesticides to control pests is gaining momentum in the market, but additional technologies are needed to enhance the performance of these products.

Introduction

Natural products chemistry actually began with the work of Serturner, who first isolated morphine from opium (*1*). Opium, in turn, was obtained from the opium poppy (*Papaver somniferum*) by processes used for over 5000 years. Since then, many similar discoveries have followed (*2*). The term natural product refers to any naturally occurring organic compounds that do not appear to participate

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directly in growth and development of the source organism. Traditionally referred to as secondary metabolites, these compounds are thought to increase the likelihood of an organism's survival by repelling other organisms. In contrast, primary metabolites (sterols, acyl lipids, nucleotides, amino acids and organic acids), are found in plants, microbes and animals, and play essential metabolic roles. Protection strategies against pests, pathogens and weeds in agriculture systems has rapidly changed due to increasing pressure to fulfill the need of food production for a growing human population. Food production increases in the past 40 years were achieved in part by spraying crops with synthetic chemical pesticides (*3*, *4*). Unfortunately, the use of chemical pesticides for higher crop yields contributed to environmental degradation due to unintentional negative affects. This led to a strong consumer push for more environmentally friendly pest control methods that were coupled with regulatory actions to reduce risk, and included the withdrawal of many synthetic pesticides from market (*5*). As a result, the European Union, the United States and several Asian countries have proposed regulatory changes in pesticide registration requirements (*6*). These new regulations, the high cost of new discovery of actives, development, registration of new synthetic pesticides, as well as the rapid emergence of pest resistance have reduced the number of new synthetic pesticides available for agriculture production. New pesticides, including natural product-based pesticides (biopesticides) are being discovered and developed to replace the synthetic compounds lost due to new registration requirements (*7*). This chapter discusses the various screening methods for the selection of strains for the development of biopesticides and details work on the discovery and development of microbial products for pest management.

Natural Products for Pest Management

Natural products are both a fundamental source of new chemical diversity and an integral component of today's inspiration for pesticide development. Many research articles and reviews have been published citing the use of natural products as pesticides (*8–10*). Most of this literature deals with compounds with promising activity that are not commercially available. Natural product-based pesticides offer advantages in that they can sometimes be specific to the target species and typically have unique modes of action with little mammalian toxicity. Moreover, many of these compounds are easily biodegradable due to environmental factors such as light, oxygen, temperature and/or biological metabolic enzymes. Application of natural chemicals for pest management provides an alternative to chemical herbicides, insecticides, nematicides and fungicides. These new methods of pest management are based on traditional observations in agriculture and forestry of the benefits obtained from naturally occurring microbial communities, which influences biological control of pests and diseases (*11–13*). Currently, several microorganisms involved in such processes are the active ingredients of a new generation of microbial pesticides (*14*, *15*). or are the basis for many natural products of microbial origin (*16*, *17*).

Why Biopesticides Are Important in Pest Management

Nature's diversity has not been efficiently explored for discovery of new natural-product pesticides (*18*). Though more than 50% of natural products are applied in the pharmaceutical industry and about 11% in the pesticide industry, only a small fraction of these are of microbial origin. Increasingly, biopesticides are needed in pest management programs to meet customer expectations for reduced residues in exported produce, SYSCO and Walmart sustainable farming requirements and protected vegetable culture systems in Europe. Most importantly, natural products registered as biopesticides have a 50-year history of safe use, starting with Bt (*Bacillus thuringiensis*) products. These products achieved exceptional market penetration due to their ability to kill a range of invertebrate pest species (*19*). The high cost of discovering, developing and registering new synthetic pesticides (over $250 million USD and a registration process of 10 years or more) and the rapid emergence of pest resistance also contribute to increased interest in biopesticides (*20*). Nearly all registered biopesticides are exempt from tolerance limits as they can be used up through harvest, and most have an LD_{50} value of >5000 mg/kg rat oral, dermal, inhalation. Many of them have a 4-hour re-entry period and generally do not contaminate ground and surface water (*21*). Due to the potential of biopesticides for pest management, many of the large chemical companies have recently jumped into natural products:

- Bayer CropScience acquires Germany-based biocontrol company Prophyta GmbH.
- American Vanguard Announces Joint Venture with TyraTech, Inc.
- Novozymes recently announces purchase of Natural Industries (Actinovate®).
- Syngenta has global distribution for Novozymes' Taegro *Bacillus* Biofungicide.
- BASF bought Becker Underwood (seed treatments) for $1 billion.
- Syngenta bought Devgen (RNAi insect and nematode control technology, rice germplasm) for $523 million.
- Syngenta bought Pasteuria Bioscience for $123 million seed-applied *Pasteuria* vs. soybean cyst nematode.
- Bayer bought AgraQuest for $425 mil USD (+$75 mil earn-out).
- Monsanto acquired several small companies in RNAi technology.
- Bayer bought AgroGreen (microbials for seed-applied nematode control; Poncho/Votivo® launched on multi million acres corn in 2011).
- FMC accesses Chr. Hansen bacterial strains for nematode control in Brazil, in-licensed Problad biofungicide, and distributing Marrone Bio Innovation's (MBI) Regalia Bioprotectant/Biofungicide in LATAM.
- Syngenta is developing/distributing Regalia® Bioprotectant in EAME.
- Scotts has global development of MBI's biopesticides for home and garden.

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Screening of Microbes for Biopesticides

The selection and development of new biopesticides involves several steps. The process starts with the isolation of pure cultures of microorganisms, followed by identification and characterization. The microbes are collected from environmental samples such as water, soils, and plants using different isolation methods and a variety of culture media. Individual fungal, bacterial, and actinomycete colonies are picked from primary agar plates and transferred until a homogeneous culture is obtained. The purity of the microbe is confirmed by carefully inspecting colony morphology on agar plates, followed by cultivation on a small scale in liquid culture media. Hundreds to thousands of isolates are evaluated for activity using *in vitro* or miniaturized *in vivo* efficacy bioassays in small-scale controlled-environment laboratory tests against targeted pathogen, insect, nematode and weed pests. The isolates showing activity are then repeatedly tested to confirm observed biological activity. These verified 'hits' are then tested in plant assays and then evaluated for a spectrum of activity across a wider selection of targeted pests. The spectrum of activity results provide important information for market placement and ultimately the selection of the isolate for development. Additional considerations for strain selection for development is based on factors such as known intellectual property claims, potential target market size and the impact on important non-targeted beneficial organisms.

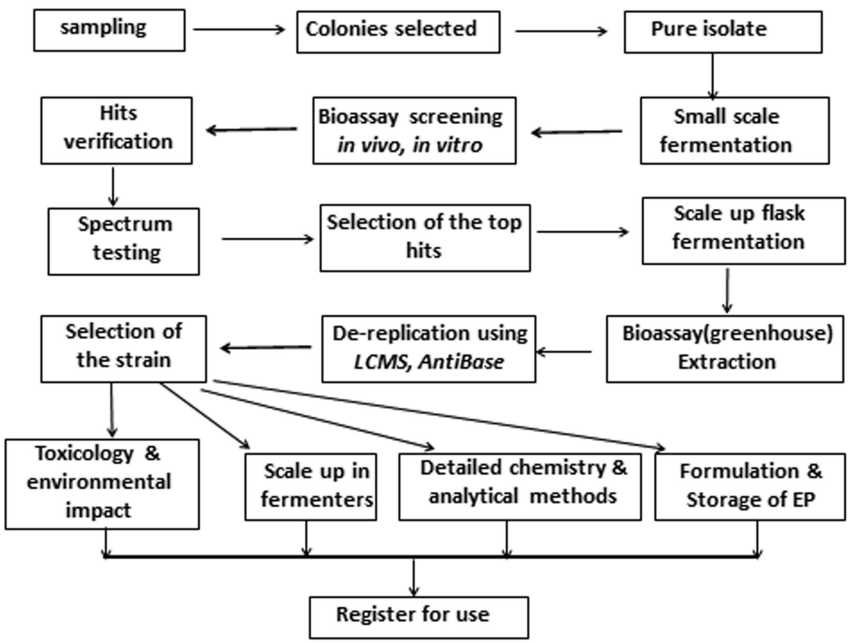

Figure 1. Method for the discovery and development of natural product-based microbial pesticides.

The most frequent toxicological tests required by regulatory agencies are rat oral acute toxicity, for determination of median lethal dose (LD_{50}), rat acute dermal LD_{50}, guinea pig skin and rabbit eye sensitivity, rat inhalation and pathogenicity. In addition, 30-day ecotoxicological feeding tests on fish, birds, *Daphnia*, honeybees, lacewings, and parasitic wasps are required. In the case of bacterial bio-control agents, an acceptable LD_{50} should be higher than 10^{11} colony-forming units/kg of animal weight (*21*). Less than 1% of candidate isolates eventually make successful products ((*22*); Figure 1).

Dereplication - Chemical Screening

Once a lead of interest is identified, the isolates are fermented or cultured using the selected growth medium in order to understand the secondary metabolite production. This process involves extraction of the whole cell broth (WCB) using appropriate solvent and binding resins to generate crude extracts. The crude extracts are then fractionated using chromatography to yield fractions that are evaluated with the appropriate bioassay to identify the active fractions. Natural product discovery programs strive to avoid nuisance compounds such as piericidin, actinomycins, aflatoxins etc. and eliminate microbes that produce these compounds. Compound isolation and structure elucidation is critically important. Microbe taxonomy is not always clear in natural product chemistry literature due to scant microbe information as well as mistaken microbe identification which is a challenge for accurate culture identification and selection. To overcome these problems we developed a de-replication method known as "smart screening system" which involves:

a) Literature search for all known chemistry based on microbial identification.
b) Liquid chromatography mass spectrometric analysis (ESI-LCMS) combined with UV detection.
c) Additional mass spectrometric analysis such as atmospheric pressure chemical ionization, both negative and positive mode.
d) Database searching using "AntiBase" (*23*) with molecular weight, and UV spectrum.

Thus, based on above analyses and results, the final decision for the selection of the isolate is made and development of the selected isolate for pesticidal use is begun. Using this approach, isolate MBI-206 (A326) was selected for detailed development work.

Bioactive Secondary Metabolites from MBI-206 (A396)

Bacterial Isolation and Identification

The isolate MBI-206 (A396) was recovered from a soil sample collected in the vicinity of a Bhuddist temple in Nikko, Japan in 2008. An extensive microbial identification by 16S rRNA analysis, DNA-DNA hybridization, fatty acid analysis,

MLST analysis, a Biolog and MALDI-TOF profiles confirmed the isolate as a non-pathogenic novel species of the genus *Burkholderia*. The detailed identification of this strain will be described in a separate publication (*24*). The isolate has been deposited with the ARS-NRRL Collection under accession code NRRL B-50319. The fermentation broth showed potent pesticidal activity.

To evaluate the toxicity of MBI-206, toxicology studies were conducted using fermentation broth i.e. TGAI (Technical Grade Active Ingredient) and the results showed no signs of pharmacologic and toxicologic effects on the tested organisms. Based on these data the test substance MBI-206 was found to be safe.

Fermentation and Extraction

The culture broth derived from the 10-L fermentation of *Burkholderia sp.* (A396) in Hy soy growth medium (Hy-Soy 15 g/L, NaCl 5 g/L, KH_2PO_4 5 g/L, $MgSO_4$ x $7H_2O$ 0.4 g/L, $(NH_4)_2SO_4$ 2 g/L, glucose 5 g/L, pH 6.8) was extracted with Amberlite XAD-7 resin (*25*) by shaking the cell suspension with resin at 175 rpm for two hours at room temperature. The resin and cell mass were collected by filtration through cheesecloth and washed with DI water to remove salts. The resin, cell mass, and cheesecloth were then soaked for 2 h in acetone/methanol (1:1) after which the acetone/methanol was filtered and dried under vacuum using rotary evaporator to produce the crude extract.

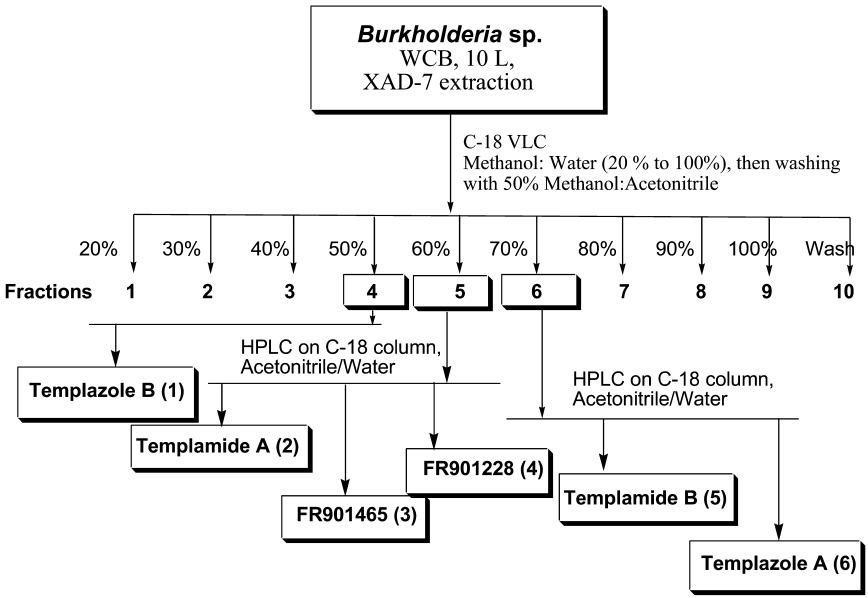

Figure 2. Schematic work-up for isolate MBI-206 (A396).

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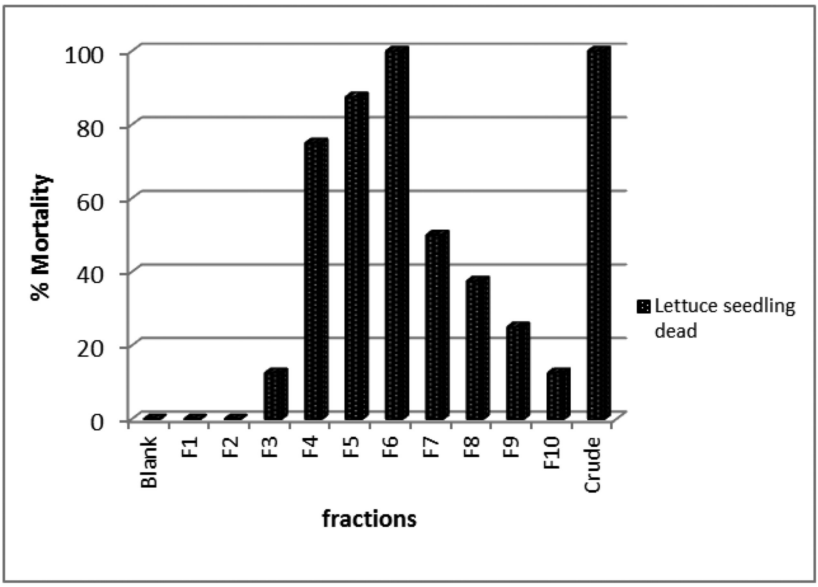

Figure 3. Herbicidal activities for tested fractions using lettuce seedlings in a 96-well plate bioassay at concentration of 10 µL/mL per well.

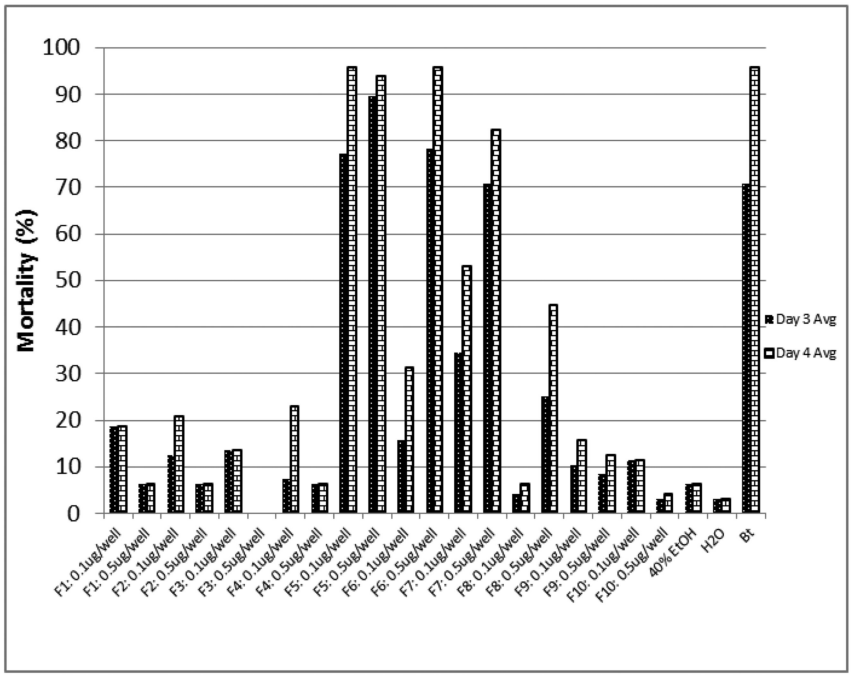

Figure 4. Insecticidal bioassay data for fractions tested against Beet Armyworm, S. exigua, 1st instars using diet overlay.

Isolation and Identification of Bioactive Secondary Metabolites

The crude extract was then fractionated in a reversed-phase C18 vacuum liquid chromatography (H_2O/CH_3OH; gradient 80:20 to 0:100%) that yielded 10 fractions (see Figure 2). These fractions were then concentrated to dryness in a rotary evaporator and the resulting dry residues screened for biological activity in herbicidal, insecticidal and fungicidal bioassays.

The herbicidal activity for these fractions is summarized in Figure 3 and for insecticidal in Figure 4. The active fractions were subjected to reversed phase HPLC (Spectra System P4000 (Thermo Scientific) to yield pure compounds, which were subsequently screened in above mentioned bioassays to locate/identify the active compounds. To confirm the identity of the compound, additional spectroscopic data such as LC/MS and NMR was obtained.

The active fraction 4 which showed only herbicidal activity was purified further by using preparative HPLC C-18 column. A new herbicidal compound was obtained and designated as templazole B (**1**). The fraction 5 showed herbicidal, insecticidal as well as fungicidal activities. The careful purification of this fraction by using repeated bioassay-guided preparative HPLC (reversed phase) resulted in the isolation of three active compounds, one new structure designated as templamide A (**2**) and the two known compounds FR901465 (**3**) (*26*) and FR 901228 (**4**) (*27*). The compound FR901465 (**3**) has been reported earlier from *Pseudomonas* sp. No 2663 and has been patented as an anticancer compound, and compound FR901228 (**4**) has been patented as an antibacterial and antitumor compound and reported earlier from *Chromobacterium violaceum* No. 968.

Fraction 6 which showed both herbicidal and insecticidal activities on bio-assay guided purification using preparative HPLC yielded two new active compounds designated as templamide B (**5**) and templazole A (**6**). The planar structures of these compounds were assigned by detailed interpretation of NMR and MS spectroscopic data and the assigned structures are shown in Figure 5 above. The structure of compound FR901228 (**4**) was additionally confirmed by X-ray crystallography. Full details of the chemical isolation and structure elucidation will be published as a separate account (*28*).

Herbicidal Activity of Templazole A (6), B (1), Templamide A (2), B (5), FR901465 (3), and FR901228 (4)

The herbicidal activity of templazole A, B, templamide A, B, FR901465 and FR901228 were tested in a laboratory assay using one-week old barnyard grass (*Echinochloa crus-galli*) seedlings and lettuce (*Lactuca sativa* L.) seedlings in a 96-well plate platform. One seedling was placed in each of the wells containing 99 microliters of DI water. Into each well, a one microliter aliquot of the pure compound in ethanol (10 mg/mL) was added, and the plate was sealed with a lid. One microliter of ethanol in 99 microliters of water was used as a negative

control. The treatments were done in eight replicates, and the sealed plate was incubated in a growthroom under artificial lights (12 hr light/dark cycles). After five days, the results were read. The grass seedlings in all eight wells that received the active compound were dead with no green tissue left, whereas the seedlings in the negative control wells were actively growing. The herbicidal activity of templamide A against lettuce seedlings was slightly lower than for the grass. On the other hand, FR901465 provided a better (100%) control of lettuce seedlings (used as a model system for broadleaf weeds) than templamide A (see Table I).

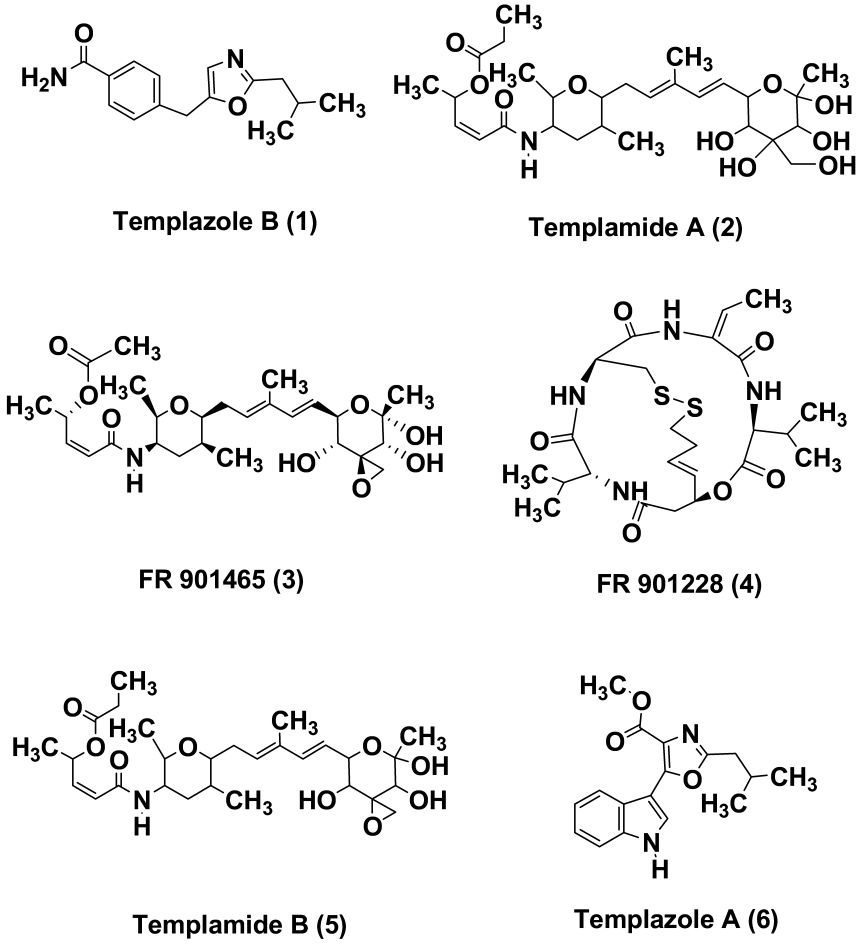

*Figure 5. Chemical structures for compounds **1-6**.*

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Table I. Herbicidal bioassay data for templazole B (1), templamide A (2), FR901465 (3), FR901228 (4), templamide B (5) and templazole A (6). Samples were tested at 10 µg/mL concentration per well.

Compounds	*Grass seedlings (% Mortality)*	*Lettuce seedlings (% Mortality)*
Templazole B (1)	ND	77
Templamide A (2)	100	88
FR901465 (3)	88	100
FR901228 (4)	100	88
Templamide B (5)	0	75
Templazole A (6)	ND	63
Control (water)	0	0

Table II. Insecticidal bioassay data for templamide A (2), FR901465 (3), FR901228 (4) and B (5) against 1st instar beet armyworm (*S. exigua*). Samples were tested at 1 µg/ well.

Compounds	*(% Mortality)*	*LC_{50} µg/well*
Templamide A (2)	40	>2.50
FR901465 (3)	60	0.2619
FR901228 (4)	90	0.0136
Templamide B (5)	80	0.0717
Bt	100	--
Control (ethanol)	0	--

Insecticidal Activity of Templamide A (4), B (5), FR901465 (6), and FR901228 (3)

The insecticidal activity of templamide A, B, FR901465 and FR901228 were tested in a laboratory assay using a 96-well diet overlay assay with 1st instar beet armyworm (*S.exigua* Hübner) larvae using microtiter plates with 200 µl of solid, artificial beet armyworm diet added to each well. One hundred microliters of each test sample was pipetted onto the surface of the diet (one sample in each well) and air-dried until the surface was dry. Six replicates of each sample was tested. Water and a commercial Bt (*B. thuringiensis*) product were the negative and positive controls, respectively. One Beet army worm first instar larva was

placed in each well and the plate was covered with a plastic Mylar cover with punched air holes. The plates with insects were incubated at 26 °C for 6 days with daily mortality evaluations. Based on the results presented in Table II, templamide A and B resulted in 40% and 80% mortality, respectively, whereas FR901228 (**4**) exhibited potent insecticidal activity (90% mortality).

Fungicidal Activity of FR901228 (4)

Fungicidal activity of FR901228 (**4**) against three plant pathogenic fungi (*Botrytis cinerea, Phytophthora capsici*, *Monilinia fructicola*) was tested in an *in vitro* PDA (potato dextrose agar) plate assay. Potato dextrose agar (PDA) plates were inoculated with plugs of several plant pathogenic fungi. After fungal growth was observed, eight sterile filter paper disks were placed on each plate about 1 cm from the edge in a circle. Ten μL ethanol solutions containing 20, 15, 10, 7.5, 5, 2.5 1.25 mg /mL of FR901228 (**4**) were added to filter paper disks, and evaporated. One disk imbedded with 10 μL of pure ethanol was used as a negative control. The assay was done with three replicates. Plates were incubated at room temperature for 5 days, after which the fungicidal activity was recorded by measuring the inhibition zone around each filter paper disk with the added concentrations of FR901228. Results showed that FR901228 had no effect on the growth of *Monilinia* but inhibited hyphal growt of *B. cinerea* and *P. capsici*. A clear dose-response inhibition was observed with threshold concentrations of 10 mg/mL and 1.25 mg/mL for *B. cinerea* and *P. capsici*, respectively.

Development of Microbial Pesticides

After passing EPA-directed toxicology and environmental studies, the next steps in the development of a biopesticide is the large scale fermentation of the identified microbe followed by formulation to stabilize and deliver the active ingredients to the targeted pest. Other critical steps are determination of shelf life stability and identification of parameters of quality specifications to assure consistent product performance. The fermentation method selected for industrial scale up of the microbes are either liquid or solid state and depends on the nature and growth habits of the selected microbe under development. Additional tests are aimed at detecting potential secondary pesticidal metabolites synthesized under evaluated test fermentation conditions. When needed, the fermentation broths are processed further to concentrate the pesticidal activity either by filtration or centrifugation. The successful commercial development of microbial pesticides is heavily dependent on the performance of the formulated product. The art of formulation chemistry is critical to enhancing efficacy of the active ingredients (AI) in the end product. Inert compounds are combined with the active ingredients to improve dispersiblity, solubility, stability and to ultimately improve efficacy in greenhouse and field applications. Rainfastness, long shelf life as well as chemical and physical characteristics of the formulated active ingredients are also tested. Based on these results, the suitable formulations are selected for further improvement and efficacy studies.

MBI-206 was scaled up to 100-L fermentation volume and then formulated using suitable formulation ingredients. The storage stability and field efficacy of several formulation prototypes were evaluated and the best formulation candidates selected for further improvement and development. The improved product formulations were tested in greenhouse and field trials. The initial results on selected formulation for MBI-206 showed excellent broad spectrum activity against flies and both sucking and chewing insects. Based on the results obtained from various laboratory, greenhouse and field trials, it was determined that the mode of action is both by contact and by feeding. Additional greenhouse and field trials are in progress to further understand the spectrum of activity. MBI-206 has been submitted to U.S. EPA for registration and the approval is anticipated in 2013.

Summary

The detailed research on one of the selected isolates from MBI discovery screens (MBI-206-A396) resulted in the development of a biopesticide that has the following interesting characteristics:

- New bacterial species of *Burkholderia*, with no relationship to human pathogenic *Burkholderia cepacia complex* species, *Burkholderia mallei* or *Burkholderia pseudomallei*.
- The bioassay guided isolation and identification of the active crude extract of *Burkholderia* sp. A396 resulted in the discovery of new bioactive secondary metabolites belonging to three chemical classes.
- Metabolites were active on contact and by ingestion – broad spectrum – on sucking and chewing insects, beetles, and flies.
- The herbicidal activity of templamide A against dicot seedlings was slightly lower than against monocot seedlings. On the other hand, FR901228 provided a better (100%) control of dicot seedlings than templazole A and B, or templamides A and B.
- Based on the results for insecticidal activity presented in Table II, FR901228, templamide A and templamide B resulted in 90%, 40% and 80% mortality, respectively. However, templazoles A and B were inactive in insecticidal bioassays.
- The LC_{50} data suggested that compound FR901228 is the most potent pesticide among all other active compounds produced by the strain.
- Through various optimization of fermentation and formulation work, a biopesticide candidate has been successfully developed from the microbial source.

Conclusion

Natural products play a very important role in pest management. For application of these natural products to pest management, knowledge of the physiology and behavior are essential to elucidate the interaction between the

host and pest. Isolation and structure elucidation of the active compounds helps understand the metabolic cycles, the enzymes that lead to their biosynthesis and the mode of action. Microbes contain a virtually untapped reservoir of natural product compounds that can be used directly or as templates for synthetic pesticides. Some of the novel compounds produced by microbe A396 could be used as leads for synthetic programs. The recent advances in chemistry and biotechnology have sped up discovery and development of microbial natural products as biopesticides. These advances, combined with increasing need and environmental pressure, are greatly increasing the interest in microbes that produce natural product compounds as future sources of pesticides. However, the success of these types of products depends on the widespread acceptance of these biopesticides as environmentally friendly and cost-effective pathogen control strategies. Added economic value and other quantifiable value such as increased yield, fruit quality, decreased pesticide residue, labor and disease resistance management by growers and other end-users. Once seen only for use in certified organic farming, biopesticides are increasingly being integrated into pest management programs with chemicals in rotations and tank mixes. The improved pest control along with all the other benefits mentioned above are driving the rapid growth and further adoption of these products.

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Chapter 4

Manipulation of Insect Behavior with Specialized Pheromone and Lure Application Technology (SPLAT®)

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SPLAT® (Specialized Pheromone and Lure Application Technology) emulsion is a unique controlled-release technology that can be adapted to dispense and protect a wide variety of compounds from degradation, including semiochemicals, pesticides, and phagostimulants, in diverse environments. ISCA Technologies, Inc., in collaboration with colleagues in academia, government, and industry, has been developing SPLAT®-based insect control products for close to a decade. This chapter provides an overview of SPLAT® technology and existing commercial formulations and describes ongoing efforts

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to develop new SPLAT® mating disruption, attract-and-kill, and repellent products for pest control in agricultural and forest environments.

Introduction

ISCA Technologies, Inc. (Riverside, CA U.S.A.) acquired SPLAT® (Specialized Pheromone and Lure Application Technology) in 2004. SPLAT® is a chemical controlled-release emulsion technology that has been used to dispense compounds to control a variety of insect pests. SPLAT® formulations have been commercialized both domestically and internationally. This chapter begins with a technical description of how SPLAT® functions and a discussion of what sets it apart from other controlled-release formulations used in semiochemical-based insect control. This is followed by three sections, each focusing on one of three semiochemical-based insect control techniques: Mating disruption, attract-and-kill, and repellents. Each section provides an introduction to the technique and summary of existing commercial SPLAT® products for insect control using that technique, and follows with one or more case studies of new SPLAT® formulations being developed to control agricultural or forestry pests using the technique being discussed.

Specialized Pheromone and Lure Application Technology (SPLAT®)

Description and Attributes

Although most semiochemical controlled-release formulations have taken the form of devices, such as aerosol dispensers (Puffer®, Suterra, LLC), polyethylene tubes (Isomate®, Shin-Etsu Chemical Co., Ltd.), and laminated polymers (Disrupt®, Hercon Environmental), ISCA has taken an alternative approach and commercialized a chemical formulation in the form of a controlled-release emulsion, SPLAT® (Specialized Pheromone and Lure Application Technology). Although adapting SPLAT® to release new compounds can pose technical challenges, the versatility of this flowable formulation provides many advantages. SPLAT® emulsions can be created to hold a range of semiochemical concentrations and additives to create a formulation that releases the optimal rate of semiochemical for the desired amount of time, while protecting active ingredients from environmental, chemical, and biological degradation. In addition, the rheological properties of SPLAT® can be adjusted to create emulsions with a wide range of physical properties. This has enabled SPLAT® products to be dispensed using a variety of manual and mechanical application techniques (Figure 1), allowing easy application to virtually any substrate or plot size. In addition, SPLAT®, unlike most other semiochemical dispensers, is not restricted to a particular point source size. Any amount of SPLAT®, large or

small, can constitute a point source. This provides yet another way to optimize the amount of volatile insect control compound released per point source for optimal efficacy. The versatility of SPLAT® has made the technology adaptable to virtually any semiochemical-based insect control application. Additional advantages of SPLAT® include the biodegradability of its inert ingredients and low manufacturing cost, which decrease environmental impacts and enable commercialization of affordable semiochemical-based control products.

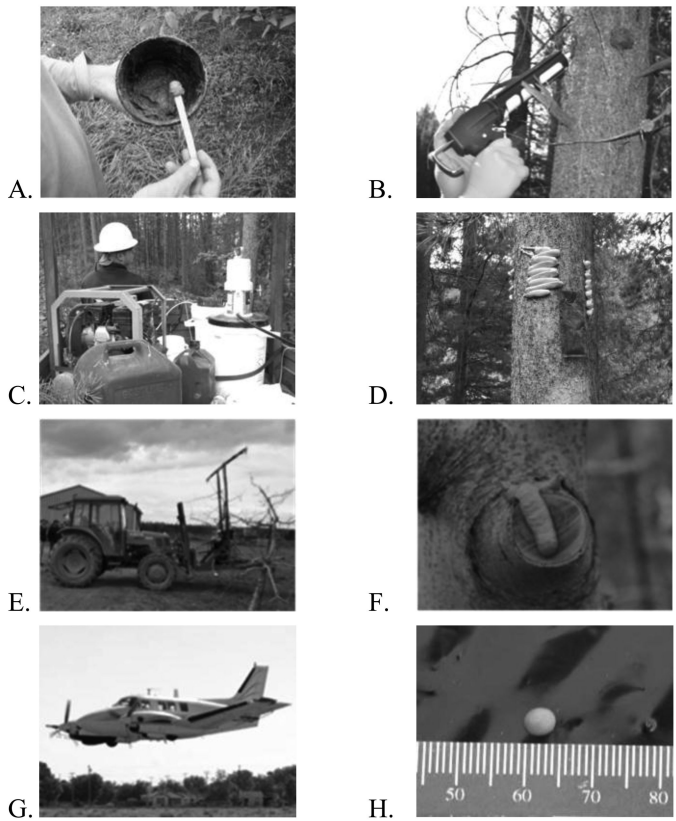

Figure 1. Application of SPLAT® using a variety of methods. A. SPLAT® applied with stick, B. SPLAT® Verb Repel applied with caulking gun, C. SPLAT® Verb Repel applied with John Deere Gator®-mounted mechanical applicator, D. SPLAT® Verb Repel dollop from application depicted in C, E. SPLAT® applied with tractor-mounted mechanical applicator, F. SPLAT® dollop resulting from application depicted in E, G. SPLAT® GM applied with airplane-mounted mechanical applicator, H. SPLAT® GM dollop resulting from application depicted in G.

Application

SPLAT® formulations typically have a paste or cream-like consistency (Figure 1). SPLAT® is a non-Newtonian, shear-thinning, thixotropic fluid, which means that SPLAT® viscosity decreases when the emulsion is placed under stress, such as when it is stirred or pumped, but increases again when the stress is removed. This property is advantageous in that the less viscous SPLAT® can easily be manipulated (e.g., stirred or pumped), but quickly thickens upon application to a surface, aiding in product adhesion. A wide variety of manual and mechanical applicators can be used to apply SPLAT®. The most basic SPLAT® applicator can be a stick, spatula, or knife. More advanced manual applicators include syringes, grease guns and caulking guns (Figure 1 A, B). Indeed, SPLAT® formulations are regularly sold loaded into standard caulking tubes and applied with off the shelf caulking guns. In addition, numerous mechanical applicators have been adapted or created specifically to apply SPLAT® with a variety of motorized vehicles, including tractors, all-terrain vehicles, and even motorcycles (Figure 1 C-F) (*1–3*). SPLAT® has also been sprayed from motorized backpack sprayers and applied aerially (Figure 1 G, H).

Controlled-Release Technology

The aqueous component of the SPLAT® emulsion gives the product its liquid character, allowing it to flow. The non-aqueous component of the emulsion is the controlled-release device. It comprises the active ingredients (e.g., semiochemical compounds or pesticides) and the additives that will protect these and fine-tune their release rates from the dispenser. Upon application, the aqueous component of SPLAT® evaporates from the dispenser within 3 hours, leaving the rainfast, non-aqueous component firmly affixed to the substrate, where it will release the active ingredients until all available molecules are dispensed (Figure 2). The longevity of the dispenser depends on the manner in which the particular SPLAT® formulation was created, its composition, how it was applied, as well as the environmental conditions to which it is exposed following application (*4*). SPLAT® products are typically formulated to release semiochemicals for 2 weeks to 6 months.

SPLAT® is a “matrix-type” or “monolithic” diffusion-controlled release device. Diffusion-controlled release devices are ones where the diffusion of the active ingredient through the device controls its release rate. Monolithic dispensers are diffusion-controlled release devices where the active ingredient is dispersed or dissolved in a matrix. If the active ingredient is dispersed in the matrix, it must dissociate from the other molecules in its crystal cell and solubilize into the matrix before release can occur. If it is dissolved in the matrix, this first step is bypassed (*4*). In the majority of cases, we expect hydrophobic arthropod pheromones to be dissolved in the SPLAT® matrix when the product is applied. The movement of the active ingredient dissolved within the matrix occurs by diffusion and follows Fick’s First Law (*5*), which states that molecules move down their concentration gradients at a rate that is directly proportional to their concentration gradient. Movement of the molecule within the matrix occurs in one of two ways. If the molecule is very small compared to the size of the

amorphous spaces in the matrix, it diffuses through the matrix by moving from one such space to another. If it is very large compared to the size of those spaces, then segments of the molecules comprising the matrix will have to be rearranged for diffusion of the active ingredient to occur. Crystalline regions in the matrix are virtually impermeable to molecules of the active ingredient. Upon reaching the surface of the matrix, the active ingredient is released into the environment. Whether the release rate of the active ingredient to the environment is zero or first order depends on the partition coefficient of the active ingredient between the matrix and the environment. If the active ingredient readily partitions to the environment, then its rate of release will be diffusion-controlled and first order. If, however, partitioning of the active ingredient to the environment is relatively slow, then its partition coefficient will determine its release rate from the matrix and the device will exhibit zero order release kinetics. The partitioning of the active ingredient to the environment is a function of the solubility of the active ingredient in the matrix; compounds more soluble in the matrix partition to the environment more slowly (*4*). SPLAT® emulsions in a field environment typically exhibit first-order release rates (*6*).

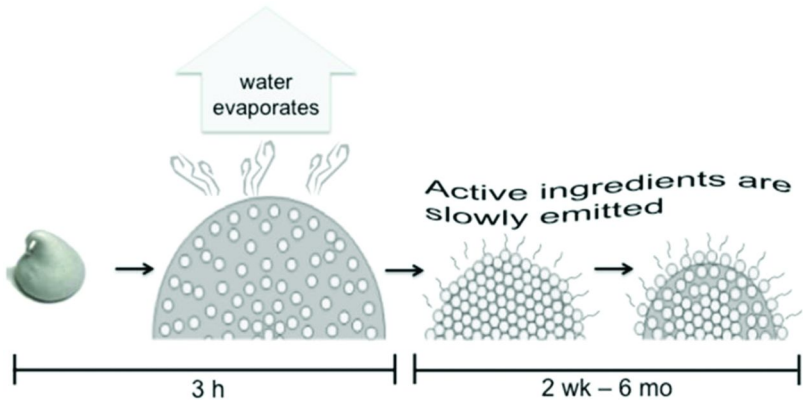

Figure 2. Following application, the SPLAT® emulsion dries and becomes rainfast within 3 hours, then releases active ingredients at a controlled rate for 2 weeks to 6 months.

Formulations

SPLAT® formulations have been developed to release a variety of compounds, including sex pheromones, kairomones, attractants, repellents, phagostimulants, and insecticides. Several SPLAT® mating disruption and attract-and-kill formulations are commercially available. SPLAT® repellent formulations will also soon become available commercially. Development of novel SPLAT® products is an active area of research. Efficacy trials conducted in the development of several of the newest SPLAT® mating disruption, attract-and-kill, and repellent formulations are described in the sections below.

SPLAT® Mating Disruption Formulations

Introduction

Mating disruption consists of dispensing a synthetic form of one or more components of the natural pheromone blend of an insect, or biologically-equivalent compounds, where host plant(s) are present. The presence of the species' sex pheromones in the environment delays or prevents mating of the insect (*7*), reducing fecundity and subsequent populations. Although using semiochemicals to disrupt communication among insects, rather than using these compounds as they are naturally used in insect communication (e.g., to attract or repel insects), was a relatively revolutionary idea when the first insect sex pheromones were identified 50 years ago, the use of semiochemicals for mating disruption was suggested and tested early on by several scientists (*8–13*). It has proven to be a powerful technique for insect control and has become the most commonly-used semiochemical-based insect control method (*14*, *15*).

The mechanisms by which mating disruption works have been discussed in several publications (*14–21*). Currently accepted mechanisms of mating disruption comprise: 1) competitive attraction (also known as "false trail following"), 2) camouflage, 3) desensitization, which includes both adaptation and habituation, and 4) sensory imbalance. Recent laboratory investigations have demonstrated that responses of females of some moth species to their own sex pheromone alters their behavior in ways that may also enhance mating disruption (*22–24*). Combinations of these mechanisms often function together in a mating disruption system and the mechanisms involved in mating disruption differ depending on both the species being controlled and the pheromone formulation used. Mating disruption research historically focused heavily on determining the efficacy of the technique for various insect species in field trials, with few studies to determine the mechanism by which mating disruption worked for the species and formulation being investigated (*14*, *16–18*). However, there has been an increasing effort by researchers in recent years not only to continue to prove the efficacy of the technique for new formulations and additional insect pest species, but also to provide evidence for the mechanisms by which mating disruption occurs for the species and formulations being investigated (e.g., (*15*, *19*, *20*, *25–27*)).

One must also be aware of the constraints of the technique to use it most effectively. Gut et al. (*14*) have provided a thorough analysis of these constraints. The success of mating disruption for a particular pest is impacted by biological and ecological factors (e.g., pest's host specificity, dispersal capacity, and population density), male response to pheromone (e.g., whether males are susceptible to adaptation or habituation), chemical characteristics of the pheromone (e.g., evaporation rate and propensity to adhere to surfaces), and the physical environment (e.g., effects of environmental conditions, such as heat and wind, plot size and shape, and site topography) (*14*). Taking these factors into account, researchers can choose the mating disruption formulations and application techniques best adapted to the target insect and treatment location.

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It is also important to keep in mind that for some pests, constraints may be too great for an economically-viable and successful mating disruption program to be designed and in these cases, alternative control techniques will need to be implemented (*14*, *17*).

The earliest SPLAT® formulations were created for mating disruption, principally of lepidopteran pests (*6*, *28–30*). Current SPLAT® mating disruption formulations include products for control of both lepidopteran and coleopteran pests (*2*, *6*, *27*, *31*, *32*). SPLAT® formulations are developed in close partnership with experts in academia, government, and industry, and often can become the first semiochemical-based control products for a pest species (e.g., SPLAT® EC, SPLAT® Tuta). SPLAT® mating disruption formulations have been commercialized for pests amenable to control using this technique and efforts have been made to determine the mechanisms by which mating disruption using some SPLAT® formulations functions (*26*, *27*). ISCA currently sells 10 SPLAT® mating disruption formulations worldwide (Table I) (*31*). Additional formulations are currently under development. The latest formulation, soon to be commercialized, SPLAT® EC for control of the carob moth, *Ectomyelois ceratoniae*, is discussed below.

Carob Moth, *Ectomyelois ceratoniae*

Ectomyelois ceratoniae (Lepidoptera: Pyralidae), the carob moth, is a widely distributed polyphagous pest that infests numerous fruit and nut crops, including dates, pomegranates, citrus, walnuts, figs, and pistachio, as well as stored nuts and seeds (*33–37*). The carob moth was first detected in the United States in 1982 in the Coachella Valley of California, the principal date-growing region of the country, and is now a major pest in this crop, with infestation levels ranging from 10% to 40% (*38*). There is also concern that the carob moth may eventually infest the Central Valley of California, a major growing region for several crops known to be susceptible to this pest (*39*). Control of the carob moth currently relies on frequent prophylactic sprays of malathion dust. There is an urgent need for safer alternative control methods.

SPLAT® EC was formulated for controlled release of (*Z,E*)-7,9,11-dodecatrienyl formate, a parapheromone (pheromone mimic) of the major component of the carob moth pheromone, (*Z,E*)-9,11,13-tetradecatrienal. Baker *et al.* (*40*, *41*) identified the sex pheromone of the carob moth to be an 8:1:1 blend of (*Z,E*)-9,11,13-tetradecatrienal, (*Z,E*)-9,11-tetradecadienal, and (*Z*)-9-tetradecenal, with the major component, (*Z,E*)-9,11,13-tetradecatrienal, primarily responsible for the attractiveness of the pheromone blend. They noted that synthetic blends of the carob moth pheromone were inferior to gland extracts in eliciting male responses, especially in field trapping studies, and postulated that this result was due to the decomposition of the highly labile triene major component of the pheromone. Todd *et al.* (*42*) synthesized (*Z,E*)-7,9,11-dodecatrienyl formate, a more stable analogue of (*Z,E*)-9,11,13-tetradecatrienal, and demonstrated that it effectively mimicked the major component of the carob moth pheromone both at the cellular and behavioral levels, and that it was equally or more effective than the synthetic blend of the natural pheromone components in field trapping studies.

Table I. Commercial SPLAT® mating disruption products

Product	*Pest*	*Availability*
SPLAT® OFM[a,b]	*Grapholita molesta*	U.S. & international
SPLAT® Cydia[a]	*Cydia pomonella*	U.S. & international
SPLAT® LBAM[a]	*Epiphyas postvittana*	U.S. & international
SPLAT® GM[a]	*Lymantria dispar*	U.S. & international
SPLAT® Tuta[a]	*Tuta absoluta*	U.S. & international
SPLAT® CLM[a]	*Phyllocnistis citrella*	U.S. & international
SPLAT® EC[a]	Ectomyelois ceratoniae	U.S.[c] & international
SPLAT® OFM/PFM[a]	*G. molesta/ Carposina sasakii*	International
SPLAT® PBW[a]	*Pectinophora gossypiella*	International
SPLAT® GRAFO/ BONA[b]	*G. molesta/ Bonagota salubricola*	Brazil

[a] Commercialized by ISCA Technologies, Inc., U.S.A. [b] Commercialized by ISCA Tecnologias Ltda., Brazil. [c] EPA registration pending.

Field trials of SPLAT® EC were conducted in two date gardens in the Coachella Valley of California. At both locations, the experiment was set up in a randomized complete block design with 1.6-ha plots, each containing 196 palms of the variety 'Deglet Noor'. There were three replicates at the first location and two at the second. Three treatments were tested at the first location: SPLAT® EC, 5% malathion dust (Gowan, Yuma, AZ), and a non-treated control. Two treatments were evaluated at the second location: SPLAT® EC and 5% malathion dust. SPLAT® EC was applied as two 2.5-g dollops per tree, one placed at the top of the tree, where date bunches are located, and the second placed on the trunk, *ca.* 1.5 m up from the ground, for a total of *ca.* 610 g SPLAT® EC per ha. A single application of SPLAT® EC was made at the beginning of the trial. Malathion dust was applied *ca.* every 2 weeks throughout the trial for a total of 4 applications.

The SPLAT® EC and malathion-treated plots were evaluated with male captures in carob moth parapheromone-baited traps. One parapheromone-baited trap was placed in the center of each plot and male moths in each trap were counted weekly. Moth capture data were analyzed by repeated measures ANOVA on square root-transformed data (PROC GLM, SAS Institute 2003). All plots were also evaluated by performing fruit damage evaluations at harvest. For this assessment, carob moth infestation was determined by collecting all of the fruit from the largest date bunch present on the 16 palms located in the center of each plot. The selected bunch was removed from the palm, the fruit were mixed, and

ca. 200 dates were collected randomly. Of these, 100 were selected randomly and evaluated for carob moth infestation by examining the fruit for moth webbing at the calyx end of the date. Fruit infestation data were analyzed with a one-way ANOVA (PROC GLM, SAS Institute 2003). Mean fruit infestation for the three treatments at location 1 were separated with Tukey's test (PROC GLM, SAS Institute 2003).

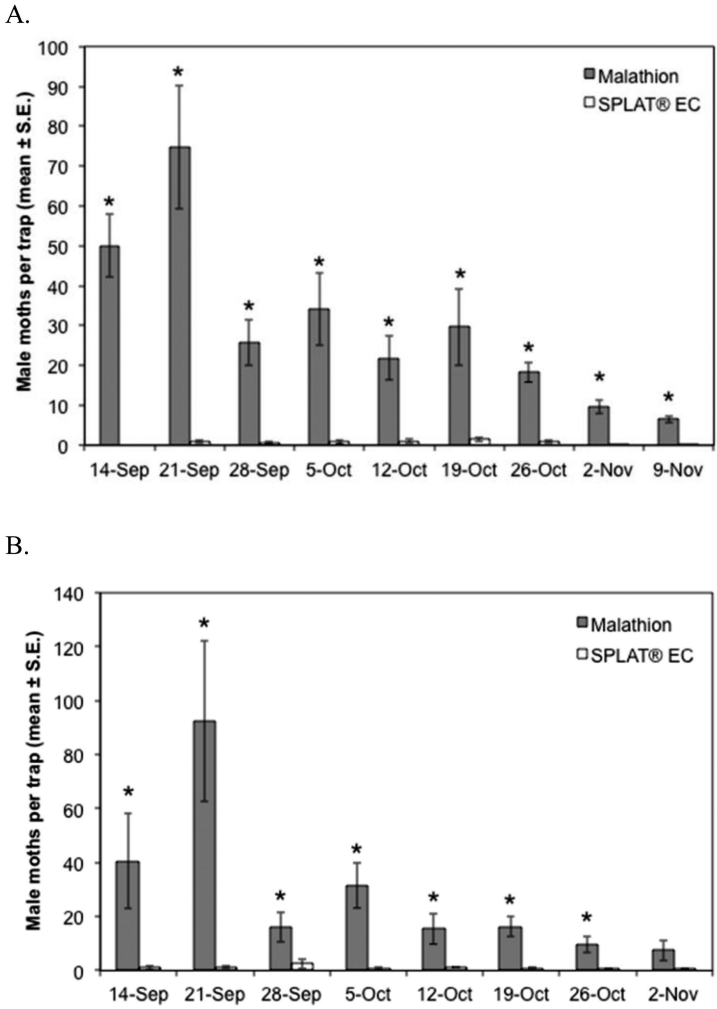

Figure 3. Field efficacy of SPLAT® EC for carob moth control in dates. Male moth captures in parapheromone-baited traps at A. location 1 and B. location 2. Malathion treatments labeled with an asterisk were significantly different from SPLAT® EC on that sampling day (ANOVA, $P \geq 0.05$).

Male moth captures in the SPLAT® EC-treated plots were lower than in the malathion-treated plots at both locations. At location 1, the differences in moth captures in SPLAT® EC- and malathion-treated plots were statistically significant throughout the trial (Figure 3). At location 2, moth captures in the SPLAT® EC- and malathion-treated plots were significantly different on all sampling dates, except November 2, when the difference was nearly significant ($P = 0.057$). The extremely low numbers of male moths captured in parapheromone-baited traps in the SPLAT® EC-treated plots demonstrated that mating disruption was nearly complete from the one-time SPLAT® EC application through harvest.

Whereas moth capture data were collected only in SPLAT® EC- and malathion-treated plots, fruit infestation data were collected in all plots, including the non-treated control plots at location 1. Carob moth infestation at location 1 was equivalent in SPLAT® EC and malathion-treated plots and significantly lower in these treatments compared to the non-treated control (Table II). Results at location 2 were similar, with no significant difference in fruit infestation in the SPLAT® EC- and malathion-treated plots. The results were economically-important because the cost of the single SPLAT® EC application (material and application) was equivalent to the total cost for all malathion dust treatments. In addition, the SPLAT® EC treatment provided the same level of carob moth control as malathion dust without negative human health and environmental impacts. Furthermore, unlike malathion dust, which is applied directly to the dates and dries out the fruit, SPLAT® EC is not applied to the fruit, yielding dates with hire water content for a superior quality product. ISCA synthesizes (*Z,E*)-7,9,11-dodecatrienyl formate in-house and is currently the only manufacturer of carob moth parapheromone lures and mating disruption products worldwide.

Table II. Field efficacy of SPLAT® EC for carob moth control in dates. Values are mean ± S.E. of fruit infestation at harvest. Means ± S.E. followed by the same letter within rows are not significantly different (location 1: Tukey, P ≥ 0.05; location 2: ANOVA, P ≥ 0.05).

	Treatment		
Location	*Pheromone*	*Malathion*	*Non-treated*
1	8.1 ± 0.6 a	10.3 ± 1.1 a	14.8 ± 1.3 b
2	4.1 ± 0.6 a	4.0 ± 0.7 a	n/a

Conclusions

SPLAT® formulations were initially developed for insect mating disruption and several have had commercial success (Table I). Developing new SPLAT® mating disruption formulations remains a focal point of ISCA's product development efforts.

SPLAT® Attract-And-Kill Formulations

Introduction

The attract-and-kill strategy is also referred to as "lure-and-kill" and "attracticide", as well as by other terms (e.g., male annihilation, lure-and-sterilize, lure-and-infect, bait spray), depending on the type of attract-and-kill strategy being used (*43*). Broadly, attract-and-kill consists of attracting males, females, or both sexes of a pest species to an insect control agent (e.g., insecticide, sterilant, or insect pathogen). Upon contact, the insect is either killed (immediately or after a delay) or sublethal effects of the control agent diminish the pest population by reducing the insect's fertility or ability to mate (*43*). The insect attractant can be a chemical attractant, a visual cue, an acoustic cue, or a combination of these. Crude baits (e.g., food lures) are also used in attract-and-kill devices and entire plants (e.g., trap crops) have been used as attractants for this technique as well (*15*, *43*, *44*). Since SPLAT® is a chemical controlled-release technology, we will focus on a discussion of attract-and-kill devices that use chemical attractants.

Attract-and-kill technology, although it has shown promise, has historically not been investigated or developed as intensively as mating disruption. Although the technique has proven effective against some species of Coleoptera, Lepidoptera, and Diptera, research efforts have been disproportionately aimed at developing the technique to manage tephritid fruit flies, which are difficult or impossible to control using other methods (*14*, *15*, *43*, *44*). However, there has been interest in recent years in developing attract-and-kill products and strategies for a wider variety of pests, including both established and emerging pest species (e.g., (*45–49*)).

Unlike mating disruption, which can control insect populations *via* a variety of mechanisms, there is only one way that attract-and-kill can achieve insect control. Pest insects (ideally both sexes) must be lured to a control agent that exerts its affect on the individual following contact. This requires the synthetic attractant to be more effective than natural attractants in the environment to successfully out-compete these. Furthermore, the attractant must not only effectively attract the target insect from a distance, but also cause the insect to contact the formulation. Thus, attractants used in attract-and-kill formulations must be highly effective for the technique to work, versus those used in mating disruption, which do not need to meet such high efficacy standards, since source contact is not necessary for mating disruption to work. An excellent attractant and appropriate insect control agent are indispensible ingredients of an effective attract-and-kill product (*43*).

Although attract-and-kill shares some of the same constraints as mating disruption, it is generally believed to be a more robust pest control technique (*43*). Just as mating disruption occurring by competitive attraction, the effectiveness of attract-and-kill is also reduced at high pest densities or when too few lures are applied to compete with natural attractants (*15*, *43*). It also must be adapted to the biology and ecology of the pest (e.g., by optimizing formulation placement and timing of application). Unlike mating disruption, however, it is less sensitive to environmental factors, such as site topography and plot size, and can successfully be used in situations where mating disruption is likely to fail. Although attract-and-kill products most often contain insecticides, which

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make them less environmentally-friendly and potentially more of a concern to the public than mating disruption products, they offer several advantages over conventional insecticides. These include the use of smaller amounts of insecticide and the option to apply the product away from the harvestable crop, increasing both worker and consumer safety. Attract-and-kill products can also be used to lure and control pests out of areas that cannot be treated with conventional sprays. Depending on the attractant used, attract-and-kill products may be very selective (i.e., if insect sex pheromones are used). However, attractants with broad effects, such as plant kairomones, should be tested to determine their impact on non-target organisms (*15*, *43*). Another important difference between mating disruption and attract-and-kill products for manufacturers is that unlike mating disruption products, these insecticide-containing formulations cannot take advantage of legislation which has simplified and reduced the cost of EPA registration of arthropod pheromone-based insect control products that do not contain insecticides (*50*).

The SPLAT® matrix is not only well-adapted for dispensing and protecting semiochemicals from degradation, but it has proven to effectively dispense and protect insecticide active ingredients as well. ISCA currently has four attract-and-kill formulations available internationally: Hook™ ME, Hook™ CL, and Hook™ ME+CL for control of *Bactrocera* sp. fruit flies and Hook™ RPW, for control of the red palm weevil, *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae). Several additional SPLAT®-based attract-and-kill formulations are currently being developed. Two of these are discussed below: Hook™ FAW for control of the fall armyworm, *Spodoptera frugiperda* and Hook™ Tuta, for control of the tomato leafminer, *Tuta absoluta*. Development of STATIC™ Spinosad ME for control of *Bactrocera* sp. fruit flies, which has been commercialized by Dow AgroSciences (Indianapolis, IN) is also discussed.

Fall Armyworm, *Spodoptera frugiperda*

Spodoptera frugiperda (Lepidoptera: Noctuidae), the fall armyworm, is native to the tropical regions of the Americas. Adults can migrate great distances, which can result in infestations as far north as Canada, although they are not able to survive the cold winters in regions north of southern Florida and Texas. Fall armyworm larvae are highly polyphagous. Although they prefer to feed on grasses, the fall armyworm has also been reported to feed on numerous other agricultural and non-agricultural plants. Larvae primarily damage plants and reduce yields through extensive defoliation of the host. Although this occurs more rarely, they are also capable of cutting plant stalks and occasionally feed directly on seeds and fruits of their hosts. Although natural enemies can significantly reduce fall armyworm populations in regions where it overwinters and some crops genetically modified to express *Bacillus thuringensis* insecticidal proteins can effectively control fall armyworms, nonetheless, large amounts of insecticides are often used to control this insect pest (*51–54*). A SPLAT®-based formulation, Hook™ FAW, containing the fall armyworm pheromone and an insecticide, is being developed as an attract-and-kill product for this pest and has been tested on field populations in Brazil.

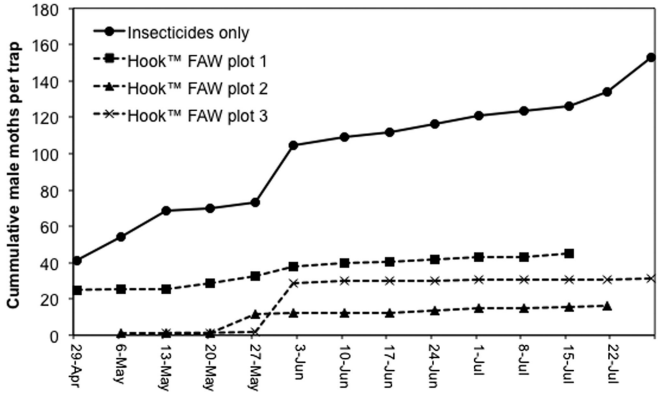

Figure 4. Field efficacy of HOOK™ FAW for fall armyworm control in corn. Grower-standard insecticide sprays were applied in all plots.

Table III. Field efficacy of Hook™ FAW for control of fall armyworm in corn. Assessment of plant damage. Values are mean ± S.E of average plant damage determined at all sampling times in each plot. Grower-standard insecticide sprays were applied in all plots. Means ± S.E. followed by the same letter within columns are not significantly different (Tukey, $P \geq 0.05$).

Plot	*Davis scale rating*	*% damage*	*n*
Hook™ FAW plot 1	5.4 ± 0.52 ab	54.8±12.01 ab	4
Hook™ FAW plot 2	4.1±0.46 bc	50.5±5.25 b	5
Hook™ FAW plot 3	2.9±0.33 c	50.7±4.51 b	7
Insecticides only	6.9±0.35 a	84.8±7.10 a	4

A preliminary study of Hook™ FAW was conducted in large corn plots in Mogi Mirim, Sao Paulo, Brazil. Hook™ FAW was applied to three 50-ha plots. A single 27-ha plot served as a control. Hook™ FAW was hand-applied using a metering device on the upper leaves of the plants, close to the stalks, at a rate of 500 g/ha when plants had three to four leaves. A second Hook™ FAW application was made at a rate of 1 kg/ha 3 weeks later. Grower-standard insecticide sprays were applied in all plots. Hook™ FAW efficacy was evaluated with three pheromone lure-baited traps per plot. In addition, fall armyworm plant damage was quantified weekly by evaluating 25 plants at each of three sampling points in each plot using the Davis scale (*55*) and by quantifying the percentage of each plant damaged. Plant damage evaluations were conducted weekly starting the week after Hook™ FAW was applied, until the plants reached the tassel stage. Moth captures per trap were reduced in plots treated with Hook™ FAW versus plots only treated

with insecticides (Figure 4). Plant damage was also significantly reduced in all plots treated with Hook™ FAW versus plots only treated with insecticides, except for plot 1 (Table III). Further development of the Hook™ FAW formulation is on-going.

Tomato Leafminer, *Tuta absoluta*

Tuta absoluta (Lepidoptera: Gelechiidae) is known by a variety of common names, including: Tomato leafminer, tomato borer and South American tomato pinworm. Larvae feed on solanaceous crops and show a preference for tomatoes. They can feed on all above-ground tissues of tomato plants, including the leaves, stems, and fruit, during all life stages of the plant. Tomato leafminers have a high reproductive potential, with up to 12 generations per year, and each female capable of laying up to 260 eggs in her lifetime. The tomato leafminer does not have an obligate diapause and can overwinter at the egg, pupil, or adult stage, which contributes to this pest's persistence and potential to develop large populations. If left untreated, tomato leafminer damage can cause 100% crop loss (*56–59*).

The tomato leafminer is native to South America, but since 2006, has spread to North Africa, Southern and Western Europe, and the Middle East (*59–61*). Control of the tomato leafminer has historically relied heavily on the use of insecticides, with up to 36 applications of insecticides per season (4 to 6 applications per week) used to control it. Due to its short generation time and high reproductive potential, the tomato leafminer is highly likely to develop insecticide resistance and indeed, in endemic regions, use of new insecticides to control the tomato leafminer has inevitably been followed by reports of resistance to these insecticides (*57*, *58*). It is highly likely that the tomato leafminer will be introduced to other tomato-growing regions of the world, including the United States. The tomato leafminer poses a major economic threat to worldwide tomato production (*59*, *61*).

A tomato leafminer attract-and-kill SPLAT® formulation, Hook™ Tuta, was created that contained the tomato leafminer pheromone and an insecticide. This formulation was tested in staked tomato plots at two locations in the municipality of Caçador, Santa Catarina, Brazil. Plant spacing in both locations was 1.5 m x 0.54 m. At the private farm, plots were 937 m^2 and each contained *ca.* 1325 tomato plants, while at the Epagri Experiment Station, plots were 212 m^2 and each contained *ca.* 260 tomato plants. At both locations, the experimental design was randomized complete block with four replicates. Two treatments were tested: Hook™ Tuta + insecticides and insecticides only. Hook™ Tuta was applied as 0.5-g dollops at a rate of 1.5 kg/ha. Grower-standard insecticide sprays were applied to both Hook™ Tuta and insecticides only plots. Adult tomato leafminer populations were monitored weekly with pheromone traps. In addition, four plants were randomly marked in each plot and fruit damage was assessed for 25 fruits on each plant 59 days post-treatment (100 total fruits per plot). Although fruit injury was similarly low in plots treated with Hook™ Tuta and plots treated with insecticides alone (Table IV), mean captures of male moths in pheromone-baited traps in plots treated with Hook™ Tuta vs. plots treated with insecticides alone over the duration of the study were reduced by 85% on the private farm and 78% at the Epagri Experiment Station (Figure 5).

Table IV. Field efficacy of Hook™ Tuta for control of the tomato leafminer in staked tomatoes. Values are mean ± S.E. of percent tomato fruit damaged. Grower-standard insecticide sprays were applied in all plots.

	Treatment	
Location	*Hook™ Tuta*	*Insecticides only*
Private farm	3.5 ± 0.6	3.2 ± 0.5
Epagri Experiment Station	3.8 ± 0.8	3.0 ± 0.7

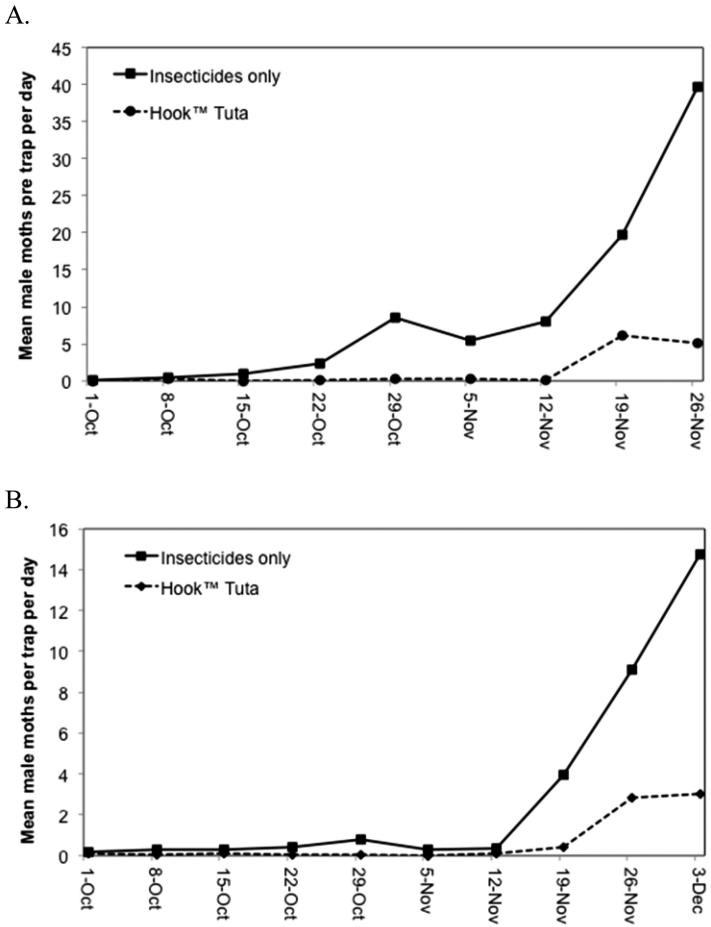

Figure 5. Moths captured in pheromone-baited traps in field efficacy trials of Hook™ Tuta for control of the tomato leafminer in staked tomatoes. A. private farm, B. Epagri Experiment Station. Grower-standard insecticide sprays were applied in all plots.

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The tomato leafminer's high level of impact on tomato production in regions where it is established, the high likelihood that it will continue to spread to other tomato-growing regions of the world, and the difficulty of controlling the tomato leafminer with insecticides, not to mention the environmental and human health impacts of relying solely on conventional insecticides to control this pest, highlight the necessity for alternative tomato leafminer control options, such as Hook™ Tuta. ISCA is currently the only United States registrant of the tomato leafminer pheromone and also holds an EPA registration for SPLAT® Tuta, the only EPA-registered tomato leafminer mating disruption product. When it becomes commercially-available, Hook™ Tuta will provide an additional control option for growers battling the tomato leafminer in endemic and newly-infested regions.

Fruit Flies, *Bactrocera* sp.

The tephritid family of fruit flies (Diptera: Tephritidae) includes numerous agricultural pests of great economic importance. Significant efforts are made to monitor and control fruit flies in countries where these pests are established, as well as in regions where the risk of introduction and establishment of tephritid fruit flies is high (*62*, *63*). Several members of the genus *Bactrocera,* including *Bactrocera dorsalis* (oriental fruit fly), *Bactrocera cucurbitae* (melon fruit fly), and *Bactrocera tryoni* (Queensland fruit fly), are of special concern because of their wide host ranges (>100 known host plants), which include numerous agricultural crops, and their capacity to cause 100% crop loss (*62*, *63*). The availability of compounds that are highly attractive to males of most *Bactrocera* species has enabled the use of attract-and-kill (often referred to as male annihilation technique - MAT) to control these fruit flies. Methyl eugenol (ME) (*64*) or cue-lure (CL) (*65*) are attractive to over 90% of males of species in the subfamily Dacinae (*66*), which comprises the genera *Bactrocera* and *Dacus*. Although attract-and-kill has been effective, it has so far relied on the use of organophosphate-based products that pose risks to worker, food, and environmental safety. In addition, most attract-and-kill control efforts have used lures placed in traps that require high cost and labor inputs to set up and maintain.

SPLAT® MAT ME and SPLAT® MAT CL, containing the reduced-risk insecticide spinosad (Dow AgroSciences) and the male fruit fly attractants methyl eugenol (ME) (*64*) or cue-lure (CL) (*65*), were created as alternative management tools to replace organophosphate-based MAT products for controlling *Bactrocera* fruit flies in area-wide fruit fly management programs. In collaboration with ISCA, Vargas and colleagues conducted a series of laboratory and field studies testing the efficacy of SPLAT® MAT ME and SPLAT® MAT CL versus organophosphate-based standards against the oriental fruit fly and the melon fruit fly in Hawaii (*67–69*) and the oriental fruit fly and the Queensland fruit fly in Tahiti (*70*). These studies demonstrated that SPLAT® MAT was as effective or superior to current MAT technologies for extended periods of time (up to 16 weeks), even though the toxicity of spinosad to the target species sometimes decreased below that of the conventional pesticides present in the standard products as the study progressed. In addition to matching the efficacy of current

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formulations, the SPLAT® formulations also provided benefits in terms of ease of application, versus current MAT techniques, which often involve applications of solid or liquid MAT formulations in traps, requiring more time and cost to apply and service than the SPLAT® products, not to mention the inability to mechanize the application of trap-based products, which greatly limits their utility for fruit fly control efforts over large areas. Weathered SPLAT® formulations also had improved longevity versus Min-U-gel (Floridin Co., Quincy, FL), a flowable organophosphate-based MAT product known to have limited field life in high temperature and high rainfall environments, such as tropical regions where Dacinae species are important agricultural pests (*63*, *71–74*). SPLAT® MAT ME is currently sold as STATIC™ Spinosad ME by Dow AgroSciences for control of *Bactrocera* fruit flies.

Conclusions

SPLAT® attract-and-kill formulations have proven to be effective and can provide an alternative control option for pests that may offer advantages over mating disruption, mass trapping, and the use of conventional insecticide sprays. Two SPLAT®-based attract-and-kill products are currently commercially-available, STATIC™ Spinosad ME is available in the United States and CIDA GRAFO/BONA is sold for control of *Grapholita molesta*, the oriental fruit moth, and *Bonagota salubricola,* the Brazilian leafminer, by ISCA Tecnologias Ltda. in Brazil. ISCA intends to continue to increase its attract-and-kill product portfolio both domestically and internationally.

SPLAT® Repellent Formulations

Introduction

We define repellents as compounds that deter or inhibit insects from finding, feeding on, or ovipositing on an attractive host substrate. Although a number of semiochemicals with repellent effects against agricultural and forest pests have already been identified, to date, they have only played a very minor role in the control of these insects. This is due to a combination of factors, including the availability of cheap and effective control alternatives for some insect pests, the lack of adequate formulations for delivery, and substantial regulatory obstacles for registering new repellent products (*15*, *75*, *76*). Although the use of repellents alone or in combination with attractants as part of a push-pull strategy has been shown to be effective in agriculture and forest systems, it requires a greater understanding of insect behavior and ecology than conventional or even other semiochemical-based alternatives, such as mating disruption and attract-and-kill (*15*, *76*). Although SPLAT® is well-adapted for delivering volatile insect repellents, the cost of bringing these technologies to market for commercial agricultural or forestry use is often prohibitive as a result of small market size and the high cost of registering products. Repellent chemicals are often best adapted for control of a limited number of insect species, in a limited number of crops, and only for growers willing and able to adopt these new techniques,

which makes these a niche product (*15*, *76*). Although the EPA has reduced data requirements, costs, and registration time for biopesticides, which generally pose lower human and environmental risks than conventional chemicals, registration of new biopesticides is still a costly and time-consuming process, especially for plant kairomones, most of which are not exempt from EPA registration and not eligible for pheromone regulatory relief (*15*, *50*, *75*, *76*). Even though the obstacles to commercialization of insect repellents are high, there are, nonetheless, situations where commercialization of insect repellents is warranted. These include cases, such as the ones described below, where repellents are effective and have the potential to provide a cost-effective alternative or significant enhancement to conventional control tactics or available semiochemical control-based alternatives (*15*). ISCA and collaborators are actively developing SPLAT® repellent formulations against several important pests, including the mountain pine beetle, *Dendroctonus ponderosae* (SPLAT® Verb Repel) and the Asian citrus psyllid, *Diaphorina citri* (SPLAT® ACP Repel). Field trials with both of these formulations have been successful and are summarized below.

Mountain Pine Beetle, *Dendroctonus ponderosae*

Dendroctonus ponderosae (Coleoptera: Curculionidae), the mountain pine beetle, is a bark beetle native to western North America that colonizes several pine species, most notably lodgepole pine, ponderosa pine, sugar pine, limber pine, western white pine, and whitebark pine. Girdling of phloem tissues by colonizing adults and developing larvae kills the host tree. The extensive and severe outbreaks that have occurred in recent years indicate that the mountain pine beetle is one of the foremost threats to western North American forests, and will remain such in the future (*77*, *78*). Extensive levels of tree mortality associated with mountain pine beetle outbreaks may result in replacement of host trees by other tree species and plant associations, with subsequent impacts on timber and fiber production, fuels and fire behavior, water quality and quantity, fish and wildlife populations, aesthetics, recreation, grazing capacity, real estate values, biodiversity, carbon storage, threatened and endangered species, and cultural resources, among others.

Like many bark beetles, the mountain pine beetle uses a complex system of semiochemical communication in host location, selection and colonization, and mating behaviors (*79*). Mountain pine beetles infest the lower and mid-tree bole in a behavioral sequence facilitated by aggregation pheromones and host kairomones. During the latter stages of tree colonization, increasing amounts of verbenone are produced by the autoxidation of α-pinene to *trans*- and *cis*-verbenol and then to verbenone (4,6,6-trimethylbicyclo[3.1.1]hept-3-en-2-one) (*80*) by intestinal and gallery-inhabiting microbes from both beetle sexes (*81*, *82*). Verbenone is considered an anti-aggregation pheromone component and believed to reduce intra- and interspecific competition by altering adult behavior to minimize overcrowding within the host tree and to provide cues as to host suitability (*83–85*).

Verbenone has been registered for management of mountain pine beetles as an alternative to standard techniques that rely on the use of conventional insecticides or tree removals to suppress populations and protect susceptible

hosts. Three formulations are currently registered, and include pouches (several registrants) and the Disrupt Micro-Flake® VBN and Disrupt Bio-Flake® VBN formulations (Hercon® Environmental, Emigsville, PA). Pouches are most commonly used and are stapled at maximum reach (*ca.* 2 m height) to individual trees prior to mountain pine beetle flight in spring, and applied in a grid pattern to achieve uniform coverage when stand protection is the objective. Although pouch formulations have been reasonably effective in reducing mountain pine beetle attacks in lodgepole pine stands (*79*), treatment failures are not uncommon, and indicate a need for improved formulations and more effective means of dispersing verbenone in forests (*86*). A pilot study of the initial SPLAT® Verb Repel prototype was conducted on the Bridger-Teton National Forest in western Wyoming. Twenty-one randomly-selected, individual lodgepole pine trees were treated with SPLAT® Verb Repel, with an additional 30 trees randomly-selected as non-treated controls. All trees in the study were confirmed uninfested by mountain pine beetles prior to treatment. Either *ca.* 32 g (15 trees) or *ca.* 39 g (6 trees) of (−)-verbenone was applied per tree in four equivalently-sized SPLAT® Verb Repel dollops with a pneumatic, John Deere Gator®-mounted mechanical application system (Figure 1 C, D). SPLAT® Verb Repel was applied in mid-July, a few weeks after the initiation of mountain pine beetle flight in the area. Each SPLAT® Verb Repel-treated and non-treated tree was baited with one mountain pine beetle tree bait (Contech Inc., Delta, BC, Canada) affixed at *ca.* 2.4 m height on the north side of the tree to challenge trees used in the study with sufficient bark beetle pressure to assess treatment efficacy. The baits were removed from all trees *ca.* 30 days later, at which time, the integrity of SPLAT® Verb Repel dollops was visually inspected. Attack densities were assessed in mid-September. Visual signs of attack (boring dust and pitch tubes) were recorded for each of the treated and non-treated trees. The following June, the presence (dead) or absence (live) of crown fade was recorded for each experimental tree to assess levels of tree mortality.

Only two attacks (pitch tubes) were observed on one of the SPLAT® Verb Repel-treated trees in September and all SPLAT® Verb Repel-treated trees were alive the following June. By contrast, 28 of the 30 non-treated trees were attacked by September and only 2 remained alive the following June (Table V). Although treatment efficacy was excellent, the SPLAT® Verb Repel dollops did not adequately adhere to the tree boles. Both the SPLAT® Verb Repel formulation and application methodology were modified to address this issue in a subsequent study (see below).

A second field study was initiated in the same area last year to determine the effectiveness of the improved SPLAT® Repel formulation and application method. Thirty randomly-selected, individual lodgepole pines were treated with SPLAT® Verb Repel using a caulking gun (Figure 1B), with an additional 30 trees randomly-selected as non-treated controls. All trees in the study were confirmed uninfested by mountain pine beetles prior to treatment. Four dollops of SPLAT® Verb Repel (7 g of (−)-verbenone per tree) were applied at *ca.* 3-m height on the tree bole. All experimental trees were baited with one mountain pine beetle tree bait (Contech Inc.) on the northern aspect at *ca.* 2.4-m height for 113 days. In October, visual signs of mountain pine beetle attack were recorded for each treated

and non-treated tree. In addition, trees within an 11 m radius of each SPLAT® Verb Repel-treated and non-treated tree were inspected for signs of mountain pine beetle attack. Whereas 28 non-treated trees and an additional 61 trees within an 11 m radius of the non-treated trees were mass attacked by mountain pine beetle, no SPLAT® Verb Repel-treated trees or surrounding trees were mass attacked (Table VI). Crown fade will be used to assess tree mortality in summer 2013.

Table V. Effectiveness of SPLAT® Verb Repel in protecting individual lodgepole pines from mountain pine beetle attack. Values are numbers of trees of 21 SPLAT® Verb Repel-treated or 30 non-treated trees.

	End of season evaluation				*Next season evaluation*	
Treatment	*No. not attacked*	*No. minor attacked*[a]	*No. strip attacked*[b]	*No. mass attacked*[c]	*No. alive*	*No. dead*
SPLAT® Verb Repel	20	1	0	0	21	0
Non-treated	2	0	3	25	2	28

[a] Two mountain pine beetle attacks on the tree bole. [b] One face of the tree bole attacked. [c] Entire circumference of the tree bole attacked.

Table VI. Effectiveness of SPLAT® Verb Repel in protecting individual and neighboring lodgepole pines from mountain pine beetle attack[a]

	Treated tree		*Trees within 11 m radius of treated tree*
Treatment	*No. not attacked*	*No. mass attacked*[b]	*No. mass attacked*[b]
Verb Repel	30	0	0
Non-treated	2	28	61

[a] N=30 per treatment. [b] Entire circumference of bole attacked.

An evaluation of SPLAT® Verb Repel for protecting 0.4-ha plots of lodgepole pine from mountain pine beetle infestation is on-going on the Caribou-Targhee National Forest in southeastern Idaho. The following treatments are being evaluated: Non-treated control, verbenone pouches (Contech Inc.), and SPLAT® Verb Repel. Preliminary results indicate that SPLAT® Verb Repel is more effective at preventing mountain pine beetle mass attacks within small plots than the verbenone pouch.

Asian Citrus Psyllid, *Diaphorina citri*

Diaphorina citri, the Asian citrus psyllid, vectors *Candidatus liberibacter* species that are the causative agents of huanglongbing (or citrus greening) disease, the most devastating disease of citrus worldwide (*87*). Although research to identify Asian citrus psyllid pheromone attractants is on-going, attractant pheromone-based control technologies, such as mating disruption or attract-and-kill are not currently available (*87–91*). Guava, interplanted with citrus, has been reported to lead to reduced Asian citrus psyllid populations (*92*, *93*), an affect that has been attributed to guava leaf volatiles (*94*). Stelinski and colleagues identified dimethyl disulfide (DMDS), a compound isolated from crushed guava leaves, as a potent repellent to the Asian citrus psyllid (*95*, *96*). Development of a SPLAT® ACP Repel formulation containing this compound has yielded promising results.

A field trial was conducted with the first SPLAT® ACP Repel prototype in a 200-ha abandoned orchard of mature, *ca.* 18-year old sweet orange trees (var. "Valencia") planted at *ca.* 284 trees per ha and heavily infested with Asian citrus psyllids. Plots were square and contained 35 trees (5 x 7 trees). SPLAT® ACP Repel applied at a rate of 50 g per tree was compared to an non-treated control. Asian citrus psyllid populations in each plot were quantified at 3, 7, 11, 14, and 21 days post-treatment by counting the number of Asian citrus psyllids in 10 trees in each plot. The experimental design was randomized complete block and there were five replicates per treatment. Asian citrus psyllid populations in the SPLAT® ACP Repel plots were reduced by at least 50% for the duration of the 3-week study (Figure 6).

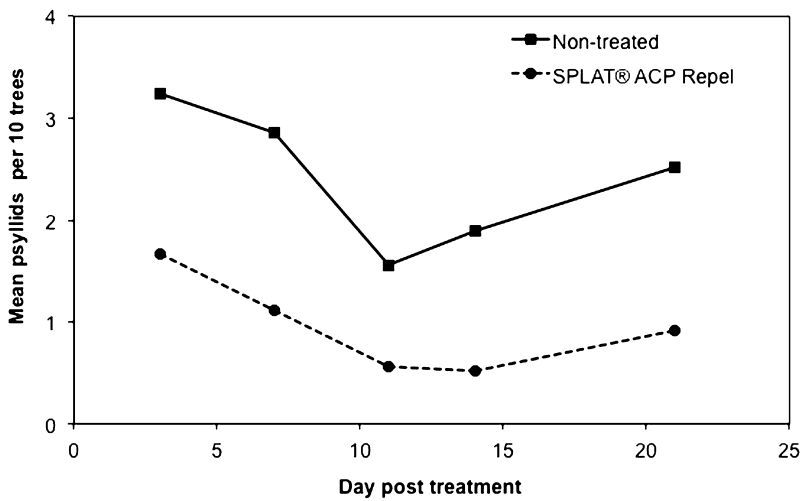

Figure 6. Field efficacy of the first SPLAT® ACP Repel prototype formulation for control of the Asian citrus psyllid in small citrus plots. Psyllid counts in trees.

In Pest Management with Natural Products; Beck, J., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2013.

Following this initial field trial, four modified SPLAT® ACP Repel formulations were created in an effort to increase the effective longevity of the formulation and to reduce the release rate of the highly volatile active ingredient. These formulations were field-tested in mature sweet orange trees (var. "Valencia"). The trees were 12 years old, planted on a 3 x 6 m spacing, and averaged 4 m in height. Each plot consisted of 20 trees. SPLAT® ACP Repel was applied at a rate of 6 kg/ha. This amounted to applying six 5-g SPLAT® ACP Repel dollops per tree. The efficacy of the prototype SPLAT® ACP Repel formulations was compared to that of a non-treated control and each treatment was replicated four times. Four yellow sticky card traps were used to assess population densities of Asian citrus psyllids in each plot. SPLAT® ACP Repel #4 provided approximately 75% repellency of Asian citrus psyllids through the five week duration of the trial (Figure 7). ISCA will continue to work with collaborators to develop this technology for use in Asian citrus psyllid integrated pest management programs.

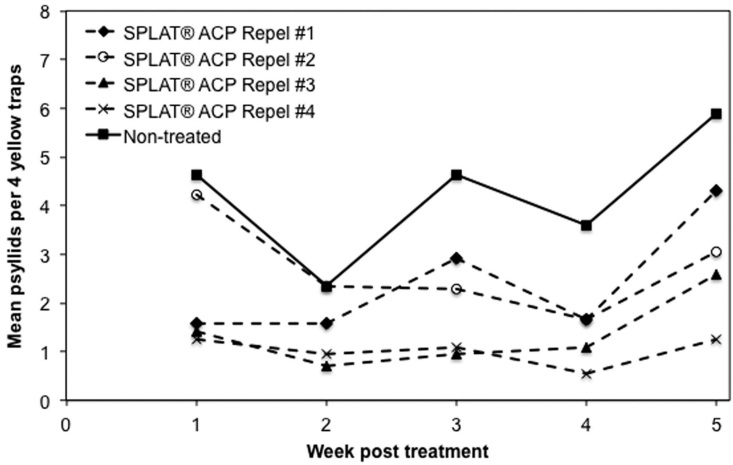

Figure 7. Field efficacy of four modified prototype SPLAT® ACP Repel formulations in small citrus plots. Psyllid captures in yellow sticky card traps.

Conclusions

Although to date, few semiochemical-based insect repellents have been commercialized for use in agriculture and forestry, we believe these products can be valuable tools for some integrated pest management programs and should be pursued in cases where existing control methods have not proven adequate. Repellent semiochemicals, when formulated in appropriate dispensing systems, can play an important role in effective control programs (*76*). SPLAT® will be useful in this regard.

Conclusions and Future Directions

SPLAT® is unique among commercial semiochemical dispensers in providing a matrix that is capable of dispensing a wide variety of compounds and can be applied using a virtually unlimited number of manual and mechanical techniques. The versatility of SPLAT® makes it adaptable for use in any semiochemical-based insect control program, regardless of semiochemical, crop, or plot size. SPLAT® mating disruption and attract-and-kill formulations have been developed for important agricultural and forestry pests both domestically and internationally. Several of the existing SPLAT® mating disruption formulations have also been certified for use in organic crop production. Repellent formulations are currently being developed for important agriculture and forestry pests, as well as attractant formulations for beneficial insects. ISCA will continue to work with collaborators to test the limits of the SPLAT® matrix for dispensing insect behavior-modifying compounds in a variety of environments as it participates in research to create new semiochemical-based insect control tools.

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Chapter 5

Volatile Natural Products for Monitoring the California Tree Nut Insect Pest *Amyelois transitella*

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The navel orangeworm (*Amyelois transitella*) is a major insect pest that inflicts serious economic damage to the California tree nut industry. Feeding by navel orangeworm larvae causes physical damage resulting in lower kernel quality; more importantly larvae are purported to vector the aflatoxigenic fungi. Aflatoxins are toxic metabolites produced by aspergilli and represent a major food safety concern. Over the years volatile natural products have played a large role in efforts to control or monitor navel orangeworm moths. The two most important sources of relevant natural products have been female navel orangeworm, which produce a complex sex pheromone blend; and, the almond host plant, which has recently been described as the source of a blend of volatiles that attract both male and female navel orangeworm. Provided herein is an overview of natural products and their role in efforts to control or monitor navel orangeworm moths in California almonds, pistachios, and walnuts.

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Introduction

Natural products have long been considered important bioactive chemical compounds with a wide variety of practical uses, including: medicinal, toxic agents, pesticides, and fungicides, among others (*1*). Volatile natural products also play a large role in the chemical cues of insects; examples include location of a food source, safe ovipositional sites, and avoidance of non-host plants (*2–8*). One example is an agricultural insect pest, the navel orangeworm (*Amyelois transitella*) (Figure 1), a major insect pest of California tree nuts (*9*) dating back to the 1960s (*10*). A blend of natural product host plant volatiles has recently been reported as an attractant for both male and female navel orangeworm moths (*11*) and the chemical components of the female sex pheromone are known (*12*). In addition to the physical damage to the tree nut kernels caused by larval feeding, navel orangeworm larvae are purported to vector aflatoxigenic fungi (*13*), thus contaminating the product and raising significant concerns regarding the safety of tree nut consumption.

Figure 1. The navel orangeworm (Amyelois transitella) moth, shown next to a whole almond, is an insect pest of California almonds, pistachios, and walnuts.

Aflatoxins

Navel orangeworm moths have been associated with aflatoxins, which are a group of compounds produced by certain mycotoxigenic aspergilli ubiquitous in California tree nut orchards (*9*, *14*). Specifically, the fungus *Aspergillus flavus* produces the aflatoxins B_1 and B_2 and *A. parasiticus* produces B_1, B_2, G_1, and

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G_2 (Figure 2), compounds **1**-**4**, respectively (*15*). Aflatoxins are considered carcinogenic and teratogenic. In addition to their food safety threat, tree nuts contaminated with aflatoxins constitute an international trade issue when exported (*9*, *15*).

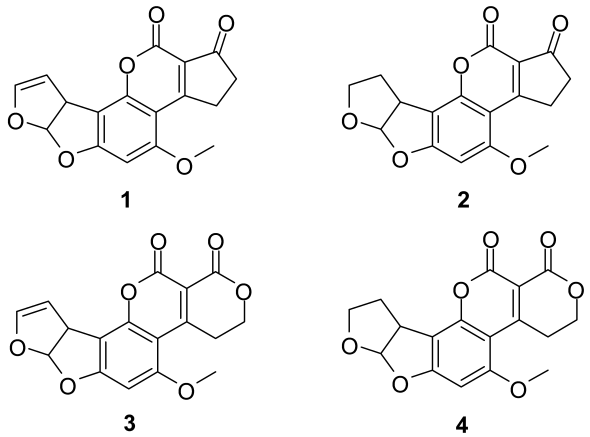

*Figure 2. Chemical structures of aflatoxins B_1, B_2, G_1, and G_2, compounds **1**-**4**, respectively.*

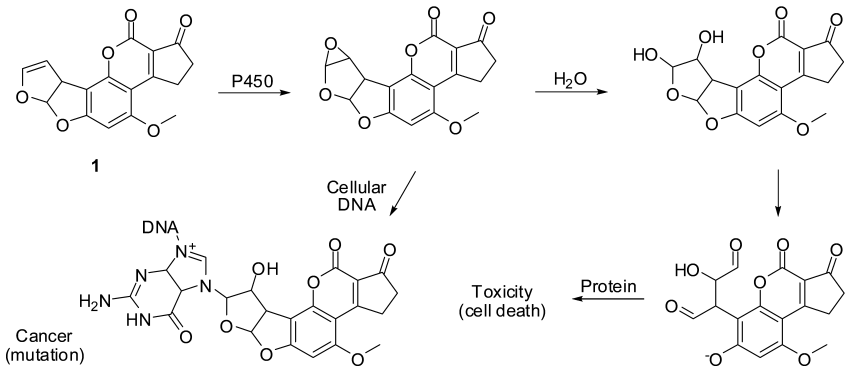

Figure 3. Mechanism of aflatoxin B_1 carcinogenicity and toxicity. (Reproduced with permission from references (17) and (18). Copyright 1998 and 2006 Elsevier.)

A mechanism for toxicity of aflatoxin has been shown to occur at the 8,9-alkene located in the furan ring of aflatoxins B_1 and G_1 (compounds **1** and **3**). Figure 3 illustrates the oxidation of this double bond by cytochrome P450 to the corresponding oxirane. The anomeric-like carbon is now highly activated toward nucleophilic attack by DNA or water. Ring opening by DNA results in the N^7-guanyl adduct which leads to mutation (*16*). An alternative pathway is oxirane

ring opening by water to form the diol, which opens further to form a dialdehyde. This dialdehyde is thought to bind with protein to form an imine adduct leading to cell death (*16*, *17*).

For several years aflatoxin contamination in almonds has been associated with feeding damage by navel orangeworm larvae (*19*, *20*). Indeed, a recent report demonstrated that navel orangeworm larvae transport the spores of *Aspergillus flavus*, thus acting as a vector for the mycotoxigenic fungus (*13*). Studies have shown the same association between navel orangeworm damage and aflatoxin contamination in pistachios (*21*). For walnuts, insect damage is assumed to be a factor for aflatoxin contamination (*9*).

Navel Orangeworm

As its name implies the navel orangeworm was originally found on navel oranges, although its geographic origin appears to be uncertain. For instance, one report from Arizona in 1922 (*22–24*) reported a new pest to oranges; however, a 1965 State of Florida Department of Agriculture document shows *Paramyelois transitella* (synonymous with *Amyelois transitella*) was first found in 1863 in the "United States, probably Florida…" (*25*). Interest in navel orangeworm in California walnuts (*26*) and almonds (*27*, *28*) appears in the literature in the late 1950s and early 1960s, respectively. These were followed by two investigations that comment on the difficulty in controlling navel orangeworm infestations (*10*, *29*). In his 1961 paper, Wade (*27*) provided nice detail of the biology of the navel orangeworm as well as its movement from southern California citrus and walnut storage areas to important fruit and nut crops in the upper Central Valley of northern California. The food safety issues, economic costs, and physical damage caused by navel orangeworm has led to numerous reports and control efforts over the years by tree nut industry, academic, and USDA-ARS researchers (*9*, *11*, *20*, *30–32*).

Host Plant Volatile Natural Products Associated with Monitoring Navel Orangeworm

Various efforts involving non-pheromonal tactics have been either investigated or implemented for control or monitoring of navel orangeworm in tree nuts – each with varying results. These efforts include either the exploration or implementation of the following: diamalt bait and terpinyl acetate in various media (*27*); pathogens of navel orangeworm (*29*, *33*, *34*); stringent orchard sanitation (*20*, *35–37*); navel orangeworm frass extracts (*38*); use of natural enemies of navel orangeworm (*39*, *40*); black light (*41*); ovipositional baits (*42*) or disruption (*43*); almond by-products (*44*); almond oil fatty acids (*45*); or, the use of the nonhost compound, phenyl propionate (*32*, *46*).

Negative results or poor performance from many of these studies prompted investigators to continue to explore other options. It was the use of almond press cake (*47*) in the early 1980s that started the more enduring utilization of almond

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parts for the monitoring of navel orangeworm (*43*, *44*, *48*). Almond press cake is "the solid…residue that remains after almond oil has been mechanically pressed or removed…" (*47*). More recently, the use of almond meal, or almond meal with small percentages of crude almond oil mixed in has been the standard tool for monitoring navel orangeworm in almond orchards (*49*). Press cake is ground to produce the almond meal (personal communication, Liberty Vegetable Oil).

There exists a lack of information regarding the chemical composition of both almond meal or press cake. Work performed in 2009 by Beck and co-workers (unpublished material) showed the majority of the headspace volatile composition of almond meal (no crude almond oil added) to be made up of several pyrazine analogues. Some of the volatiles detected during the survey of almond meal via solid phase microextraction analysis (tentative identifications for pyrazines) included limonene, methyl pyrazine (unknown isomer), 2,5-dimethyl pyrazine, 2-ethyl-5-methyl pyrazine, 3-ethyl-2,5-dimethyl pyrazine, among other alkyl pyrazines. Other compounds tentatively identified included two methyl butanol isomers, small chain alkanals, and benzaldehyde. Subsequent electroantennographic (EAG) analysis and limited field studies of a few available isomeric components (similar to related work ref. (*11*)) did not provide reason for the pyrazine compounds to be considered further as possible attractant candidates by these researchers.

Other studies have explored various host plant materials to determine the chemical composition and possible association to navel orangeworm. For instance, Buttery and co-workers (*50*) studied the chemical composition of steamed almond hulls and postulated association of similar compounds from navel oranges as having possible relation to navel orangeworm. A large number of compounds detected included alkyl aldehydes typical of fatty acid oxidation/breakdown, among others (*51*).

Another volatile investigated for its ability to attract navel orangeworm was phenyl propionate (*32*, *46*, *52*). In field trapping studies, this compound attracted navel orangeworm moths and held the interest of researchers for a number of years. However, its origin was not divulged (*52*) and thus its classification as a natural product related to navel orangeworm hosts is unsubstantiated.

In 2009 a study (*31*) using EAG analysis was used to screen a large number of volatile natural products for potential attractiveness to the navel orangeworm. The volatiles were detected *in situ* from whole almonds and were studied under the hypothesis that female navel orangeworm use the background volatiles as a way to help distinguish a site for oviposition (*53*). Based on their antennal responses during EAG bioassay a number of compounds were identified as potential candidates, however none have been demarcated as having significant navel orangeworm behavioral activity.

In 2012 a blend of volatiles based on damaged almond hulls and almonds undergoing hull split was reported to attract both male and female navel orangeworm during field trapping studies (*11*). The navel orangeworm attractant blend comprised the structurally simple natural products (±)-1-octen-3-ol (**5**), (±)-(*E*)-conophthorin (**6**), acetophenone (**7**), ethyl benzoate (**8**), and methyl salicylate (**9**) (Figure 4).

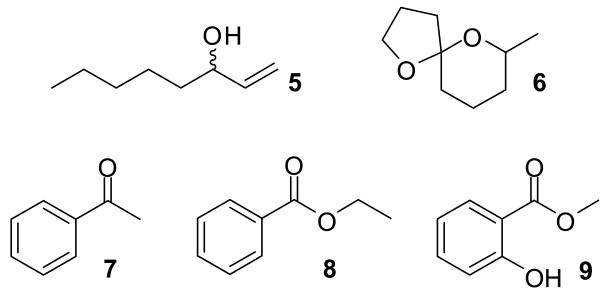

Figure 4. Chemical components of the host plant volatile blend that attracts adult navel orangeworm.

In the report, the blend of almond host plant volatiles underwent field-trapping studies over the course of a growing season (*11*). Table I shows the total number of male and female navel orangeworm moths captured in California almond and pistachio orchards in 2011 and compared to the standard for monitoring, almond meal. See also Beck et al. 2012 (*11*) for full statistical analysis of almond trap captures; Table I also provides unreported trap capture data for pistachio orchards in 2011.

Table I. Navel orangeworm moths captured in delta-sticky traps baited with a host-plant volatile blend (compounds 5-9), almond meal, and blanks in California almond and pistachio orchards in 2011

		Navel Orangeworm Moths Captured		
Orchard	***Treatment***	***Total***	***Female***	***Male***
Almond	Blend	155	59	96
	Meal	20	19	1
	Blank	2	1	1
Pistachio	Blend	32	20	12
	Meal	2	2	0
	Blank	0	0	0

A surprising result for moth captures in both almond and pistachio orchards was the relatively large number of males captured by the host plant blend. Almond meal is known for its ability to attract gravid female navel orangeworm, but is not an attractant for males. The host plant blend attracted numerically greater number of males in almonds, yet fewer males than females in pistachios. This phenomenon of male/female capture ratios is being examined further in trapping studies in both

orchards. Correspondingly, almond meal in pistachio orchards is known to become less effective in the month of June and by July the almond meal-baited egg traps are not attractive (personal observation, Bradley Higbee) to female navel orangeworm.

Four of the five components of the attractive host plant blend are derived from the volatile emissions of *in situ* almonds undergoing hull split (*11*) – the three benzenoids and the spiroketal conophthorin. The fifth component, 1-octen-3-ol, is generally considered to be a volatile associated with fungal contamination (*54*). All of the components have a history of semiochemical activity with other insects, yet none of the individual compounds elicited strong behavioral responses from navel orangeworm in field trapping studies (*11*).

Since the time of the study that reported on the host plant volatile blend's ability to attract navel orangeworm, there have been two other reports of conophthorin detected from sources other than hull split almonds (*11*) or from non-host angiosperms in relation to scolytid bark beetles (*55*). Conophthorin was recently detected from fungal spores on fatty acids (*56*) and from bacteria on varying laboratory media (*57*). These studies broaden the complexity of the origin of this particular spiroketal and add to the rich history of this natural product as a semiochemical.

Sex Pheromone Volatile Natural Products for Navel Orangeworm

Sex pheromones are important tools for monitoring and potentially controlling insect pests and need to be accurately identified and synthesized before their use as a tool to monitor or control insect populations. Sex pheromones are particularly valuable for techniques such as mating disruption, lure and kill, or mass trapping (*58*). In 1979, the major component of the sex pheromone emitted by female navel orangeworm moths was identified as (11*Z*,13*Z*)-hexadecadienal (compound **10** in Figure 5) by Coffelt and co-workers (*12*). Using their results from laboratory-based male behavioral bioassays, which demonstrated wing-fanning, orientation, and some upwind movement, Coffelt and co-workers (*59*) believed that this aldehyde would be sufficient as a monitoring lure. This supposition was supported by numerous examples in the literature that major components of lepidopteran sex pheromones were sufficiently attractive for use as a monitoring tool in various trapping schemes (*60–62*). However, efforts to develop a monitoring lure for navel orangeworm using only (11*Z*,13*Z*)-hexadecadienal were unsuccessful (*63*, *64*). It was demonstrated that relative to traps baited with virgin female moths, very few male navel orangeworm moths were captured in traps baited with synthetic (11*Z*,13*Z*)-hexadecadienal (*63*). This result suggested that additional components may be present in the natural pheromone blend produced by female moths. Additional studies that focused on purity, dosage, formulations on various substrates (e.g. rubber septa), and stabilizers confirmed that the synthetic form of (11*Z*,13*Z*)-hexadecadienal alone was so much less attractive than virgin females that ultimately its use as a field lure was not feasible (*65*).

As previously mentioned, a number of species of lepidopteran pests have been successfully managed using synthetically derived sex pheromones as mating disruptants (*65*). Since the discovery of the major sex pheromone component for navel orangeworm, the possibility of developing a management strategy based

on pheromone-mediated mating disruption has been of great interest (*66*). This reflects the importance of navel orangeworm as a pest to the almond and pistachio industries and also the shortcomings of the conventional insecticidal approach. Groups of studies over several years demonstrated that (11*Z*,13*Z*)-hexadecadienal had biological activity on males in the field; more specifically, interference of the orientation of male moths to unmated female moths used as bait (interpreted as trap shutdown) and damage reduction effects in small (1-8 ha) almond plots (*67–69*). Methods of dispensing pheromone into orchard systems can be divided into three broad groups based on the number of dispensing units and amount of pheromone emitted by each unit. In 2006, Sarfraz and co-workers (*70*) categorized formulations as microencapsulated, hand-applied and high-emission dispensers. Results were mixed for initial almond trials, which used a variety of hand-applied dispensers. Although complete trap shutdown was achieved, damage levels were unacceptable in some trials due to high levels of egg deposition by mated females within the plots, likely due to immigration of mated females from the surrounding area which was not permeated with (11*Z*,13*Z*)-hexadecadienal (*69*). Technical problems with the pheromone chemistry and release of the pheromone were also suspected to contribute to the inconsistent reduction in damage (*69*).

These difficulties remained unsolved until subsequent studies, which used larger plots (16 ha), high emission rates, and metered and timed mechanical devices (puffers) (*71*, *72*). In 1996, Shorey and Gerber (*71*) placed puffers around the perimeter of each plot and demonstrated that trap shutdown could be achieved as effectively as the more numerous hand-applied dispensers (with lower emission rates) applied throughout the smaller plots in previous trials. In the 1996 study, relatively few (5/ha) puffers rather than many (200-400/ha) passive dispensers were tested in almonds. Complete trap shutdown could be achieved in almonds, but not walnuts. The potential problems of dispersal of mated females, air movement impact on pheromone dispersion, pheromone loss through adsorption on foliage, and vertical mixing were identified as potentially interfering with the ability of navel orangeworm males to orient to females used as bait in a sticky trap. In 2008, Higbee and Burks (*72*) compared biological and damage effects in a series of experiments using 8 and 16 ha plots in almonds and pistachios. Puffers deployed peripherally, puffers gridded evenly throughout the plot, and hand-applied membrane dispensers were compared to control plots receiving no treatments in 16 ha plots. The puffers in the gridded deployment were superior to peripherally placed puffers and hand-applied dispensers on both biological (trap shutdown and suppression of mating in sentinel females placed in the center of plots) and damage reduction impacts in almonds and biological impacts in pistachios. In addition, data on estimation of release rates for the puffers and membrane release dispensers indicated that the release rate of the membrane dispensers, which is temperature dependent, was much more variable than the puffers over the season. Whereas the puffer provides a stable and protected environment for the pheromone formulation and emits pheromone only during the hours navel orangeworm are active (*73*). In these later studies, the use of larger plots was able to overcome the problems of immigration of mated females, and puffers solved the problems of pheromone instability and complete release of the pheromone formulation.

More than 25 years after the discovery of (11*Z*,13*Z*)-hexadecadienal and many attempts by chemical ecologists to discover additional navel orangeworm sex pheromone components, a combination of approaches (including molecular biology and sensory physiology) was successful in identifying a number of minor pheromone natural product components (*74*). These natural products include analogs of the major aldehydic component (compound **10**), but in different oxidation states – (11*Z*,13*Z*)-hexadecadien-1-yl acetate and ethyl-(11*Z*,13*Z*)-hexadecadienoate (compounds **18** and **21**, respectively in Figure 5), in addition to two unusual polyunsaturated hydrocarbons – (3*Z*,6*Z*,9*Z*,12*Z*,15*Z*)-tricosapentaene and (3*Z*,6*Z*,9*Z*,12*Z*,15*Z*)-pentacosapentaene (compounds **12** and **22**, respectively in Figure 5). Subsequent studies suggested that many of these minor constituents were not important in the attraction of male navel orangeworm, while two- to three of the compounds when mixed with the major component resulted in a highly attractive blend in wind-tunnel assays and field experiments (*64*, *75*). Although this blend of natural products was highly attractive in the field, this attraction was short-lived and it was suspected that degradation products and/or impurities interfered with the response of male moths.

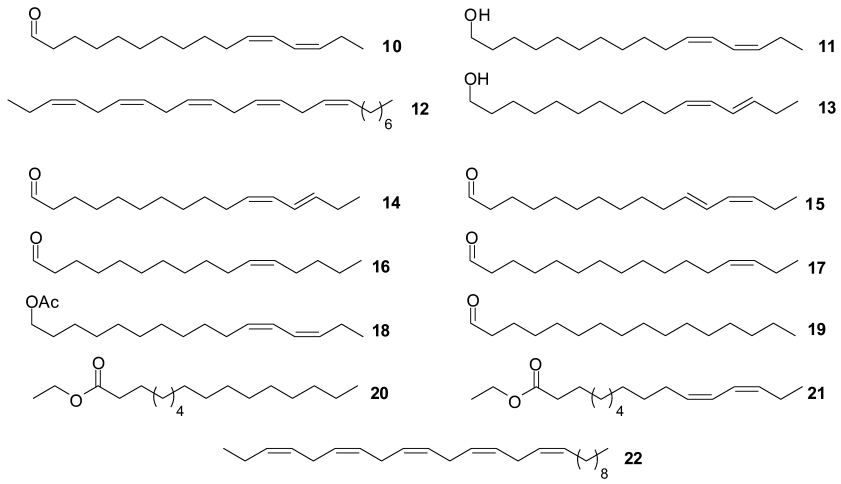

*Figure 5. Components of the current pheromone-based lure (**10-13**) and the identified female navel orangeworm sex pheromone components (**10-12, 14-22**).*

With the discovery and optimization of the complete sex pheromone blend for navel orangeworm, it seemed that an attractive lure that could be used for monitoring this pest would be immediately forthcoming. However, despite the use of stabilizers and various methods of release, such as specially treated plastic vials along with conventional rubber septa, attractiveness of lures decreased rapidly after placement in the field.

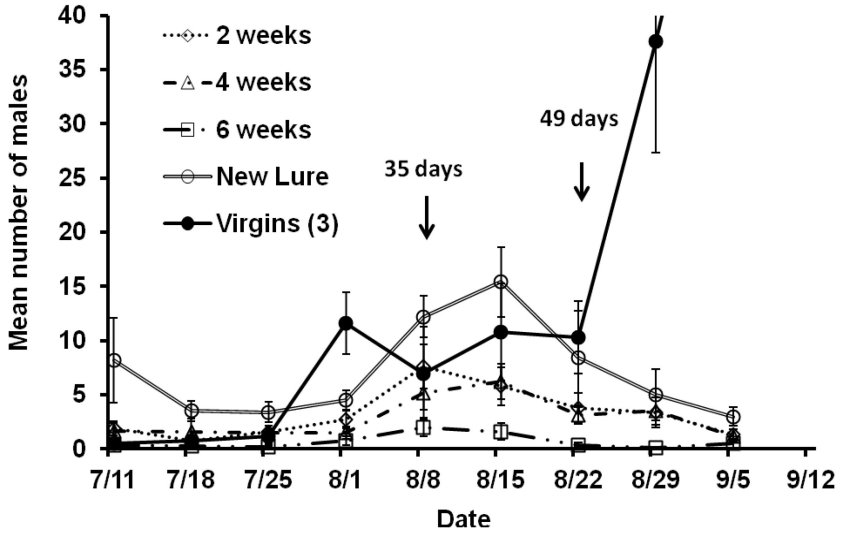

*Figure 6. Male navel orangeworm captures in delta-sticky traps baited with lures aged for 2, 4, and 6 weeks prior to deployment and compared to traps baited with fresh lures in addition to traps baited with three virgin female moths. Lures were formulated with a four-component blend of sex pheromone natural products (compounds **10**-**13**). Trials conducted by B. Higbee in Kern County, CA, 2012. Error bars represent standard errors.*

A breakthrough occurred when a four-component blend comprised of the sex pheromone natural products, (11*Z*,13*E*)-hexadecadienal, (11*Z*,13*Z*)-hexadecadien-1-ol, (3Z,6Z,9Z,12Z,15Z)-tricosapentaene, and (11Z,13E)-hexadecadien-1-ol (compounds **10**-**13**, respectively in Figure 5) was formulated with *tert*-butylhydroquinone and castor oil in a membrane system (Suterra LLC, Bend, Or). The result was a lure that lasted 4-6 weeks under field conditions and was as attractive to male navel orangeworm as virgin-baited traps in almond orchards (Figure 6; unpublished information).

In addition to the natural products mentioned above, Leal (*74*) and later Kuenen (*64*) and co-workers identified other minor components of the natural sex pheromone blend in 2010. The full suite of compounds is shown in Figure 5. While a number of the identified compounds from the natural sex pheromone mixture do not play a role in attraction of male navel orangeworm, the compound (11**Z**,13**Z**)-hexadecadien-1-yl acetate (*18*) antagonizes attraction of another Pyralidae species, the meal moth, *Pyralis farinalis* (*64*, *74*). Thus, this compound, and possibly other minor components may function as behavioral antagonists, thereby mediating interspecific interactions (*75*).

It should be mentioned that the history of the navel orangeworm and the subsequent control efforts in California orchards is plentiful and complex. Moreover, numerous researchers from industry, academia, and government laboratories have contributed vastly to this history. This current overview and further explanation of the relationship between natural products and the navel orangeworm only touches briefly on the overall history, thus it is not a comprehensive review of the chemical ecology of the navel orangeworm.

This overview of natural products and the California tree nut insect pest, navel orangeworm serves as just one example to highlight the important role volatile natural products play in chemical ecology. Moreover, this example of natural products emphasizes the critical relationship between results from laboratory-based experiments and results generated from field-based experimentation (*76*). Ongoing investigations by scientists from several disciplines continue to contribute important knowledge regarding natural products and their role in the chemical communication of navel orangeworm. Important to the California tree nut industry is what appears to be the forthcoming transfer of positive results to technology applicable to the successful monitoring of navel orangeworm.

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Chapter 6

Volatile Organic Compounds (VOCs) for Noninvasive Plant Diagnostics

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Plant systems are frequently important agriculture commodity crops, and it can be critical to track emerging pathogen infections or nutrient deficiencies that can limit or prevent food production. While some methods have been developed for in field monitoring, they are frequently invasive and not rapid. Volatile organic compounds (VOCs) are produced by all plant systems, and present a possible noninvasive window through which we can monitor plant health. Here we review different methods of VOC sampling and detection and elucidate potential of VOC monitoring for precision agriculture.

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Introduction

The volatile organic compounds (VOCs) emitted by a plant are a significant and important part of the plant's life cycle, and are reflective of the physiological status of the plant. The emission of VOCs may occur as a side effect of ongoing metabolic processes or certain chemicals could be emitted for a specific purpose, such as defense or signaling. For example, when plants are feed upon by insects or herbivores, their direct defensive response is frequently to release VOCs (*1*). These emitted volatiles are often referred to as induced VOCs (IVOCs), and these compounds can be released from the surface of the plant's leaves, fruits, and roots. It has been reported that the IVOCs play an important role in plant-to-plant communication (*2–4*), herbivore defense (*1*, *5*, *6*), and has been shown to aid resistance to biotic (biological) stress. This change in a plant's VOC profile is not necessarily only induced under biotic stresses, but can be caused by abiotic (non-biological) factors as well. There are thousands of emitted compounds, and the composition of these released VOC mixtures may relate important biochemical information of the underlying metabolic processes within the plant system (*7–9*). The expressed VOCs can be collected and measured using analytical methods to provide a momentary snapshot of the plant's health status at any given time. Therefore, measuring and interpreting the VOCs is an attractive avenue as a noninvasive and rapid way of monitoring the physiological processes in plants, including: flowering (*10*), ripening (*11*), maturation (*12*) and stress (*13*, *14*). Plant VOCs can be sampled *in situ* from whole plants, fruits, and leaves, or directly from detached plant tissues (*15*).

Due to the extreme complexity of VOC patterns emitted by plants, it is challenging to directly correlate VOC production with a specific cause. Four main aspects of this analysis need to be comprehensively addressed in order to discern specific changes in VOC patterns and attribute them to possible causes. First, the alterations in the biological state of the plant need to be recognized and attributed to some cause. Multiple biotic and abiotic causes of VOC alteration may be occurring at the same time. Nutrition status, presence of a pathogen, infestation, diurnal cycle, geographic location, plant varietal, and other factors can all have an effect on VOC production. Furthermore, various parts of the plant may be producing VOCs at different rates. For example, young flush can be expected to be more metabolically active than older foliage. While some of the factors may be not significant, others will greatly affect VOC production. Certain factors affect VOC production differently in conjunction with other conditions, such as when nutrient stressed plants are exposed to a pathogen. It is critical that the study design take all of these aspects into account, so that causality of the VOC production can be attributed to the correct factor responsible for the compounds being released.

VOC sampling is a second important aspect of noninvasive diagnostics that needs to be addressed for practical field use. Biogenic variations that are indicative of certain changes in the plant may be subtle, and the use of improper sampling techniques can introduce large error. It is also possible for a sampling method itself to bias the VOC collection towards certain compounds, which may or may not be

important in that particular plant system. Either of these cases could ultimately result in correlations of interest being obscured due to sampling variation or error.

The third aspect important to the measurements is the selection of an appropriate analytical chemistry technique employed in the VOC study. It is essential to choose an appropriate chemical detection method that enables the identification of sampled chemical differences. Ideally, detection and quantification of all of the chemicals in a very complex mixture is sought. However, due to technological limitations as well as cost and time of analysis considerations, a method that provides only limited information might be chosen. For example, some devices such as the electronic nose ("E-nose") do not identify the actual chemicals released by the plants. These devices only output a "fingerprint" pattern of the sensor response. This can be problematic if confounding chemicals are later present in unknown sample, because the sensor has rather limited chemical information. This highlights the importance of using methods that unambiguously identify the biomarker VOCs of interest.

Finally, the information provided by a sensor needs to be processed, interpreted and correlated to a specific biological status, or sets of conditions that are being studied. Often a very sophisticated data analysis is required to extricate complex correlations, especially if the signal is "noisy" or the biological variability is substantial. Many signal processing methods and algorithms have been developed to attempt this for VOC research in plants, animals and humans. In plant VOC research and biological studies, it is essential to address all the above aspects for a robust study. Failure to appropriately address any of the factors may lead to negative testing results and/or overlooked cause-effect relations. Despite the obvious challenges, great progress has been made to date. In the following sections, we will consider each of the four key factors needed for VOC detection success, discuss specific issues, and recommend future advances needed for state-of-the-art plant VOC monitoring technologies.

Existing Visual Strategies of In-Field Plant Health Assessment

There are several methods of assessing in field plant health. Visual inspection by the human eye is the most common method, while an alternative strategy is to use sensing technologies. Visual inspection and assessment of overall plant health status or specific issues in the field is called "scouting." Scouting is the most commonly used procedure for monitoring overall crop health. Scouting is done on foot, while riding on all-terrain vehicles, while standing on raised platforms mounted on a vehicle, or by a combination of these methods (*16*). Scouts identify plant stress symptoms as either abiotic or biotic. Abiotic stress symptoms include nutrient deficiencies, water stress, freeze damage, and lightning damage. Biotic stress symptoms can include bacterial diseases, viral diseases, fungal diseases, and blight. Scouts use a guide prepared by plant pathologists or field managers to identify symptoms specific to the crop being inspected. Symptoms may be located on leaves, branches, flowers, fruits or roots. Often pathogen stress can mimic nutritional stress, or result in nutritional stress. For example, the Huanglongbing disease in citrus could be easily confused with zinc deficiency as both cause leaf

blotching. The training of scouts determines their efficiency, and over time scouts develop experience in identifying plant stress symptoms.

In specialty crops, especially tree fruits, global positioning systems (GPS) are used to register the position of stressed trees for additional monitoring and disease control (*17*). Scouting is usually performed to search for incidence of diseases rather than abiotic stress conditions. Typically, the crops are well maintained with respect to nutrients and water to achieve maximum productivity. The biotic stress conditions are more unpredictable and can have greater economic impact. Scouting for plant health usually accompanies scouting for other reasons, such as presence of insects/vectors, weeds, immature fruit fall, and others. The economics of field scouting rely on the crop of interest, location of field sites, total acreage to be monitored, availability of scouts, and their experience. Sometimes, scouts are paid more based on their level of expertise. Scouting is prone to human error caused by fatigue or effects of environmental factors on human sensing system. It can also be a complex, time-consuming and expensive process, depending on the crop acreage scouted. Alternatively, minimally-invasive sensing technologies can offer a less expensive and more accurate means to rapidly determine plant stress.

Recent advancements in noninvasive sensor technologies have led to new methods of sensing plant health. The best type of sensor is one that is accurate, rapid, specific and sensitive, and will detect the early onset of plant stress symptoms (*18*). Optical sensors are probably the most utilized technique for monitoring plant health. In the field of optical sensing, several spectroscopic and imaging techniques are being investigated to create a practical tool for a large-scale, real-time, in field assessment of plant health. Examples of optical techniques include: fluorescence imaging (*19–22*), multispectral or hyperspectral imaging (*23–26*), infrared spectroscopy (*27*, *28*), fluorescence spectroscopy (*29–32*), visible/multiband spectroscopy (*33–36*), and nuclear magnetic resonance spectroscopy (*37*).

Optical sensors accurately detect specific health conditions because different regions of the electromagnetic (EM) spectrum are sensitive to certain physiological changes that occur in plants. For example, the visible region of the EM spectrum is sensitive to chlorophyll and other pigments. Unhealthy canopies reflect less radiation, while healthy canopies reflect higher radiation in the near-infrared region due to the scattering of light caused by the leaf structures. Similarly, the mid-infrared region can be used to identify the chemical signature based on the molecular vibrations of different leaf components, such as carbohydrates. Thermal infrared and terahertz frequencies work well in detecting water-related changes in plant canopies.

The desired spectral features, field-of-view and data resolution can be achieved by selecting the appropriate sensors and sensor platform that mechanically support the sensors and carry the sensors in the field. The sensor system can be ground-based or aerial in nature. Ground-based sensors can be handheld or mounted on different types of mechanical support platforms (Figure 1a-c). The selection of a suitable platform depends on the purpose of the sensor application, crop characteristics, and crop distribution. Another sensor support system is to mount the sensors on tractor components that are used for regular agricultural operations.

Figure 1. Sensor platforms. (a) Sensors mounted in front of a utility vehicle, (b) sensors mounted on a retractable mast connected to an agricultural vehicle, (c) high-lift platforms attached to a vehicle, and (d) unmanned aerial vehicle (not to scale). [Source: Reza Ehsani]

Several different types of aerial methods are used for monitoring crop health. These include use of satellites, fixed-wing planes, helicopters and unmanned aerial vehicles (UAVs). The major benefit of UAV-based systems is the ability to acquire high-resolution aerial images by adjusting the flying altitude (*23*). Different types of UAVs, also referred to as unmanned aerial systems, have been described in literature (*38–40*).

Some of the commonly used sensing techniques such as visible-near infrared spectroscopy, fluorescence spectroscopy and thermal imaging can be used to remotely sense plant health in field conditions (*41*). There are a few commercial handheld sensors available that can be utilized for agricultural applications such as the Green Seeker RT200, SPAD-502 Leaf Chlorophyll Meter, Multiplex®, Dualax®, and Crop Circle ACS-430 Active Crop Canopy Sensor. These sensors are based on visible-near infrared or fluorescence spectroscopy. The major advantage of visible-near infrared spectroscopic sensors is that they can be adapted for detection of specific stress conditions. Fluorescence spectroscopy is suitable for identifying the overall stress conditions based on fluorescence from chlorophyll and other leaf structures.

The aerial sensor platforms are often integrated with visible-near infrared multispectral imaging and thermal imaging systems, although fluorescence imaging remains challenging. The thermal imaging systems are most applicable

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for water-stress detection, although they have been applied for other stress conditions. One of the major challenges in the application of optical sensor technology is the need for proper calibration and corrections. However, the technology is constantly improving, and presently several sensors have auto-calibration or active detection. In auto-calibrated sensors, the sensors are integrated with light-sensitive sensors to calibrate the instruments for existing light conditions. Another way to compensate for varying sunlight intensity is by using a light source with the sensors. Each sensing technique has its unique advantages and limitations. For example, although mid-infrared spectroscopy offers the benefit of identifying chemical signature related to plant physiological changes, the method requires some form of sample preparation. One method to overcome challenges associated with each individual sensor could be the application of sensor fusion approach. Multiple sensors systems can be integrated to complement each other, so that one method can eliminate the limitations of the other; thus creating a robust ideal sensor system with enhanced capabilities. Another critical step in plant health monitoring is the development of pattern recognition algorithms for proper analysis and interpretation, and selecting the right sensor system along with development of robust statistical models will allow early in field detection of plant stress.

Nucleic Acid-Related Methods of Plant Disease Detection

Analytic biochemistry assays are a very important diagnostic tool in plant pathology. Enzyme-linked immunosorbent assays (ELISA) with polyclonal and monoclonal antibodies have been used for plant disease detection in vector and plant tissues (*42*). However, the lack of suitable commercial ELISA kits, the low titers and the non-homogeneous presence of systemic (vascular) pathogens in plant tissues strongly limits their application on a large scale. DNA-based techniques have been developed for the detection of a variety of pathogens in insects and plants (*1*) using DNA hybridization methods. However for woody plants, the reliability of these methods is lower when the pathogen titers are extremely low and non-homogeneously distributed. Restriction fragment length polymorphism (RFLP) analysis of the amplicons has also been employed using appropriate restriction enzymes to detect subgroup relationships, such as for the detection of Flavescence dorèe (*43*). Tremendous improvements have been made using polymerase chain reaction (PCR)-based techniques (*44*), allowing the development of universal PCR assays for the detection of many pathogen types, or specific pathogens associated with different diseases (*45*). Another current diagnosis method relies on real time PCR (RT-PCR) detection of a putative pathogen candidate, and this is now commonly used to confirm diagnosis made with less specific and sensitive methods (such as symptom visualization and ELISA). Different PCR strategies such as conventional PCR, nested PCR, and semi-quantitative PCR have been developed, although the qRT-PCR analysis (SYBR Green real time PCR and Taqman assays) are more sensitive and have been used for both qualitative results and for quantifying pathogen load. The

analysis is easy, rapid (from DNA extraction to qRT-PCR results can take a minimum of 2-3 hours), cheap (few dollars per sample), extremely sensible and with low false positive results.

However some disadvantages exist. First, qRT-PCR methods rarely identify pathogens during the early asymptomatic stage of the disease. Although PCR methods have tremendously helped diagnosis confirming visual symptomatic observations, they are not reliable at asymptomatic stage. Yet, early identification of asymptomatic plant and trees is desirable to detect primary infection sources and thus control secondary spread by infected plant removal. As occurs for other vascular-limited pathogens (*46*), long-distance, asymptomatic primary spread of plant disease are caused by pathogens with very low titers, undetectable with current real-time PCR technologies. PCR-based detection methods usually allow reliable and sensitive detection of the putative pathogen only when symptoms are present (*47*, *48*). At the asymptomatic stage, different PCR methods may also often yield contrasting results. This is due to the technology's detection limits (down to 4.6 x 10^2 L/g) and the presence of PCR inhibitors, as shown for the detection of *Candidatus liberibacter asiaticus*. A second substantial issue is related to the high mutation rates of all pathogens (especially viruses). Real time assays frequently cannot detect emerging or mutated pathogen strains, and new primers need to be continuously designed for them. A third important issue is when qRT-PCR is used to detect a systemic pathogen having non-homogenous distribution in plant organs. Sampling will greatly affect diagnostic results, and this exponentially increases the number of samples required for proper detection. This will substantially increase costs for reagents and labor, and can also lead to long turnaround times for results. Sampling different part of plant (such as different points in the foliage) can have effect on results as pathogens can be unevenly distributed, be not present in all parts of a plant or concentrated higher in some parts compared to others. Sampling errors can not be avoided in such cases. A forth disadvantage is the need for expensive lab infrastructure and operation costs due to the high costs for equipment and the need of highly-skilled personnel. Finally, every DNA-based methods would detect the pathogen without giving any information on its living status and the progression of the disease. On contrast, in vivo methods such as the analysis of plant volatile emissions are closely linked with disease status. An alternative microarray technology (lateral-flow microarray) has been used to directly detect pathogens in the orchard, although the range of sensitivity is lower and improvements in this technology are still needed.

Rapid and specific induction of messenger and small RNAs is also a potential early diagnostic biomarker for disease. This complexity can now be analyzed to an unprecedented depth using new DNA sequencing methods which reveal very rare mRNA, splice variants, allelic variants, and single-nucleotide polymorphism (SNPs). Analysis of the deep transcriptome using network theory will help define gene regulatory networks and identify key disease-specific biomarkers usable for in field sensor devices that detect not only transcripts, but also volatiles. In any biological system there is a complex relationship between genes, RNA, proteins, metabolites, and emitted volatile organic compounds (VOCs) that we expect network analysis to reveal. Changes to individual elements may trigger

an expression of others or alter signal transduction pathways, forming a complex interdependent network. Comparing interaction networks of healthy and diseased plants will identify pathways that allow identification of underlying early disease biomarkers. In the short term, pathogen biomarkers can assist plant removal decisions for infected trees. In the medium-term, this can help define pathways to target for therapeutic treatments. All plants organs (leaves, seedlings, bud, flower, fruit) are likely early biological warnings of pathogen infection, and all are a source of the biomarkers that can be monitored in order to detect pathogen at the earliest possible infection stage to limit disease spread.

Volatile Organic Compound (VOC) Sampling Methodologies

A number of different VOC sampling methodologies exist, and the most suitable method for a specific experiment should be chosen according to the biological system under investigation. In all of the existing methodologies, most sampling methods aim to minimize the number of artifacts from the environment. These methods also try to obtain the "best" VOC profiles that reflect the naturally occurring VOC distributions of the plant. These methodologies can be divided into two broad categories, namely active and passive sampling. In both cases, plant VOCs are typically transferred onto a chemically stable sorbent trap in the field, and then later returned to a laboratory for chemical analysis. Newer miniature analytical chemistry methods are allowing this to be done directly in the field, and they are still under development.

In active sampling, air is forced over the plant to an instrument sampling manifold or sorbent trap by a pump. Passive sampling relies on the diffusive motion of the VOC molecules as they come into contact with a sorbent sampling surface and are then trapped by their chemical interaction with that sorbent material. Further differences among the methodologies include the specific design of the enclosure to route the VOCs from the plan to a trap or chemical analysis tool, and types of sorbent phase that are employed. The choice of sorbent is often dictated by the chemical properties of the analyte of interest, such as polarity or molecular size. Detailed descriptions of sorbent options and information for guidance of specific sorbent selection have been previously published (*49*, *50*).

With either active or passive sampling, an enclosure is typically required to support the sorbent and to isolate the VOCs originating from the plant or specific part of the plant, such as roots or leaves, from the surrounding environment. The enclosure is normally made out of an inert material to minimize the impact of the enclosure material on the sampled VOC composition. Therefore, materials with very low VOC off-gassing such as glass or Teflon are used to avoid sample contamination. These materials also offer minimal absorptivity which reduces the changes to the local environment. Other properties of the selected materials can also be important as they affect the sampling environment in other ways. For example, Stewart-Jones and Poppy found that during dynamic sampling in direct sunlight with glass and polyester enclosures led to an increase in the internal temperature of the enclosure by an average of 7.5 °C and 5.2 °C, respectively (*51*). Such temperature increases will affect plant metabolism and alter the released

VOCs. Furthermore, both of these materials were found to release and adsorb some of the VOCs produced by plants (*51*). Commercially available enclosure devices have been successfully used to isolate parts of the plant while maintaining control over temperature and photosyntetically active radiation (PAR) during active sampling (*52*).

In addition to the pre-concentration of VOCs onto sorbents, they can be directly collected by pumping the air off the plant into a canister or bag. The sorbent or VOC-containing vessel can then be stored, and VOC analysis may be performed at a later time given the proper storage conditions of low humidity and low temperature. Pre-concentration onto a sorbent is not always necessary, as certain analytical instruments can either receive direct injections of a VOC air sample or a steady stream of sample. However, it is often necessary to increase VOC concentration levels or to interface the analyte source with an instrument operating in a pulse mode. In these cases, the preconcentration/sorption step is essential, and there are numerous types of sorbent available (*53*).

Certain sorbents are commonly used in almost all VOC analysis. These sorbents are capable of collecting a broad range of organic molecules in order to achieve the most comprehensive collection of the volatiles emitted by plants, as opposed to sorbents designed to retain certain specific analytes and discriminate against many other compounds that may comprise certain chemical backgrounds. Since the majority of the VOCs emitted by plants, such as terpenes/terpenoids, esters, long-chain aldehydes and hydrocarbons are relatively non-polar, the sorbents that most efficiently collect non-polar compounds are often used. For example, PDMS polymer sorbent was used to monitor the emissions from in vitro mechanically and herbivore-wounded plants as well as diurnal differences in volatiles emissions (*54*).

Tenax® TA is a commonly employed sorbent for biological VOC sampling. This material is a porous polymer which is primarily used for active sampling. Tenax® TA is often pre-weighed and placed in a silicone-coated thermal desorption tube, where the sorbent held in place with glass wool. Additional sorbent phases, such as Carbopack® D, may be used in order to enable collection of a wider range of VOCs. Volatiles collected on Tenax® TA are then released with thermal desorption. Tenax® TA has been successfully applied in the characterization the VOC response of birch trees to larvae and the resulting bird predation (*55*), VOC plant response to water deficit stress (*56*), and VOC emission from trees with varying resistances to aphids (*57*).

In addition to using sorbent as pre-formed pellets of powder, it can also be coated or formed onto another surface. A very popular version of this approach is the solid phase microextraction (SPME) method, which was first developed in the mid-1990s (*58*, *59*). SPME sampling is primarily used with static headspace sampling, although in some cases it was also used in dynamic sampling devices (*60*). The SPME unit consists of a fiber tip coated in a sorbent that can be retracted into a narrow case to protect the tip and minimize adsorption of extraneous chemicals during transportation and storage. Different sorbent phases and thicknesses can be applied to enable better selectivity towards different classes of analytes such as nonpolar and polar compounds. SPME have been extensively used in biological research. For example, SPME were used to study

the differences in volatiles emitted from flowers and flower organs from different sexes and cultivars of carob trees (*61*), and the differences in VOC emissions from certain species of trees in the Sierra Nevada mountains (*62*).

Another example of a sorbent-coated device is the stir bar sorptive extraction (SBSE) sampler. This device is comprised of a magnetic stir bar coated in PDMS (*63*). One key advantage of SBSE over SPME is a greater amount of sorbent coating, leading to an increased capacity to collect VOCs. The stir bar has approximately 24-100 μL sorptive capacity compared to SPME, which only has 0.5 μL capacity (*64*). While SBSE was primary designed for aqueous solutions extraction, it has also been used for static VOC sampling with thermal desorption. SBSE has been used to study VOC emissions from various biological surfaces (*65*) as well as plant volatiles specifically: plant VOCs induced and released during wounding by herbivorous arthropods (*66*), volatile triggers in plant defense response (*67*), and the relationships between wine aroma and grape development (*68*).

An important consideration in VOC sampling studies is the abundances of sampled chemicals. In certain cases, when target or matrix compounds are present in large quantities, it is possible to overload the sampling device which will result in the deterioration of data quality. Such chemical overloading may present a problem for both *in situ* VOC measurements and VOC preconcentration on sorbents with laboratory-based analysis. Possible sources of high abundance volatiles are certain plants (e.g. citrus) that produce volatiles in relatively high amounts, plant parts such as blossoming flowers, or extraneous environmental sources. In addition, physical damage to the plant may lead to extensive production of chemicals such as "green leaf volatiles", e.g. cis-3-hexenal (leaf aldehyde) and methyl salicylate. The contamination and high chemical background will lead to difficulties in data analysis and interpretation. Optimization of sampling time and selection of appropriate sorbents that allows discriminating against the prevalent background compound(s) are, therefore, necessary. In addition, thorough sorbent conditioning and baking of surfaces that were exposed to the sample are essential in order to diminish "memory" effects and reduce the consequences of overexposure.

Analytical Instrumentation for Plant VOC Detection and Biomarker Chemical Characterization

A number of analytical techniques can be used to analyze global VOC fingerprints from various plant systems (*69*). Gas chromatography mass spectrometry (GC/MS) is the most common analytical technique used to characterize plant VOCs (*70*). It is the method of choice for such studies due to the high amount of chemical information it provides. With further advances such as GCxGC (*71*), even the most complex VOC mixtures can be separated and individual chemicals can be identified. Certain types of MS instrumentation are particularly suitable for plant VOC analysis. For example, a special ion source based on proton transfer process from hydronium ions can be directly connected to an analyzing system such as quadrupole mass analyzer. This technique, called

Proton-Transfer-Reaction Mass Spectrometry (PTR-MS) (*72*) can be used for real-time monitoring of chemical composition of a (usually gaseous) sample due to a very short response time (~100 msec) of the instrument. Indeed, PTR-MS is most commonly used in VOC monitoring in ambient air in environmental and biological research (*73*). Another example of a MS technique that is particularly suitable for VOC analysis is Selected Ion Flow Tube Mass Spectrometry (SIFT-MS) (*74*). This technique is based on the chemical ionization of trace volatile compounds by selected positive precursor ions such as H_3O^+, NO^+ and O_2^+ in a flow tube connected to a mass spectrometer. SIFT-MS can be used for quantification of trace compounds in the gas phase and it has shown a great potential for use across a wide variety of fields, particularly in the life sciences, such as biology and medicine (*75*). Other techniques such as nuclear magnetic resonance (NMR) and liquid chromatography mass spectrometry (LC/MS) are commonly used to analyze the semi-volatile and higher molecular weight plant metabolites (*76–80*).

Both MS- and NMR-based methods are capable of providing the most comprehensive chemical information, but possess several drawbacks. First, all of these methods require time-consuming sample preparation and handling. Even though currently there are some available options for portable in field mass spectrometers (*81*), the MS analysis is almost exclusively done in laboratory conditions. Consequently, the analysis is very expensive, and requires complex and bulky equipment that needs to be operated by highly skilled personnel. This, in turn, makes the MS and NMR methods impractical for routine applications suitable for users outside communities of researchers in universities or designated facilities.

On the opposite side of the analytical information and metabolite content that a method measures, there is a family of lower-resolution VOC detection methods named the electronic nose or "E-nose" (*82*, *83*). E-nose devices are comprised of an array of partially-selective chemical elements that are sensitive to various types of chemical compounds (similarly to odor receptors in biological olfactory systems) (*84–88*). E-nose devices are not capable of identifying and quantifying specific compounds, but instead provide the overall profile of a VOC mixture as a "fingerprint". If the device is "tuned" to perform a specific discrimination task, it can distinguish among varying VOC signatures from different samples (*89–92*). Successful applications of E-nose to plant status monitoring were reported (*93*, *94*). Compared to MS and other bench-top methods, the E-nose technology is rapid, inexpensive and easy-to-use as it does not require pre-concentration and sample preparation. Unfortunately, it has performance issues such as poor reproducibility, signal drift and difficulty in correlating E-nose responses with other analytical sensors outputs. This has limited their wide-spread use at the present time.

A family of ion mobility-based methods is positioned somewhere in between mass spectrometry and the E-nose methods, in terms of ease-of-use and information provided by the sensor output. An example of such technology is high Field Asymmetric waveform Ion Mobility Spectrometry (FAIMS), which is also called Differential Mobility Spectrometry (DMS). DMS is a gas phase separation and detection technique. It operates by exploiting the non-linear behavior of charged ions at rapidly alternating high and low electric fields to induce separation

and subsequent detection of chemical species (*95*). DMS operates at ambient pressure with routine sensitivity at the parts-per-billion (ppb) level. It has low power consumption, a small size and has the potential for further miniaturization (*95*, *96*). At the same time, specific analytes can be identified by DMS if standards are run prior to field experiments, and quantitative measurements of chemical abundances are also feasible, sometimes down to even the parts-per-trillion (ppt) levels. Furthermore, hyphenation with gas chromatography (GC) provides additional chromatographic separation and further enhances the method's analytical capabilities. For example, GC/DMS has been applied to characterize and distinguish volatile compounds emitted from peel sections of normal healthy citrus fruit and those with citrus "puff" disorder (*97*) and trees affected by citrus greening (*98*). DMS is a relatively new analytical technique, but it already has been extensively applied to characterization of VOCs in complex systems, such as discrimination of proliferating bacterial samples (*99*).

Data Analysis and Signal Processing Approaches

Data analysis is one of the most important steps in determining chemical VOC biomarkers of significance in plant and biological systems. As discussed previously, the VOC mixtures emitted from plants are extremely complex and vary greatly in composition and abundance (*100*). Therefore, one of the main challenges is the high dimensionality of data sets (*82*, *101–105*). It is also important to note approaches that may be used to numerically analyze the data from different types of analytical instrumentation in order to attribute biomarker chemical identities to specific biological processes, such as a disease or disorder.

Gutierrez-Osuna (*82*) describes the steps to be considered for the pattern analysis which could potentially be extended to many VOC analysis data types. The process consists in four main steps: signal preprocessing, normalization and dimensionality reduction, analysis, and validation.

Data analysis is divided in two main categories: supervised and unsupervised methods. Unsupervised methods refer to finding a hidden structure in unlabeled data while supervised methods relate to finding a mathematical transformation which correlates the data with their respective classes or categories. The supervised methods, in turn, are split into two groups: (i) explanatory, where the mathematical transformation performed by the algorithm is transparent; or (ii) inductive, where the aim is to find which metabolites are most important in separating different classes.

Signal Preprocessing

A preprocessing step is the application of different compression algorithms to obtain information regarding the change in the signal. As discussed previously, GC/MS and the E-nose are two of the most commonly employed analytical

chemistry techniques used to analyze plant volatiles, both in the laboratory and in the field. Each of these tools also produces different types of data outputs that are unique to the instrument used. Since signal preprocessing is specific to a given type of data structure, variations of traditional mathematical analysis are applied to the data from these instruments.

The signal from an E-nose sensor, either custom-built or commercially available, is often a time-varying response. The signal is comprised of a transient and steady state signal where the waveform changes according to presence of measured VOCs and their concentrations. The main signal preprocessing issues that need to be addressed are a baseline correction and compensation for sensor drift. Three types of baseline correction can be applied to E-nose signals: (i) difference correction, which is used to eliminate additive noise by subtracting the baseline; (ii) relative correction, which normalizes the signal by dividing by the baseline with the objective of removing multiplicative drift; and (iii) fractional correction, which is a combination between difference and relative corrections where a dimensionless and normalized response is obtained. There are some algorithms dedicated to extracting the information from the steady state response. However, in the last decade, the focus has shifted to evaluation of the transient response (*106–108*).

Preprocessing of GC/MS data is a critical procedure required to remove non-relevant information and to resolve overlapping peaks. In general, there are several main steps involved in GC/MS preprocessing (*109*): noise removal, peak deconvolution and identification, and chromatogram alignment. For GC/MS noise removal, different approaches have been developed, such as: median filtering (*110*); filtering based on a polynomial regression (*111*); wavelet transformation (*112*, *113*); and the most popular, moving window filters (*114*).

After noise removal, a GC/MS signal is deconvolved to discern two or more coeluting (overlapping) components in the GC/MS data. The most popular software used for GC/MS deconvolution is called Automated Mass Spectral Deconvolution and Identification system (AMDIS) (*115*). AMDIS extracts qualitative and quantitative information such as peak height, an estimation of the total abundance of each deconvoluted compound, and identification of each compound using mass spectroscopic libraries such as NIST (National Institute of Standards and Technology Gaithersburg, MD, USA).

Normalization and Dimensionally Reduction

Normalization is the next step of VOC data analysis that should be applied after preprocessing and independently of the analytical technique used for data collection. Normalization procedures compensate for sample-to-sample variation caused by analyte concentration differences and/or ensure that the sensors output signal magnitudes are comparable within a data set. Normalization can frequently be carried out by correcting the signal using a single or multiple internal standards spiked into the sample (*116*), but this is not always feasible when analyzing ambient gas phase VOCs in field conditions.

In addition to normalizing the data, frequently dimensionality reduction techniques are applied (*117*) to remove irrelevant information (noise) thus enhancing the classification/regression performance. This also provides easier data interpretation, while improving computational and data storage costs. However, dimensionality reduction may lead to loss important features/information. There are especially salient concerns for VOC analysis, because we ultimately desire to know the identity of the biomarkers from our studies. To deal with this, there are two approaches that can be applied to VOC metabolite data: (i) feature extraction, and (ii) feature selection.

The aim of feature extraction is to extract important biological signal features via mathematical transformations of the original data vector. Principal Component Analysis (PCA) (*118*) is the most widely used technique for this process. It is an unsupervised method that is mostly used as a representation technique, and it projects the original data into a subspace along the directions of maximum variance. As this technique does not use prior information regarding data classes to generate a model, a supervised method needs to be used to validate discrimination of the data classes (e.g. health status). PCA is optimal under unimodal Gaussian assumptions or normally distributed systems. The new projected dimensions are called scores and loadings. The scores are a projection of each sample over the new subspace and the loadings represent the variables projected over the new subspace. Therefore, if any discrimination is found in the scores, the loadings will represent which variables in the data are responsible.

Linear Discriminant Analysis (LDA) or Fisher Discriminant Analysis (*118*) is also a supervised method which maximizes class separation in one dimension. LDA assumes unimodal Gaussian classes with different mean and class-covariance. However, LDA as dimension reduction technique requires that the number of samples must be at least 3 times greater than the number of features to avoid overfitting. Furthermore, LDA may fail if discriminating information is in the variance of the data but not in the mean. There are some variations of LDA such as non-parametric LDA (*119*), Orthonormal LDA (*120*), and Generalized LDA (*121*). Sammon's maps (*122*, *123*), multilayer perceptrons (*124*), Kohonen self-organizing maps (*125*), Kernel PCA (*126*, *127*), projection pursuit (*128*) and Independent Component Analysis (*129*) are used when the data are not normally distributed. Multivariate orthogonal projection methods (OP) aim to reduce the original data to a new subspace in which the new dimensions represent maximum variance related or unrelated to the information of interest. There are different techniques used to achieve this objective, and the final purpose of these algorithms is to improve the data and minimize the influence of non-desired variation in the data.

The main objective of *feature selection* (FS) is to find "the best" subset of VOC signal features maximizing prediction accuracy for a given plant data set. FSS evaluates the whole space of possibilities combining different features, either individually or by feature sets to determine the optimal feature combination. The simplest algorithm is an exhaustive search where all possibilities are evaluated, but this approach is time consuming and carries a high computational cost. Exponential, sequential and randomized search strategies are designed to explore the feature space more efficiently.

Data Interpretation and Analysis Methods

Once the complex VOC data sets from the plant systems have been simplified and normalized, various approaches can be applied to interpret the meaning of the data. Typically, these rely on having enough raw data from the biological experiments to divide it into two categories: a training data set to build models of the data, and a testing data set to understand accuracy of the models that we generate of the biological response to disease or disorder.

Classification Methods

Quadratic Classifiers (QC) (*82*, *118*) are a simple way to separate measurements of two or more classes. K nearest neighbor classifiers (k-NNs) (*130–132*) are also powerful techniques for classifying VOC data sets based on the *k* closest examples in a training dataset. Even though k-NN generates highly nonlinear classifications with limited data, it is very sensitive to non-scaled data. Therefore, the selection of the *k* neighbors is crucial to avoid local boundaries and misinterpretation of the data. Multilayer Perceptron Classifiers (MLPs) (*133–135*) are the most popular example of artificial neural network (ANN). The Support Vector Machine (SVM) technique (*136*) has also been successfully used in different metabolomics fields. Although SVM is mainly used to solve classifying problems, recently their applications were extended to solving regression problems and density estimations. Partial Least Squares Discriminant Analysis (PLSDA) (*137*, *138*) is especially powerful when dealing with high-dimensional data which are frequently obtained in VOC metabolite monitoring.

Regression Methods

Ordinary Least Squares (OLS) (*139*) is the simplest method to perform regression. OLS assumes that the dependent variable Y (such as the biomarker VOC analyte concentration) can be predicted from a linear combination of the independent variables X (the sensor response for a biological condition). It assumes that X and Y are mean-centered and the covariance matrix of Y is not singular. Partial Least Squares (*140*, *141*) is the most used method in calibration problems due to its ability to handle collinear data and reduce the number of calibration observations. PLS maximizes the correlation between X and Y in sequential steps. PLS attempts to find new factors called latent variables which are linear combinations of X. The first latent variable is a projection of X along the eigenvector Y. Then the subsequent latent variables are defined by regression between X and the current PLS latent variable. This procedure is repeated until a criterion, such as a pre-set number of latent variables, is met.

Clustering Methods

Three main steps are involved in clustering algorithms (*142*, *143*): (i) definition of a pattern proximity or distance metric measure between observations, typically Euclidean distance or Mahalanobis distance, (ii) optimization of clustering or grouping criterion, and (iii) data abstraction or/and data assessment.

Hierarchical Clustering methods (*144–147*) use a tree structure called a dendogram which represents the biological states produced by hierarchical clustering. C-Means (*148–150*) is another one of the simplest methods to solve clustering problems. This algorithm attempts to classify a data set into C disjointed clusters (number C is defined prior). Self-Organizing Maps (SOM) (*151*, *152*) are the most popular neural network models. SOM performs data clustering without knowing the class membership of the input data

Validation of Data Analysis

The validation procedure is one of the most important steps in the data analysis process used to attribute VOC biomarkers to specific biological states. The validation methodologies assess how well a statistical analysis of metabolite data (classification/prediction) will generalize to an independent data set. The simplest approach is frequently called the "hold out" strategy. The original raw data is separated into a validation set with 30% of the original raw data and an initial training set with the rest of the data from a study. This break down works well when the number of replicate biological samples is sufficiently greater than the number of variables in the biological question being studied.

One of the potential problems with this approach is the possibility of a non-uniform distribution of the data that can lead to misleading results. Certain variations of the "hold out" method are designed to overcome such problems. In the K-fold cross-validation method, the data are split into K partitions, with each partition used for both training and validation set. Another method is random subsampling cross-validation. This method is similar to K-fold cross-validation, but in this case each split is chosen randomly. When the number of folds is equal to number of samples, the method is called "leave-one-out." This method is also valid when the number of samples is small. In these three cases, the performance of the model is calculated by the average over the K data partitions. The K value could be critical: when K is large, the variance across partitions will be large and the bias of the estimate will be small making the final prediction overly optimistic. On the other hand, if K is small, the bias will be large and the variance of the estimate will be small, therefore the final relationship we infer may be underestimated.

Concluding Remarks and Future Trends

In the present chapter, we have reviewed approaches to monitor and measure plant health via emitted volatile organic compounds (VOCs). Compared with traditional methods and visual inspection methods, these techniques

have great promise, although there is still much work to be done. Major advances in our understanding of plant physiology coupled with developments in analytical instrumentation and sampling techniques have continuously pushed the boundaries of these VOC-based diagnostic possibilities. While many examples of successful applications of plant VOC analysis in biological, ecological studies and agricultural applications are given, the full potential of VOC monitoring in plants has not yet been realized.

Ultimately, we envision VOC-based agriculture sensing that can be performed on plants in the field to interpret a chemical "signature" that reflects the biological/physiological status of the entire organism. This could include information on the presence of a pathogenic infection, changes in nutrition status, or any other relevant information about plant's health status. In turn, this information can be used to guide decisions for actions such as plant removal, pesticide spray levels, watering, or administering fertilizer and nutrients. Apart from being reliable, fast, reasonably inexpensive and environmentally safe, such VOC monitoring also needs to be amenable for monitoring of large acreages. The output of VOC sensors needs to be easily interpretable by non-scientists which would allow growers to take advantage of technology directly, and not rely on scientific personnel.

At the present time, documenting the baseline variability of VOCs that emanate from plants is still a challenge. It is also important to understand how much diversity exists between commonly cultivated varietals of the same plant. Also, we still need to investigate how factors such as weather, soil quality, and geographic location will affect the VOC output. A part of the challenge in establishing such a baseline is a lack of standardization of sampling procedures for VOCs in the field. Concerted research efforts and standardization of sampling protocols by the community of dedicated plant VOC researcher may ultimately lead to further breakthroughs in our understanding of the plants' "chemical language" of VOC production and signaling.

Also, great advances have been made in analytical instrumentation for complex VOC analysis. However, there is still a lack of instrumentation immediately suitable to assess and monitor the plant VOCs directly in field conditions. Current in field sampling and analysis approaches are still somewhat rudimentary and often have many manual steps. Consequently, laboratory-based analytical chemistry equipment is often heavily relied upon. A miniature, portable, easy-to-use VOC detection system that is highly sensitive and capable of quantifying the amount of specific VOCs that are released from plants is not yet widely available. At the same time, the application of advanced data analysis in conjunction with the constant stream of improvements in experimental design and more robust equipment will eventually lead to VOC analysis taking its rightful place as one of the most important tools at the disposal of growers around the world. Finally, understanding of VOC production in the larger framework of biochemical pathways will further advance the field. A host of applications beyond precision agriculture may also be possible. These applications may include, but are certainly not limited to: pest management, post-harvest monitoring, biodiversity and ecology studies. For example, an opportunity to find and study new and interesting endophytes in situ would be

fascinating from a basic science perspective (*153*). The full potential of plant VOC monitoring and the realm of future applications are still yet to be realized.

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Chapter 7

Quantitative Structure-Activity Relationships (QSARs) of Monoterpenoids at an Expressed American Cockroach Octopamine Receptor

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Monoterpenoids are found in essential oils from numerous plant families. Octopamine is a biogenic monoamine found within various invertebrates, including insects. Octopamine exerts its physiological effects through the activation of G-protein-coupled receptors (GPCRs). GPCRs are an under-utilized receptor target in the agrochemical industry. Here we report the expression of an octopamine receptor from the brain of the American cockroach (*Periplaneta americana*) in *Saccharomyces cerevisiae,* creating a ligand-independent histidine-auxotrophic assay. The majority of monoterpenoids acted as an inverse agonist in this system. Three QSAR models show that electronic properties are most important for monoterpenoid interaction with this octopamine receptor in this yeast assay.

Introduction

Concern about the adverse health and environmental effects of conventional synthetic insecticides is evident through governmental restrictions, like the Food Quality Protection Act of 1996, limiting the availability of traditional synthetic insecticides. Therefore there is the need to identify safe but effective

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compounds to control insect pests. Natural products, like essential oils and essential oil components, are becoming a valuable source for lead compounds for insecticide development. Essential oils have been known since the Middle Ages for their antibacterial properties. Essential oils can be derived from multiple tissues of plants by steam or hydro-distillation (*1*). Essential oils are complex mixtures, primarily composed of terpenoids (mono- and sesquiterpenoids) at various concentrations. The complexity of the terpenoid mixture is enhanced by the presence of various functional groups, stereochemistry, and carbon skeletal structures (*1*). The toxicity of essentials oils and their terpenoid constituents to insects has been the focus of several studies (*2–4*). While these terpenoids have been shown to be toxic to insects, their precise mechanism of how they exert this toxic action is not fully understood. Various studies have indicated that monoterpenoids may have several mechanisms of action. Mechanisms that have been reported include: inhibition of acetylcholinesterase (*5*, *6*), binding at the GABA receptor (*7–11*), binding at the nicotinic acetylcholine receptor (*12*), the octopamine receptor (*13*, *14*), and the tyramine receptor (*15*). Previous studies have also described quantitative structure-activity relationships (QSARs) to describe the toxicity of monoterpenoids and their interaction at the GABA receptor (*11*).

Octopamine is found in numerous invertebrates and functions as a neurohormone, neurotransmitter, and neuromodulator. Octopamine has been shown to have numerous physiological actions in the insects' nervous system and several peripheral target sites. Octopamine in insects is believed to be comparable to norepinephrine in vertebrates. This is because of its similarities in its chemical structure, but also its physiological action (*16–18*). An octopamine receptor has previously been isolated and characterized from the American cockroach, *Periplaneta americana* (Pa oa1) and was used to describe monoterpenoid interactions here (*19*).

GPCRs have been studied for their possible involvement in human disease and as targets for pharmaceutical intervention. It is estimated that 30-45% of current pharmaceuticals target GPCRs (*20*). However, GPCRs have been an under-utilized target in the agrochemical industry. *Saccharomyces cerevisiae*, referred to as yeast hereafter, has emerged as an important organism for the study of heterologously expressed GPCRs (*21*, *22*). Functional expression of heterotrimeric GPCRs can be achieved by linking the expressed receptor to the endogenous pheromone response pathway, which has been performed for analysis of multiple mammalian GPCRs (*22*) and some invertebrate GPCRs (*23–25*). Previously, we have reported the expression of Pa oa1 in yeast, which resulted in a ligand-independent (constitutive) expression system (*25*). Constitutively expressed GPCRs can still yield important results about ligands interacting with the expressed receptor (*26*, *27*). For instance, constitutively active expression of GPCRs can identify compounds that act as inverse agonists or potentially as allosteric modulators (*27*). A constitutively expressed system can show the possibility of a compound interacting with a receptor resulting in various conformations of activation or inactivation. Here we expand on previously screened monoterpenoids against Pa oa1 expressed in yeast to prescribe physicochemical properties that are important for this interaction.

Materials and Methods

Insects

American cockroaches (*P. americana*) were maintained in an established colony on a 14:10 light:dark photocycle at 23 ± 2°C. These insects were provided with an unlimited supply of dry cat food and water.

Chemicals

All monoterpenoids and related aromatic compounds were purchased from Sigma (St. Louis, MO) with the exception of pulegone, which was purchased from Eastman Chemical Company (Miami, FL). For screening purposes the compounds were dissolved in certified dimethyl sulfoxide (DMSO) and serially diluted to a screening concentration of 1 x 10^{-4} M. The final concentration of DMSO to which the cells were exposed was less than 1%.

Isolation and Functional Expression of Pa oa1

The isolation and expression of the American cockroach octopamine receptor Pa oa1, was performed as previously described (*25*). Briefly, RNA was extracted from adult American cockroaches. cDNA was prepared with gene-specific primers based on the previous sequence of Pa oa1 (*19*). *NcoI* and *XbaI* restriction sites were added to the Pa oa1 open reading frame and amplified. Amplicons were ligated into the yeast expression vector, Cp4258. Yeast cells were transformed using a lithium acetate method. Specifically, yeast cell line CY 14083 (genotype: MATα PFUS1-HIS3 GPA1-Gαo(5) can1 far1Δ1442 his3 leu2 lys2 sst2Δ2 ste14::trp1::LYS2 ste18γ6-3841 Ste3Δ1156 tbt1-1 trp1 ura3) was transformed with Cp4258 carrying the Pa oa1 open reading frame (yeast cells were kindly provided by J. Broach, Princeton University). Comparisons were made to mock-transfected yeast cells that had the expression vector, Cp4258, but lacking Pa oa1. Cp4258 contains a constitutively active leucine gene, which allows for selection of yeast cells transformed with the appropriate vector.

Histidine-Auxotrophic Assay

The histidine-auxotrophic assay is the expression of Pa oa1, or an exogenous GPCR, which couples to the yeast's endogenous pheromone-response pathway (the yeast's reproductive system). This is a modified auxotorophic yeast strain, which carries a *His3* reporter gene under transcriptional control of the pheromone-responsive *Fus1* promoter. *His3* expression results in the synthesis of histidine. Therefore, when the receptor is in the active state, histidine will be produced, and yeast cells will grow when they are present in histidine-deficient medium. The histidine-auxotrophic assay was performed similarly to previous reports from our laboratories (*23*, *25*).Briefly, 2 mL of selective medium (-Leu) was inoculated with transformed yeast cells and allowed to grow overnight on an orbital shaker (30°C and 250 RPM (OD_{600} 1.0-2.0)). Cells were pelleted at 5,000 x *g* at room temperature and washed three times with medium which was deficient in leucine

and histidine. The pellet was resuspended in 1 mL of leucine/histidine-deficient media supplemented with 10 mM 3-amino-1,2,4-triazole (3-AT;Sigma) to help control growth by interfering with histidine synthesis. Cells were dispensed into a 96-well clear Costar® plate with a total volume of 200 µL at 15-20 cells/µL (OD_{600} of 0.01), which included 10 µL of vehicle or test compound solution. Cells were allowed to grow at 30°C and 98% humidity for 24 hr at which time optical density readings (absorbance of 600 nm) were taken using a Spectramax 190 (Molecular Devices, Inc. Sunnyvale, CA). Experiments were performed in quadruplicate on 96-well plates for one experiment, with a total of five experiments performed for statistical analysis.

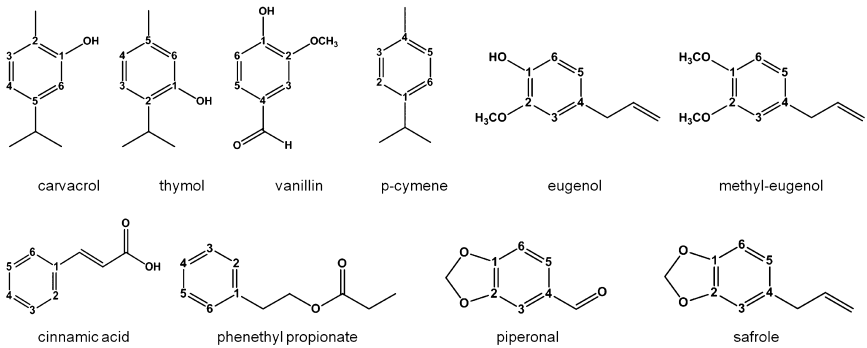

Figure 1. This figure shows numbering of the aromatic monoterpenoids used in the yeast assay and used for Model 1.

QSAR Calculations and Analysis

Descriptors were chosen to measure classical and semi-empirical quantum parameters. Descriptors included the water-octanol partition coefficient (*Log P*), lowest unoccupied molecular orbital (LUMO), highest occupied molecular orbital (HOMO), dipole moment, Mulliken charge, Lwdin charge, and polarizability. Calculations were performed using GAMESS interfaced with ChemBio3D Ultra 12. (Cambridge Software Corporation, Cambridge MA). The energy and geometry of candidate monoterpenoids were optimized and analyzed using a split valence basis set and a polarization function (6-31*d). Electrotopological state of candidate monoterpenoids was calculated with E-Calc (Scivision INC., Burlington, MA). Prior to calculation of selected descriptors, six of the monoterpenoid carbons were numbered, and this was focused on a six-member ring (Figure 1 and Figure 2). In aromatic monoterpenoids, the six carbons of the aromatic ring were numbered (Figure 1). In acyclic monoterpenoids, carbon skeleton structures were drawn in a conformation that gave a structure similar to cyclic monoterpenoids, and carbons were numbered 1-6 (Figure 2). Carbon numbering was based on substituents. For monoterpenoids that did not contain a

heteroatom (oxygen), the lowest number was assigned to the largest substituent (i.e. propyl versus methyl). The next lowest number was given to the carbon bonded to the next closest substituent; priority was always given to the next largest substituent, if applicable. A monoterpenoid that contained a heteroatom that was directly attached to one of the six numbered carbon atoms was given a lower number than a heteroatom attached to a carbon that was not directly numbered 1-6 (carbonyl). In compounds that contained two heteroatoms, a lower number was given to a hydroxyl versus an ether.

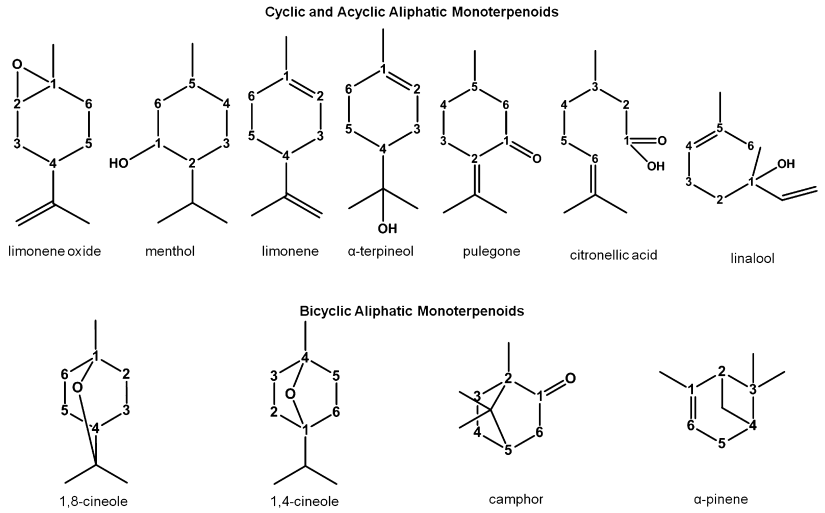

Figure 2. This figure shows the numbering of the aliphatic acyclic, cyclic, and bicyclic monoterpenoids used in this assay to produce Model 2 and Model 3.

Selected descriptors and the growth results, log transformed, from the ligand-independent yeast histidine-auxotrophic assay were analyzed with simple linear and multiple linear regressions. The square of the correlation coefficient (R^2) of ≥ 0.8 was required to describe activity. Regression models were validated using the leave-one-out method (Q^2), shown in the equation below. Simple and multiple linear regression models that had an R^2 of ≥ 0.8 and a Q^2 of ≥ 0.6 were suggested to have a non-random relationship (*28*).

$$Q^2 = 1 - \frac{PRESS}{SSTO}$$

where,

$$PRESS = \sum_Y (Y_{predicted} - Y_{actual})^2$$

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Statistical Analysis

Statistical analysis of data obtained from the yeast growth assay was obtained using Analysis of Variance (ANOVA) with α=0.05, using SAS 9.2 (SAS Institute Inc., Cary, NC). Yeast growth was normalized with respect to the vehicle control, and log transformations were performed to achieve an accurate fit model. Linear and multiple regression models were obtained with SAS 9.2.

Results

Yeast Histidine-Auxotrophic Assay

Monoterpenoid activity at Pa oa1 was determined using a histidine-auxotrophic yeast functional expression assay. However, expression of Pa oa1 in a modified auxotrophic yeast strain CY 14083 resulted in a 35-fold increase in growth over yeast cells not expressing Pa oa1 (*25*). When cells expressing Pa oa1 were exposed to octopamine and its immediate synthetic precursor, tyramine, there was not a significant result from the vehicle (Table 1). However, three octopaminergic compounds, phentolamine, synephrine, and chlordimeform, resulted in decreases in yeast growth (Table 1).

Table 1. The effect of octopaminergic compounds on yeast growth by using a yeast expression of a heterologous Pa oa1. Growth that was significantly affected is shown in bold with an asterisk (ANOVA, α = 0.05).

	Octopaminergics
Compound	***% Yeast growth ± SEM***
Vehicle (control)	100%
Octopamine	99 ± 2%
Tyramine	96 ± 4%
Phentolamine	**63 ± 2% ***
Synephrine	**65 ± 3% ***
Chlordimeform	**76 ± 2% ***

The expressed Pa oa1 was screened against 21 monoterpenoids with various effects on its activity (Table 2 and Table 3). In the ligand-independent system, all of the aliphatic and most of the aromatic monoterpenoids acted as inverse agonists. Only one of the tested monoterpenoids, carvacrol, resulted in an increase in yeast growth (Table 2).

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Table 2. The effect of aromatic monoterpenoids on yeast growth by using a yeast expression of a heterologous Pa oa1. Growth that was significantly affected is shown in bold with an asterisk (ANOVA, α = 0.05).

Aromatic Monoterpenoids	
Compound	***% Yeast growth ± SEM***
vehicle (control)	100%
carvacrol	**130 ± 7%***
Thymol	103 ± 2%
Safrole	**93 ± 3%***
Cymene	**80 ± 3%***
phenethyl propionate	**80 ± 4%***
cinnamic acid	**65 ± 4%***
Eugenol	**30 ± 2%***
Methyl eugenol	**17 ± 10%***

MODEL 1

$$Y = 1.55(\pm 0.23) + 7.56\ (\pm 1.00)[\mathrm{MCC3}] + 0.72(\pm 0.24)[\mathrm{MCC1}] - 0.12(\pm 0.02)[\mathrm{DM}]$$

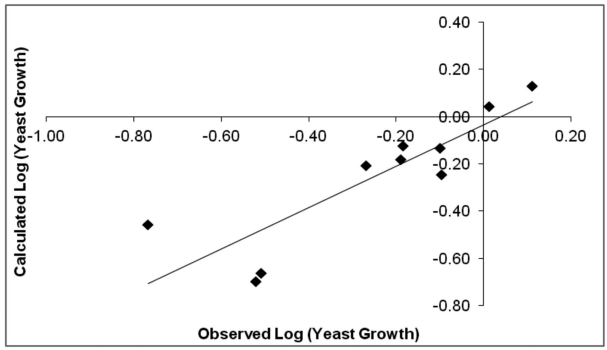

Figure 3. This figure shows the observed log of yeast growth versus the calculated log of yeast growth for the aromatic monoterpenoids. It resulted in Model 1: Y = 1.55(±0.23) + 7.56 (±1.00) [MCC3] + 0.72 (±0.24) [MCC1] – 0.12 (±0.02)[DM] with an R^2 = 0.9109, F=20.45, and a Q^2 = 0.8582.

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QSAR Analysis and Models

The biological data along with the output calculations of physicochemical properties allowed for simple and multiple linear regressions to be performed. Initially, all screened terpenoids (aromatic, cyclic, acyclic, and bicyclic) were placed in the same training set; however, it did not result in an $R^2 > 0.8$ or $Q^2 > 0.6$, which were the preset guidelines. Therefore, an aromatic monoterpenoid (Figure 1) training set was used to identify a model that predicts growth activity as a result of aromatic monoterpenoids interacting with the expressed Pa oa1. A multiple linear regression model (Model 1), within the identified limits ($R^2 \geq 0.8$ and $Q^2 \geq 0.6$), for 10 aromatic monoterpenoids (Figure 1) was determined (Model 1, Figure 3, Table 4). Model 1 resulted in an R^2 of 0.9109, F=20.45, and a Q^2 of 0.8582. Model 1 shows that an increase in the Mulliken charge at carbon-3 (MCC3) and the Mulliken charge at carbon-1 (MCC1) (indicating an increase in electron density at these two carbons) cause a greater interaction with expressed Pa oa1. Furthermore, a decrease in the dipole moment (DM) of the aromatic monoterpenoids is also important to the interaction of aromatic monoterpenoids with expressed Pa oa1.

Table 3. The effect of aliphatic monoterpenoids on yeast growth by using a yeast expression of a heterologous Pa oa1. Growth that was significantly affected is shown in bold with an asterisk (ANOVA, α = 0.05).

Aliphatic Monoterpenoids	
Compound	***% Yeast growth ± SEM***
vehicle (control)	100%
limonene oxide	99 ± 3%
α-terpineol	92 ± 5%
linalool	91 ± 2%
1,8-cineole	89 ± 5%
1,4-cineole	**78 ± 6%***
citronellic acid	**74 ± 4%***
pulegone	**73 ± 2%***
limonene	**68 ± 5%***
camphor	**56 ± 4%***

Data from the 11 aliphatic monoterpenoids (Figure 2) resulted in a multiple linear regression model (Model 2 Figure 4, Table 5). This multiple linear regression model was within the identified model limits ($R^2 \geq 0.8$ and $Q^2 \geq 0.6$)

for all of the aliphatic compounds. Model 2 resulted in an $R^2 = 0.8175$, $F = 5.60$, and $Q^2 = 0.6358$. This model shows that a decrease in the Mulliken charge around carbon-1 (MCC1) with an increase in the Lwdin charge at carbon-1 (LCC1), an increase in the HOMO, and the electrotopological state at carbon-6 (ES6) are important factors in aliphatic monoterpenoids interacting with Pa oa1 and thereby affecting the growth of the yeast cells. While Model 2 fits within the parameters initially identified, removal of the four bicyclic aliphatic monoterpenoids resulted in a model with a higher R^2 and Q^2 (Model 3 Figure 5, Table 6).

Table 4. This table shows the values for the observed and predicted yeast growth to form Model 1. It also shows the residual for the observed and predicted yeast growth.

Aromatic monoterpenoid	*Observed yeast growth*	*Predicted yeast growth*	*Residual*
carvacrol	0.11	0.13	0.02
thymol	0.01	0.04	0.03
cinamic acid	-0.19	-0.18	0.01
cymene	-0.10	-0.13	0.03
eugenol	-0.52	-0.70	0.18
methyl-eugenol	-0.77	-0.46	0.31
phenethyl propionate	-0.18	-0.12	0.06
piperonal	-0.27	-0.21	0.06
safrole	-0.10	-0.25	0.15
vanillin	-0.51	-0.66	0.15

Model 3 is produced by reducing the training set from 11 to seven aliphatic monoterpenoids, which increased the R^2, Q^2, and F-value to 0.9211, 0.8716, and 29.94, respectively. Model 3 shows that a decrease in the Lwdin charge at carbon-3 (LCC3), along with an increase of the Mulliken charge at carbon-4 (MCC4) and an increase in the electrotopological state at carbon-3 (ES3) causes an increase the growth of yeast, presumably by the monoterpenoid interaction with Pa oa1. Again, this is showing the electron density around certain carbons to be important in describing the interaction of the aliphatic monoterpenoids with Pa oa1.

MODEL 2

$$Y = 1.10(\pm 0.56) - 1.52\,(\pm 0.39)[MCC1] + 1.57(\pm 0.54)[LCC1] + 0.08(\pm 0.04)[HOMO] + 0.08\,(\pm 0.03)[ES6]$$

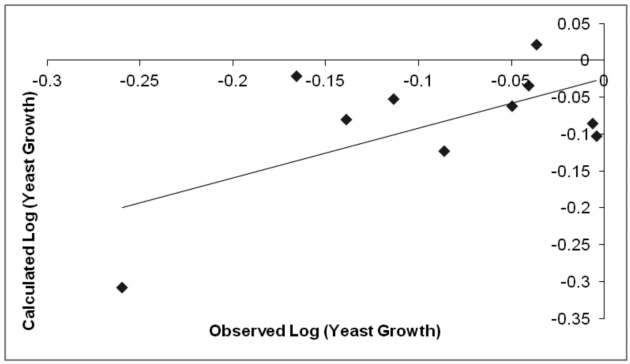

Figure 4. This figure shows observed log of yeast growth versus calculated log of yeast growth for all aliphatic monoterpenoids. This was used to form Model 2: Y = 1.10 (±0.56) – 1.52 (±0.39)[MCC1] + 1.57 (0.54)[LCC1] + 0.08(±0.04)[HOMO] + 0.08 (±0.03)[ES6] with an R^2=0.8175, F = 5.60, and Q^2 =0.6358.

Table 5. This table shows the values for the observed and predicted yeast growth to form Model 2. It also shows the residual for the observed and predicted yeast growth.

Aromatic monoterpenoid	*Observed yeast growth*	*Predicted yeast growth*	*Residual*
1,8-cineol	-0.05	-0.06	0.01
1,4-cineol	-0.11	-0.05	0.06
α-terpineol	-0.04	0.02	0.06
α-pinene	0.00	-0.10	0.10
camphor	-0.26	-0.31	0.05
limonene	-0.17	-0.02	0.14

Continued on next page.

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Table 5. (Continued). This table shows the values for the observed and predicted yeast growth to form Model 2. It also shows the residual for the observed and predicted yeast growth.

Aromatic monoterpenoid	*Observed yeast growth*	*Predicted yeast growth*	*Residual*
limonene oxide	-0.01	-0.09	0.08
menthol	-0.09	-0.12	0.04
pulegone	-0.14	-0.08	0.06
linalool	-0.04	-0.04	0.01

Model 3

$$Y = -5.41(\pm 0.73) - 20.30(\pm 2.71)[LCC3] + 2.01(\pm 0.31)[MCC4] + 0.16(\pm 0.04)[ES3]$$

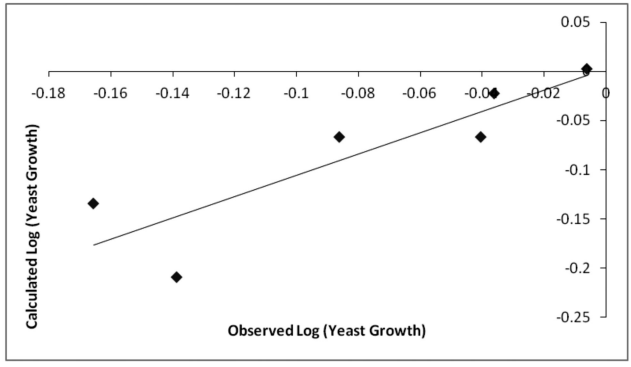

Figure 5. This figure shows observed log of yeast growth versus calculated log of yeast growth for acyclic and cyclic aliphatic monoterpenoids. This was used to form Model 3: Y = -5.41(±0.73) – 20.30(±2.71)[LCC3] + 2.01(±0.31)[MCC4] + 0.16(±0.04)[ES3] with an R^2 =0.9211, F-value 29.94 , and Q^2=0.8716.

Table 6. This table shows the values for the observed and predicted yeast growth to form Model 3. It also shows the residual for the observed and predicted yeast growth.

Aliphatic monoterpenoid	*Observed yeast growth*	*Predicted growth yeast*	*Residual*
α-terpineol	-0.04	-0.02	0.01
limonene	-0.17	-0.13	0.03
limonene oxide	-0.01	0.00	0.01
menthol	-0.09	-0.07	0.02
pulegone	-0.14	-0.21	0.07
linalool	-0.04	-0.07	0.03

Discussion

The expression of Pa oa1 in yeast resulted in a ligand-independent expression system; the lack of response to the previously identified ligand, octopamine, suggests that the receptor is fully activated (Table 1). However, other octopaminergic compounds interacted with the Pa oa1, which is constitutively active, and decrease the response of this receptor in this system. This probably is not related to the *in vivo* function of these ligands but shows an interaction with Pa oa1 (Table 1). Several octopaminergic compounds and monoterpenoids were shown to interact with the octopamine receptor, significantly altering the growth rate of yeast cells. It has been previously suggested that a ligand-independent screening system is beneficial in identifying molecules that can block this activity. Further, this type of assay is advantageous in the detection of compounds that can regulate the function of Pa oa1 independent of its ligand, octopamine (*27*). Previously, several constitutively active human GPCRs, formed by over-expression in *Xenopus laevis* melanophores, were used to search for potential new drugs (*26*).

In this study, octopaminergic compounds were shown to affect the growth of yeast by interacting with Pa oa1. In a ligand-independent system octopaminergic compounds acted as inverse agonists. Therefore, octopaminergic compounds are interacting in some manner with Pa oa1 that changes the conformation of the receptor, decreasing the affinity for the endogenous G-protein, thereby decreasing the signaling through the endogenous pheromone response pathway and decreasing the production of histidine and therefore decreasing yeast cell growth. However, one of the monoterpenoids tested, carvacrol, was shown to increase growth of the yeast cells (Figure 2). This suggests that this aromatic monoterpenoid interacts with Pa oa1 altering the conformation of the receptor and increasing the affinity for the endogenous G-protein. This increases the

production of histidine and therefore an increase in yeast cell growth. The current system provides a response that can be characterized as interacting with Pa oa1 at various degrees of efficacy (at one concentration). Therefore, we suggest that this assay is good for providing an initial indication of the interaction of a monoterpenoid with the receptor, but further analysis should be performed to determine the exact efficacy.

Twenty-one monoterpenoids were tested in this study and used to create models to predict activity at a constitutively active Pa oa1 receptor. It has previously been suggested that octopamine receptor activity is enhanced with the presence of an oxygen atom (*13*). However, p-cymene, which lacks an oxygen substituent, showed a significant result in our assay, but was not as effective as other monoterpenoids that contained an oxygen substituent. Methyl-eugenol showed the best response in this assay. Methyl-eugenol contains a hydroxyl and ether directly attached to the aromatic ring. In contrast, the bicyclic aliphatic monoterpenoid, camphor, which contains a ketone, was the most active aliphatic monoterpenoid tested. Limonene was the next best aliphatic monoterpenoid and does not contain an oxygen substituent; this again shows that an oxygen atom was not necessary for activity in this system.

Various structural features of the tested monoterpenoids were quantified using physicochemical properties. This resulted in the formation of three models to describe the activity of monoterpenoids at the ligand-independent octopamine receptor (Pa oa1). It was evident that electronic parameters are important in prescribing this interaction. Electronic parameters were also important in QSAR models using similar compounds at the insect GABA receptor (*11*). In the training set composed of all aromatic monoterpenoids: as the electronic density at carbon-3 and carbon-1 increased, a compound's interafction with Pa oa1 also increased (a decrease or increase in growth). A decrease in the molecule's dipole moment also increased aromatic monoterpenoids' interaction with Pa oa1. Electronic parameters were demonstrated to be important in acyclic, cyclic, and bicyclic aliphatic monoterpenoids with increases in the HOMO and increases in the electronic accessibility (E-state) at carbon-6 causing greater interaction with Pa oa1. However, removing bicyclic aliphatic monoterpenoids from Model 2 resulted in a better model (Model 3). In Model 3, electronic parameters at carbons 3 and 4 were important in the interaction of acyclic and cyclic aliphatic monoterpenoids.

Conclusion

Electronic parameters are important in determining the effect of various monoterpenoids' activities at the octopamine receptor. Decreasing the aliphatic training set to exclude bicyclic monoterpenoids increased the quality of the model but limited the size of the training set. Future studies should focus on increasing the number of molecules within a training set to get more comprehensive models. This may include the introduction of synthetic derivatives of naturally occurring monoterpenoids. Expression of Pa oa1 in a mammalian-based cell system also may result in a more effective testing plateform.

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Chapter 8

Novel Synthetic Ligands Enhance the Behavioral Responses of Asian Citrus Psyllid to Naturally Occurring Host Plant Volatiles

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Huanglongbing, a devastating disease of citrus, is spread by the Asian citrus psyllid (ACP) (*Diaphorina citri* (Kuwayama)). Area wide management plans aimed at reducing the incidence and spread of Huanglongbing rely upon sampling ACP with yellow sticky cards, which are not always reliable. The development of highly effective scent attractants may improve trapping efficacy, which, in turn, is needed to increase the reliability of vector detection and monitoring and to reduce costs associated with these activities. The protein components of the insect olfactory system can control and alter insect behavior; thus, these proteins are suitable targets for the development of novel control products using rational design, as has been accomplished for drug discovery in the pharmaceutical industry. Odorant binding proteins (OBPs) from ACP were recombinantly expressed in vitro and screened for their ability to bind to petitgrain oil, an essential oil from sour orange foliage known to attract ACP, in order to identify OBP(s) responsible for attraction behavior. The OBP that bound petitgrain oil was

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then screened with a combinatorial chemical library comprising 30,000 small molecules in order to isolate synthetic ligands; these ligands were subsequently evaluated for their behavioral effects on ACP. A synthetic ligand named 'Titan' was found to be behaviorally active in preliminary tests; Titan was tested further with respect to its abilities to stimulate two foraging behaviors of ACP and to interact with limonene, a monoterpene emitted by many ACP host plant species. The addition of Titan to limonene increased the amount of probing by ACP into an artificial midrib composed of an emulsified wax relative to the probing level elicited by limonene alone. Probing level to Titan alone was influenced by the concentration of Titan in the wax midrib, with the lower concentration being more stimulatory than the higher concentration. In olfactometer tests, response to Titan was similar to the odor of young orange jasmine foliage. Olfactory response was also higher to a mixture of limonene and Titan than to limonene alone. These results demonstrated that Titan is highly stimulatory and modifies psyllid response to limonene. Further tests will determine whether Titan can be used in concert with naturally occurring terpenes to develop potent scent attractants for ACP.

Introduction

The Asian citrus psyllid (ACP), *Diaphorina citri* (Kuwayama) (Hemiptera: Psyllidae) transmits *Candidatus* Liberibacter asiaticus, the causal agent of citrus greening disease, also known as Huanglongbing. This is the most devastating disease of citrus trees in the world today and has resulted in the loss of hundreds of thousands of hectares of orchards and billions of dollars in productivity (*1–4*). ACP can move over large distances in a relatively short time (*4–6*) and inhabits citrus trees growing in both commercial groves and residential areas (*4*).

Area wide management plans aimed at reducing the incidence and spread of Huanglongbing rely upon detection and monitoring of ACP to determine its spatial distribution and rate of spread. The heterogeneous distribution of ACP across landscapes comprised of commercial and residential citrus trees makes it challenging to effectively sample the psyllid. Current detection and monitoring protocols for ACP rely upon visual inspection of citrus foliage for the presence of adult and immature ACP, tap and vacuum sampling of foliage, and the use of yellow sticky card traps placed on citrus trees. These methods are labor intensive and expensive; yellow sticky cards may be inconsistent in attracting and trapping psyllids and are ineffectual at low population densities (*7*). Improvements in trapping efficacy are needed to increase the reliability of ACP detection and monitoring and to reduce costs. Moreover, traps sensitive enough to detect low populations of ACP would greatly facilitate discovery of ACP as it spreads into new areas.

The addition of scent lures based on pheromones, host plant odors, or a combination of the two may be a means of increasing ACP capture on traps. There is evidence of an ACP sex pheromone in which males are stimulated at close range to females, and work is proceeding to determine whether scent attractants using a psyllid sex pheromone can be developed (*8–10*). Laboratory studies have shown that ACP is stimulated by host plant odors (*11–13*). Because ACP mates, reproduces, and develops only on the growing shoots of *Citrus* and related genera (*1*), it is likely that the psyllid uses volatiles emitted by young foliage to locate actively growing trees. The foliar odor emitted by ACP's rutaceous host plants is comprised of a wide variety of volatiles, primarily monoterpene, sesquiterpene, and terpene esters (*11*, *14*). The profile of ACP host plant volatiles change as a function of leaf age, physiological condition, and agronomic factors (*15*, *16*). The great degree of variation inherent in the volatiles emitted by host plants makes formulating volatile mixtures with the proper composition, proportion, and concentration to effectively attract ACP a daunting task (*17–23*). In terms of improving trapping efficacy, scent lures must be able to compete with the growing trees in attracting psyllids.

In insects, odorants are detected and differentiated by olfactory sensory hairs (sensilla), which are primarily located on the antennae and palps (*24–26*). Odorants enter through cuticular pores and interact with a variety of olfactory proteins within the sensillar lymph. The odorant molecules attach to extracellular chemosensory proteins (CSPs) that transport them to receptors located on membranes of the olfactory neurons, which, in turn, then initiate downstream signaling. CSPs solubilize ligands and facilitate transport of hydrophobic molecules through the sensillar lymph. The specificity and sensitivity of an insect species' olfactory response to various types of odorant molecules is strongly influenced by the physical and chemical characteristics of the ligand binding sites of the CSPs present in its antenna and palps (*24–26*). Thus, CSPs are a key component in insect detection of odorants.

Attention has recently been focused on identifying potential ligands of the CSPs present in the olfactory sensilla of insects and developing means of using these compounds as surrogate scent attractants, repellents, or confusants for disease vectors such as malarial mosquitoes (*24–31*). In the case of ACP, these ligands could enhance psyllid response to host plant odorants by stimulating the olfactory proteins in its antennae. In this scenario, the presence of additional CSP ligands should lead to an increase in the number of bound CSPs in the sensillar lymph, which, in turn, would enhance olfactory response in one of two ways: either increased triggering of a specific type of olfactory receptor or triggering two or more types of receptors. If this were the case, then the addition of CSP ligands could provide a level of stimulation sufficient to enable mixtures of naturally occurring terpenes to compete with living trees for the attention of ACP.

In this study, a binding assay was used to determine which CSPs expressed in ACP antennae bind petitgrain oil. Petitgrain oil is an essential oil extracted from the leaves of sour orange (*Citrus aurantium* L.) and is typically comprised of monoterpene esters, such as linalyl acetate, and monoterpenes, such as linalool and limonene (*13*, *16*). Petitgrain oil was selected as a test material in the binding

assay to ACP CSPs after it was shown to be attractive to flying ACP using the methods described below.

Once the CSP responsible for binding petitgrain oil was identified, this protein was used as a screening target in a proprietary fluorescence assay developed by Inscent, Inc. in order to isolate novel synthetic ligands for the protein (*29*). A number of these compounds were subjected to an initial screening for behavioral activity. This so-called 'rational approach' to discovering effective ligands has been applied successfully in the pharmaceutical industry, where combinational chemical libraries are routinely screened for binding partners to proteins of interest (*24*).

Using this approach, candidate compounds, putatively identified as CSP ligands, were subsequently subjected to an initial evaluation to test their ability to stimulate specific foraging behaviors of ACP. One putative ligand, nicknamed 'Titan', induced the highest level of behavioral response and was selected for further testing. Two assays were used to determine whether Titan modified ACP host plant selection behavior. One assay used probing level as a response variable (*11*); a second assay was used to determine whether ACP response was due to olfactory reception of Titan. Since ACP response to odorants is influenced by volatile concentration (*11*), Titan was tested at a low and high concentration. To determine whether Titan influenced behavioral response to naturally occurring host plant volatiles, psyllid response was also measured to a mixture of the Titan and limonene, a stimulatory monoterpene present in the foliage of a variety of ACP host plants (*10*, *13*). This test was necessary since it was not known whether Titan would interfere, synergize, or have a neutral influence on ACP response to natural volatiles.

Experimental

Study Insects

For the petitgrain oil test, ACP were collected from local citrus trees 1-2 hrs prior to testing and held in plastic vials. For the 'leaf dish' assay and olfactometer tests, ACP were collected with an aspirator from a colony maintained at the former USDA-ARS laboratory in Weslaco, TX. The psyllids were reared on orange jasmine (*Murraya paniculata* (L.) Jack.) and curry leaf (*Bergera koenigii* L. Sprengel) plants grown in 2 L pots in a growth chamber (14:10 L:D, 26°± 2°C). The plants were illuminated with CF full spectrum lamps (25 W, 120 V, 60 Hz, 6400 K) (Interek) and infrared grow lights (90 W) (Prosource Worldwide, Inc.) for maximum plant sustainability. The plants were rotated every two weeks from a greenhouse to maintain plant vigor.

Petitgrain Oil Test

The attractiveness of petitgrain oil to ACP was tested with free-flying psyllids in no-choice tests conducted in a greenhouse (25 m x 12 m x 6 m) located at the USDA-ARS laboratory in Weslaco, TX. The greenhouse was maintained at 60% relative humidity via the cool pad and overhead misters. It had a system of sliding

benches with wire mesh decks that served as a platform for anchoring the supports of the traps used in the tests. The traps were made by placing two sections (each 20 cm x 2.5 cm) of sticky card traps (ISCA Technologies, Riverside, CA), one atop of the other, at the top of a 1 m long bamboo stake support. Bamboo stakes were anchored in concrete-filled flower pots. A 4 x 4 array of stakes were deployed in the greenhouse. Stakes within the same column were separated by a distance of 1 m while stakes within the same row were separated by a distance of 2 m. A dispenser for the petitgrain oil aroma was made by placing the cut end of a cotton applicator into a hollow rubber latex septum. A single dispenser was attached via staple to the top of each card. A 1 mL aliquot of petitgrain oil (Ananda, Inc., Boulder, CO.) was added to the cotton at the beginning of each test. In the control tests, only blank dispensers were used while in the petitgrain oil tests all of the dispensers were charged. Five replicated tests were conducted for each control and petitgrain oil test.

Psyllids were released from a screened cage placed downwind of the trap array. Thirty minutes prior to the start of each test, ca. 1000 psyllids were placed inside of a screened cage (45cm x 45cm x 45cm) (BioQuip, Rancho Dominguez, CA) with a hinged lid. Subsamples of 50 psyllids showed an even sex ratio present in the field collected insects. At the time of this study, Huanglongbing had not yet been detected in Texas, so the insects were pathogen-free. Tests were conducted between 11:00 and 12:00. At the beginning of each test, the lid was opened slightly (ca. 2.5 cm). The narrow opening of the lid allowed psyllids to fly from the cage but retarded escape flight. The traps were checked at 15 min intervals for 60 min and the numbers of psyllids caught on each trap were recorded. Tests were conducted in June 2009. The response variable was the percentage increase in the numbers of psyllids captured in the control versus petitgrain oil-scented traps.

Probing Assay

A test arena (‘leaf dish’) that mimicked a leaf surface was used to measure the psyllids’ probing responses to test stimuli (*11*) (Figure 1). The leaf ‘blade’ was made by stretching plastic paraffin film (Parafilm® American National Can, Inc., Chicago, IL) across the opening of a 52 mm dia plastic Petri dish (Becton Dickenson Labware, Franklin Lakes, NJ). The ‘midrib’ was made with a thin line of an emulsified wax (SPLAT®, ISCA Technologies, Inc.) placed along the middle section of the dish. SPLAT is used to dispense insect pheromones for the control of agricultural pest insects (*32–34*). The SPLAT line was made with a 3 mL syringe fitted with a 20 G needle.

The ‘leaf dish’ was placed inside a larger plastic Petri dish (9 cm x 1.5 cm) that served to confine the psyllids (Figure 1). Prior to the start of each trial, the psyllids were collected with an aspirator, held in a plastic vial for 60 min and then anaesthetized by chilling. Five anaesthetized individuals were placed onto the membrane of each leaf dish. During the experiment, the dishes were placed in an incubator kept at 28 ± 1 °C illuminated with fluorescent lights. The experiments were begun between 10:00 and 13:00. After 2 hr, the dishes were removed from the incubator and the psyllids were killed by chilling. Post-mortem examinations were conducted to determine the number of male and female psyllids in each

dish. Following removal of the psyllids, the leaf dishes were submerged in 0.1% Coomassie blue solution for 5 min to stain the salivary sheaths left in the SPLAT when the psyllids probed the wax line (*7*). After rinsing in distilled water, the SPLAT lines were air dried and then examined with a dissection microscope. All of the probing holes along the length of the 'midrib' were counted and recorded.

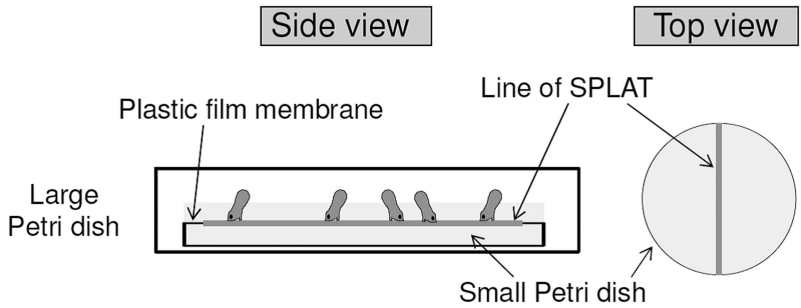

Figure 1. Schematic diagram of 'leaf dish' assay set-up.

A previous study showed that ACPs were moderately responsive to SPLAT lines that were colored pale yellow-green (*11*). To prevent attraction to visual stimuli from interfering with interpretation of data on olfactory response, only SPLAT lines colored pale yellow-green were used in the study (*11*). The SPLAT was colorized by mixing 6 µL neon green food coloring (McCormick & Co., Inc) into 10 mL of white SPLAT.

Preliminary leaf dish assays were conducted on 11 proprietary compounds identified by fluorescence tests (*25*) as ligands to CBPs isolated from the ACP antennae. Of the 11 compounds tested, one, nicknamed 'Titan', elicited probing activity and thus all subsequent tests were conducted with Titan. Two concentrations of Titan were tested, 'low' (1.667 mg Titan/10 mL SPLAT) and 'high' (5 mg/10 mL SPLAT). Psyllids in the control treatment were exposed to blank SPLAT. Psyllid probing response was also measured to limonene, a monoterpene present in the foliage of many *Citrus* species (*10*, *13*). In earlier tests, ACPs were stimulated by D-limonene (*10*) and mixtures containing limonene (*11*). In this study, D-limonene (Sigma-Aldrich) was tested at a concentration previously shown to be stimulatory (20 µL/10 mL SPLAT) (*11*). It was also tested with Titan, at both the low and high concentrations, to determine whether Titan influenced ACP response to terpenes emitted by their host plants.

Olfaction Test

The probing responses observed in the leaf dish assay may be elicited by perception of stimuli through multiple chemosensory mechanisms; i.e., olfaction and gustation. To measure a behavioral response to the test treatments that was purely olfactory, an olfactometer was designed in which the response variable was retention time in a target area exposed to an odor plume (Figure 2). The body

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of the olfactometer was made from a tapered 24/40 glass inlet adapter with a compression cap and O-ring seal at one end and an 18 mm i.d. opening at the other end (Kontes, Inc.). The target area was comprised of an aluminum foil cup placed into adapter opening. The foil cup had 25 perforations, made with a dissection needle, through which the odor plume flowed. A strip of Parafilm was wrapped around the perimeter of the foil cup to secure it to the tube and to prevent extraneous airflow from the edge of the cup. Flexible Teflon tubing was inserted into the compression cap and O-ring. The tubing was connected to an impinger tube (ARS Inc.) containing the odor sample. Airflow was provided by a personal air sampler pump (SKC, Inc.) at a rate of 33 mL/min. The laboratory air used in the experiment was passed through a charcoal filter (ARS, Inc.) prior to entering the odor source tubes. The olfactometer was supported by a clamp attached to a ring stand.

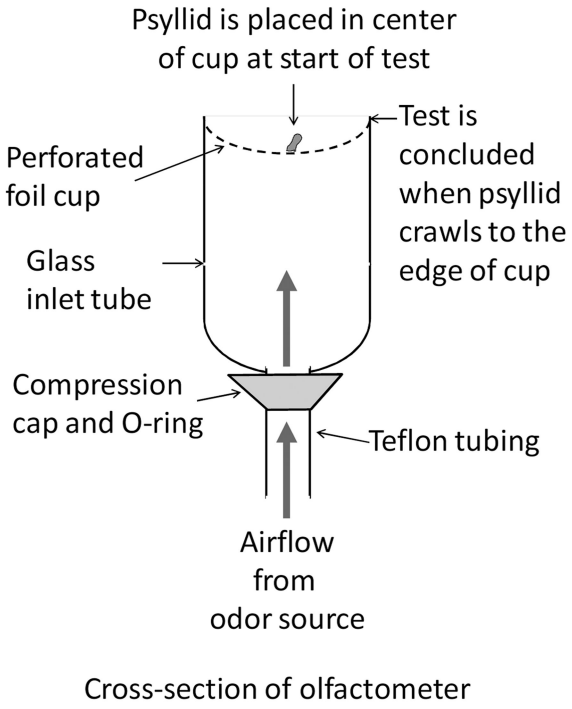

Figure 2. Schematic diagram of olfactometer (not to scale). Olfactometer was held upright with a clamp attached to a ring stand (not shown).

The SPLAT treatments were administered by applying 1 mL SPLAT to a glass microscope slide with a paintbrush. After a 30 min drying period, the slide was inserted into the odor sample tube. Three orange jasmine sprigs, ca. 10 cm long, were collected immediately prior to testing from greenhouse-grown plants. The sprigs were rinsed with reverse osmosis water and dried before insertion into the odor collection tube. Sixty replicates were conducted for each treatment.

Prior to the start of each test the psyllid to be tested was anaesthetized by chilling for 60 sec at -4 °C and then placed into the center of the aluminum foil cup. The test began 20 sec after the psyllid recovered from the chilling and assumed a normal foraging position. The 20 sec delay was used to exclude individuals that engaged in escape behavior immediately following recovery from anaesthetization. The test ended when the psyllid moved to an ink line drawn around the rim of the cup. Lower retention times indicated that individuals had been stimulated and were actively searching while longer retention times were indicative of a lack of stimulation. Each test lasted for 300 sec; psyllids that did not move to the rim line within this time frame were scored as unresponsive and omitted from the analysis. Preliminary tests indicated that females were non-responsive in the olfactometer to any of the treatments. This may have been due to the fact that the tests were conducted in the winter and the psyllids were reared under artificial conditions. Because of the female's lack of responsiveness, only males were tested in the olfactometer.

Statistical Analysis

The percent increase of psyllids on petitgrain oil-scented and blank traps between sampling intervals was compared with t-tests following arcsin transformation (*35*). Probing data from the leaf dish assay were analyzed with planned comparisons of treatments with t-tests with α adjusted for the number of comparisons. One group compared blank SPLAT individually with the other five treatments ($\alpha/n = 0.05/5 = 0.01$); a second group compared blank scented with the pooled data of all scented treatments ($\alpha/n = 0.05/2 = 0.025$); the third group consisted of pair-wise comparisons of all scented treatments ($\alpha/n = 0.05/6 = 0.008$). Retention times of psyllids exposed to different odor treatments in the olfactometer were evaluated using Kaplan Meier log rank analysis (SigmaPlot 11.0) with pairwise comparisons.

Results

Evaluation of Asian Citrus Psyllid Attraction to Petitgrain Oil

In the interval between the 15 min and 30 min checks, the mean percentage increase of psyllids on the petitgrain oil scented traps was significantly greater ($t = 2.361$; $P = 0.046$) than on the blank traps (Figure 3). The mean percentage increase on the scented traps was twice as high as on the blank traps in the intervals between the 30 min and 45 min censuses and the 45 min and 60 min censuses, but these differences were not statistically significant. A total of 279 psyllids were captured on the blank traps while the petitgrain oil scented traps caught 355 psyllids, a 21% difference.

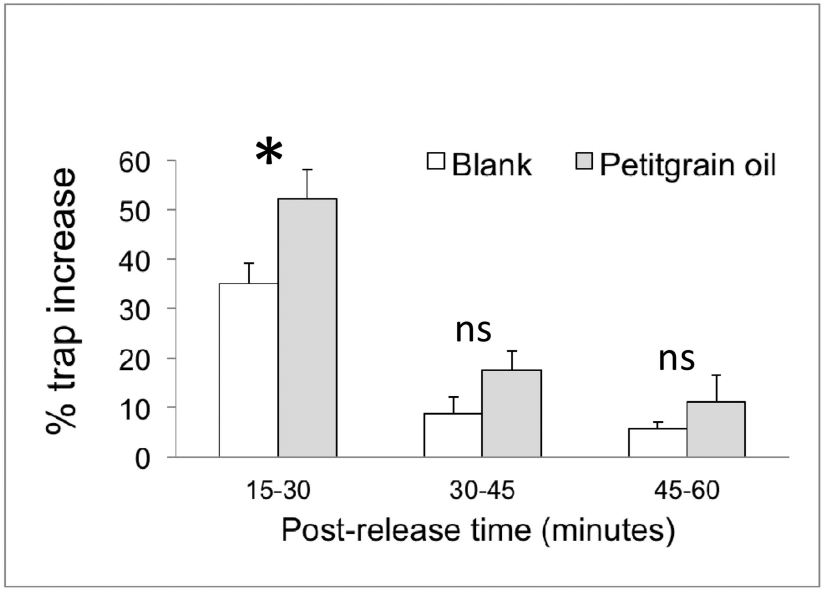

*Figure 3. Petitgrain oil attraction test. Bars show percent increase of psyllid captures on traps relative to previous census period. Pairwise samples analyzed with t-test. Bars marked with * are different at $P \leq 0.05$, ns = not significant, n=5 replicated tests per treatment.*

Probing Response to Limonene and Titan

Overall, the scented treatments induced higher levels of probing than the blank SPLAT control (Figure 4). Response to the limonene and high concentration Titan treatment was relatively low (mean number of probes: limonene = 5.8, high concentration Titan = 3.6); the response to the low concentration Titan treatment was relatively higher (mean = 7.5 probes) though statistically similar to limonene ($P = 0.24$). Addition of Titan to limonene resulted in higher amounts of probing than in the treatments containing only single compounds (Figure 4), with the greatest amount of probing occurring in the mixture containing limonene and low concentration Titan (mean number of probes: high concentration Titan + limonene = 10.6, low concentration Titan + limonene = 12.3).

Olfactory Response to Titan

In the first test, male psyllids left the cup significantly faster when exposed to the odors of Titan or orange jasmine foliage than to the controls (air v. foliage, $P = 0.002$; air v. Titan, $P = 0.008$; blank SPLAT v. foliage, $P = 0.02$; blank SPLAT v. Titan, $P = 0.03$) (Figure 5). The responses to Titan were similar to those of orange jasmine ($P = 0.9$). In the second test, male psyllids left the cup significantly faster when exposed to the odor of Titan than to air or limonene alone (air v. Titan, $P =$

0.004; Titan v. limonene, $P = 0.03$) (Figure 6). However, the response to Titan + limonene was not different than the response to air or limonene (Figure 6).

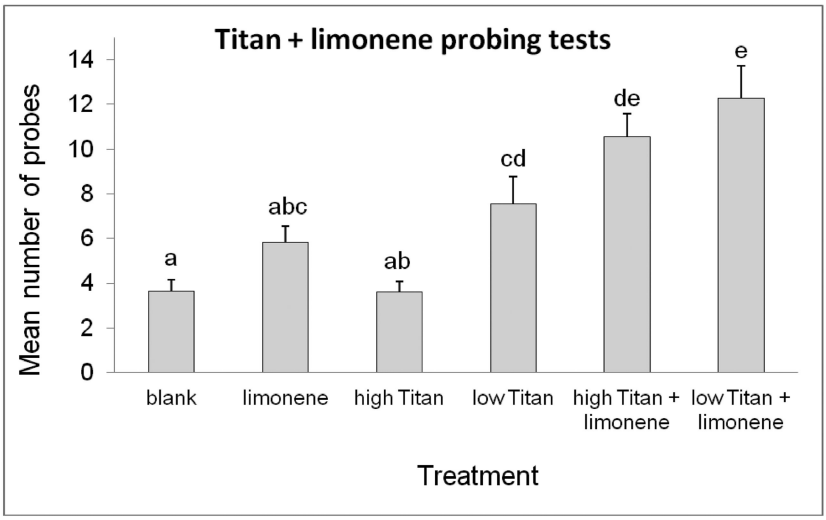

Figure 4. 'Leaf' assay test measuring probing response to limonene and Titan. Bars show mean number of probes psyllids made into lines of emulsified wax containing test treatments. Bars with different letters are different at $P \leq 0.05$, n=30 replicated tests per treatment.

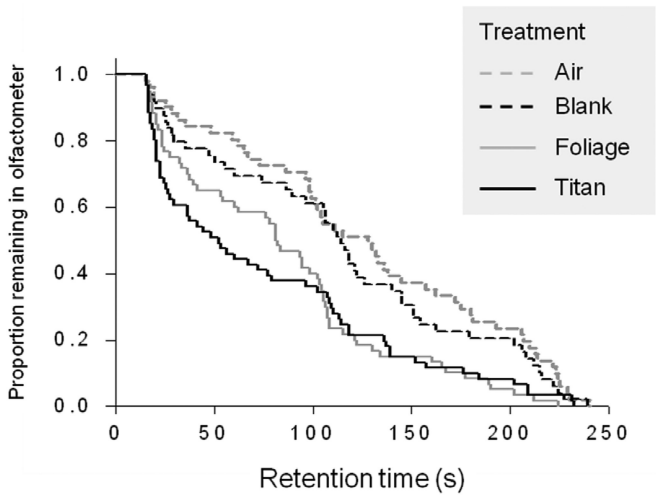

Figure 5. Olfactometer test results showing retention time of male psyllids in olfactometer cup when exposed to Titan, the odor of young orange jasmine foliage, blank SPLAT, and air. Psyllids exposed to Titan had a retention time similar to those exposed to the odor of orange jasmine foliage. Kaplan-Meier Analysis. $P \leq 0.05$, n=60 replicated tests per treatment.

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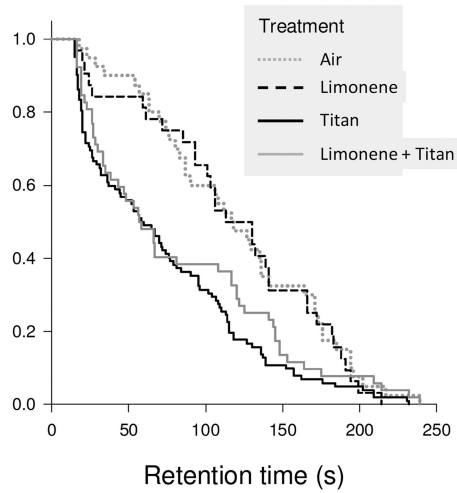

Figure 6. Olfactometer test results showing retention time of male psyllids in olfactometer cup when exposed to limonene, Titan, a mixture of Titan + limonene, and blank SPLAT. Psyllids exposed to Titan or Titan + limonene left the cup significantly faster than those exposed to limonene or filtered room air. Kaplan-Meier Analysis. $P \leq 0.05$, n=60 replicated tests per treatment.

Discussion

The results showed that Titan, a synthetic ligand of a CSP of ACP, is biologically active alone and enhanced the behavioral responses of psyllids to limonene, a naturally occurring monoterpene emitted by psyllid host plants. Both probing response and host plant searching behavior were positively influenced by the presence of Titan while the addition of Titan to limonene enhanced probing response. These results indicate that synthetic ligands have the potential to greatly enhance the attractiveness of naturally occurring terpenes. This, in turn, may make it possible to formulate highly attractive scent mixtures that will be effective in luring ACP to traps placed in orchards and residential citrus.

As has been observed in a previous study (*11*), scent concentration influenced psyllid probing response. The low concentration Titan treatment was more stimulatory than the high concentration Titan treatment, which, in turn, elicited a response similar to the limonene treatment and the blank control. Both high concentration Titan + limonene and low concentration Titan + limonene elicited high probing responses, but only the low concentration Titan + limonene treatment elicited significantly more probing responses than the low concentration Titan alone treatment. It is interesting that Titan concentration was a factor in

influencing the psyllids' probing response to limonene, which was held at a constant concentration, because it indicated that the CSPs and olfactory neuron receptor(s) (ONR) that interact with Titan are different than those that interact with limonene. If the CSPs and ONRs were the same for both compounds then it would be expected that a stronger response would be obtained with the high concentration level of Titan. An alternative explanation is that the higher concentration level resulted in complete binding at both the CSP and ONR levels. As well, it may be that concentration and mixture ratio is more fully processed in the glomeri of the antennal lobes and higher order brain centers (*19*) rather than at the level of the OBPs and ONRs. Further experiments with varied limonene and Titan concentrations are needed to determine whether this is the case or not.

The results of the olfactometer experiment demonstrated that ACP can detect Titan via olfaction. The comparable response of Titan versus orange jasmine odor was surprising given that orange jasmine is a favored host plant of ACP; it is a further indication that Titan has a strong stimulatory effect on ACP. The addition of Titan to limonene enhanced ACP response in the olfactometer further verified that Titan modified behavioral response to a naturally occurring terpene.

Ongoing laboratory tests are being conducted to measure the behavioral responses of ACP to Titan and Titan mixed with naturally occurring monoterpenes and sesquiterpenes. These tests will determine the extent to which Titan may influence psyllid behavior in the presence of other terpenes. Since Titan is a ligand of olfactory binding proteins that react with the volatiles present in petitgrain oil, it is likely that it will influence most strongly the constituents of the oil, and perhaps compounds with chemical structures that are very similar. Further work is needed to determine the types of molecules with which Titan will interact in modifying psyllid behavior. Electrophysiological and other studies are needed to elucidate the neurological mechanism underpinning modification of behavioral responses.

Pathogenic organisms have been shown to modify the odorant profiles of infected host plants as a means of attracting their psyllid vectors ((*36–39*); see also Aksenov et al., non-invasive diagnostics this book volume). Infection induces the release of a specific volatile signal that renders infected plants more attractive to the psyllids than non-infected plants. Psyllids tend to leave infected plants after acquiring the pathogen and move to nearby healthy plants, which appears to be a mechanism that escalates pathogen spread (*38*). The addition of OBP ligands to the natural odorant complex may function in a similar manner as the modifications to the host plant odor profile induced by pathogenic organisms. That is, both the pathogen induced odorants and OBP ligands may trigger an additional set of OBP-ONRs leading to a stronger overall olfactory response. Clearly a better understanding of the physiological underpinnings of reception and perception of the OBP ligand and pathogen-induced odorant systems would contribute significantly to the development of effective scent attractants for plant pathogen vectors whose host plants emit a dizzying array of odorants. Such research could provide a way for determining the necessary factors (i.e, chemical classes, minimum numbers of representative compounds, proportions, and concentration) for the formulation of effective scent attractants.

Acknowledgments

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Chapter 9

Irregular Terpenoids as Mealybug and Scale Pheromones: Chemistry and Applications

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Mealybugs are widely distributed pests of numerous agricultural crops and ornamental plants. In addition to causing direct damage, they are known vectors of plant pathogens such as grape leafroll viruses. The identification and several alternate syntheses of the irregular terpenoid pheromones of three important mealybug species (obscure, longtailed, and grape mealybugs) are described, along with the development of practical applications of the pheromones for detection and monitoring of these major pests. A stereoselective synthesis of the sex pheromone of an invasive species, the passionvine mealybug, and identification and synthesis of the sex pheromone of a closely related species, the invasive scale *Acutaspis albopicta*, also are described.

Mealybugs (Order Hemiptera: Family Pseudococcidae) are scale insects with waxy, "mealy" covers. These small sucking insects are widely distributed pests of numerous agricultural crops and ornamental plants. Besides causing direct feeding damage, they excrete honeydew as they feed, which consists of undigested plant sap and sugars. This honeydew serves as an excellent medium for the growth of sooty mold and other fungi. In addition, mealybugs can transmit important plant pathogens, some of which are lethal. This disease transmission is particularly important in long-lived perennial crops such as grapevines, tree fruits, and ornamental trees, where it can take several years for plants to

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reach maturity. Over the past decade, crop damage and economic losses from mealybug infestations have increased dramatically. Until recently, there were no effective methods to detect and monitor many of the mealybug species that are most important to agriculture in the western United States because mealybugs tend to live in protected areas of the plant. Monitoring usually consists of painstaking and laborious examination of plant material for live insects, including destructive sampling to find mealybugs under the bark. However, over the past decade, this situation has changed substantially due to the identification of the female-produced sex attractant pheromones for several of the most important pest species. These pheromones are extraordinarily powerful; lures loaded with a few micrograms have field lifetimes of at least 2-3 months. These lures have now been commercialized, providing simple and very sensitive methods for detecting mealybug infestations. Pheromone-baited traps are now being used to detect and monitor mealybugs in numerous areas of the world.

The vine, obscure, longtailed, and grape mealybugs are the four most important mealybug pests in vineyards in the western United States. Our work on the identification, synthesis, and applications of vine mealybug pheromone has been previously reported in some detail (*1*, *2*), independently verified (*3*), and thoroughly reviewed (*4*), so it will not be discussed further here. Since then, we have identified and synthesized sex pheromones of the obscure mealybug **1**, the longtailed mealybug **2**, and the grape mealybug **3**. We also developed a stereoselective synthesis of the sex pheromone of the passionvine mealybug **4**, and identified and synthesized the pheromone of the scale insect *Acutaspis albopicta* **5** (Figure 1). The latter two invasive species have the potential to cause significant damage to U.S. agriculture should they become established in the United States.

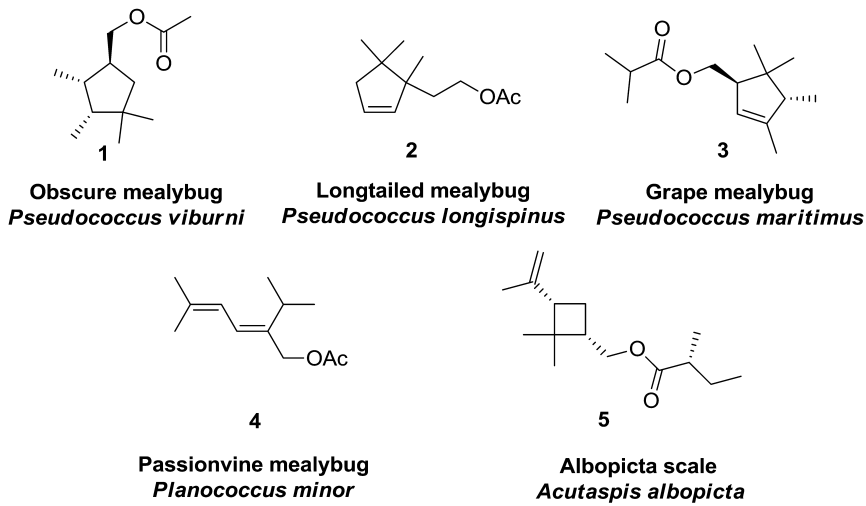

*Figure 1. Mealybug and scale pheromones identified (except **4**) and synthesized by Millar group.*

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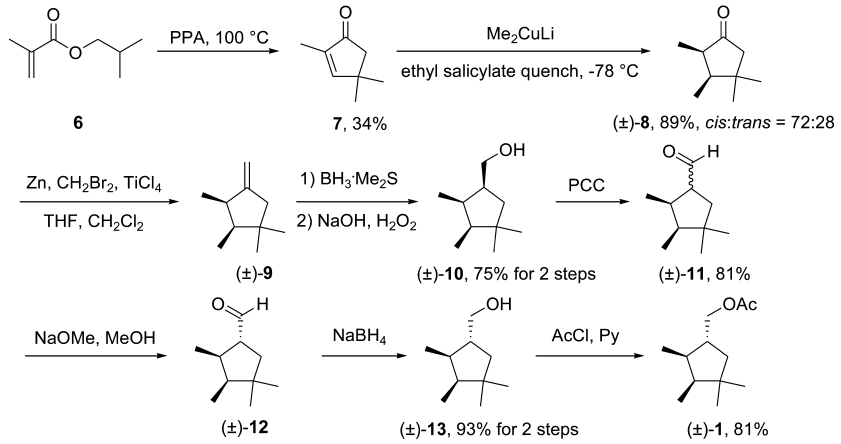
Sex Pheromone of the Obscure Mealybug

The irregular monoterpenoid (1*R**,2*R**,3*S**)-1-acetoxymethyl-2,3,4,4-tetramethylcyclopentane **1** was identified as the female-produced pheromone of the obscure mealybug, *Pseudococcus viburni* (*5*). The basic carbon skeleton was determined by a combination of microchemical reactions and mass and microscale NMR spectrometry, but because of the very small amount of purified material available, we could only partially and tentatively determine the relative stereochemistry of the molecule. The NMR data suggested that the methyl groups on carbons 2 and 3 were on the same side of the ring, but their relationship to the CH_2OAc group on C-1 was uncertain. Thus, our first synthesis was specifically designed to be non-stereoselective, to provide access to all four possible diastereomers for use as standards so that the relative stereochemistry could be determined unequivocally (*5*). These were separated and purified by liquid and preparative gas chromatography, providing milligram quantities for detailed NMR studies that allowed conclusive assignment of the relative stereochemistry of each diastereomer, and confirming the natural pheromone to be (1*R**,2*R**,3*S**)-**1**. The synthesis was then modified to produce a preponderance of the desired diastereomer (*6*). Thus, as shown in Scheme 1, isobutyl methacrylate **6** was cyclized in hot polyphosphoric acid to the trisubstituted cyclopentenone **7** (*7*, *8*). Conjugate addition of lithium dimethylcuprate to **7** and quenching the resulting enolate at low temperature with ethyl salicylate, a chelating proton donor (*9*, *10*), gave the desired, thermodynamically disfavored *cis*-isomer **8** as the major product (*cis*:*trans* = 72:28). Whereas methylenation of ketone **8** under standard Wittig conditions was not satisfactory due to poor yields, **8** was cleanly converted to **9** with no epimerization at C-2 using the Takai/Lombardo conditions (*11*, *12*). Hydroboration followed by oxidation gave alcohol **10** with the wrong configuration at C-1. This center was readily inverted by a simple and efficient three-step sequence of oxidation, base-catalyzed epimerization, and reduction to furnish alcohol **13** with the correct relative configuration. The synthesis was completed by straightforward acetylation.

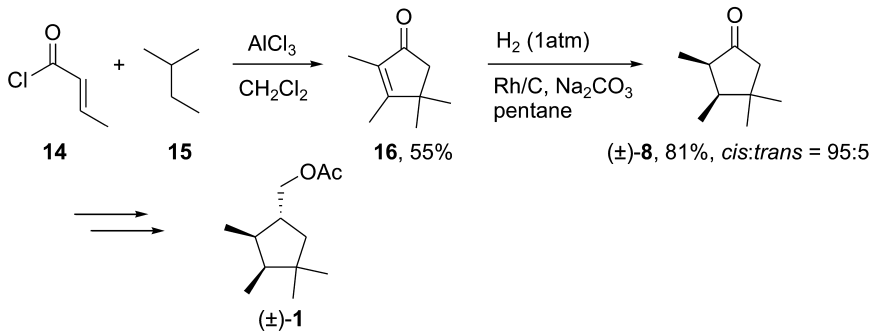
A lingering problem with this synthesis was that the key step to establish the relative stereochemistry at C-2 and C-3 (**7** → **8**) gave only modest *cis*/*trans* selectivity, and in practice, was tedious to perform.

The absolute configuration of the natural enantiomer was determined to be (1*S*,2*S*,3*R*) by kinetic resolution of the racemate, followed by vibrational circular dichroism analysis to assign the absolute stereochemistry of each enantiomer (*13*). The assignment was subsequently confirmed by independent enantioselective syntheses of both enantiomers on milligram scale (*14*, *15*).

In field tests, the racemic compound was as attractive to male mealybugs as the natural enantiomer, so for practical purposes, there was no need for an enantioselective synthesis. Furthermore, the pheromone was extraordinarily powerful; rubber septum lures loaded with 25 micrograms of the racemate remained attractive for 2-3 months or more under field conditions (*16*).



*Scheme 1. Synthesis of the obscure mealybug pheromone **1**. (Reproduced with permission from reference (6). Copyright 2007 Elsevier Ltd.)*



*Scheme 2. Improved synthesis of the obscure mealybug pheromone **1**. (Adapted with permission from reference (17). Copyright 2011 Elsevier Ltd.)*

However, a more efficient diastereoselective synthesis capable of being scaled up to produce multigram quantities of racemic **1** was still desirable. Our improved synthesis, based on a relatively uncommon stereoselective reduction of a tetrasubstituted alkene, is shown in Scheme 2 (*17*). Thus, reaction of (*E*)-crotonyl chloride with isopentane in the presence of $AlCl_3$ furnished the key precursor **16**. Presumably, acylation of the alkene generated in situ by hydride transfer from the isoalkane gave the divinylketone intermediate, which underwent Nazarov cyclization (*18*). The *cis* relative stereochemistry then was secured by rhodium-catalyzed diastereoselective catalytic hydrogenation of tetrasubstituted alkene **16**. The speed of the reaction and the ratio of the *cis*- to *trans*-isomers proved to be very sensitive to the reaction conditions. In our first attempts,

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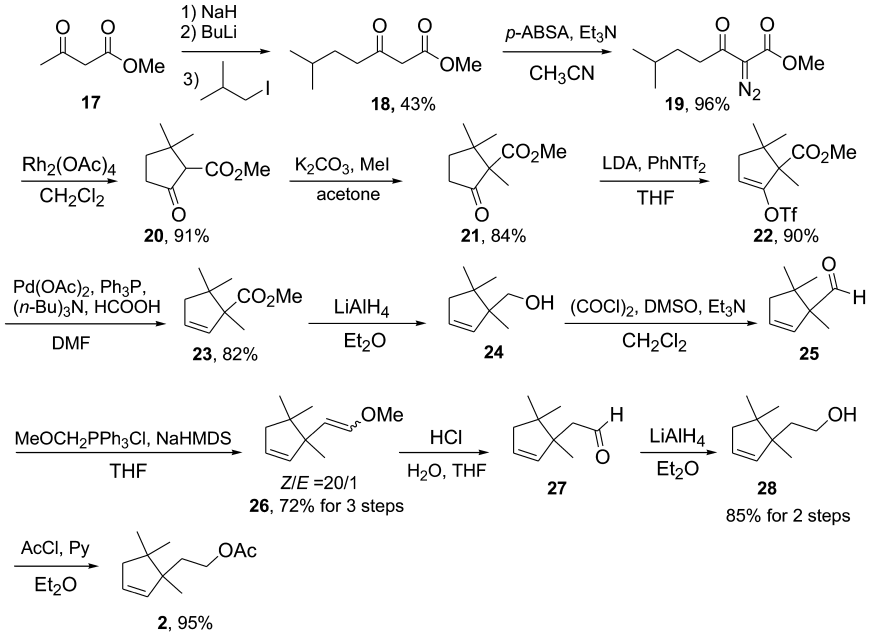
using **16** purified only by distillation, the reaction was very slow. After careful experimentation, it was found that the purity of **16** was crucial to the rate of hydrogenation, and the general conditions developed by Paquette and coworkers (*19*, *20*) using rhodium on carbon as catalyst proved successful. With **16** purified by flash chromatography (GC purity > 99.5%), hydrogenation was complete in several hours, giving *cis*-isomer **8** with good stereoselectivity (*cis*:*trans* = 95:5) and yield. The synthesis was then completed as described in Scheme 1 above.

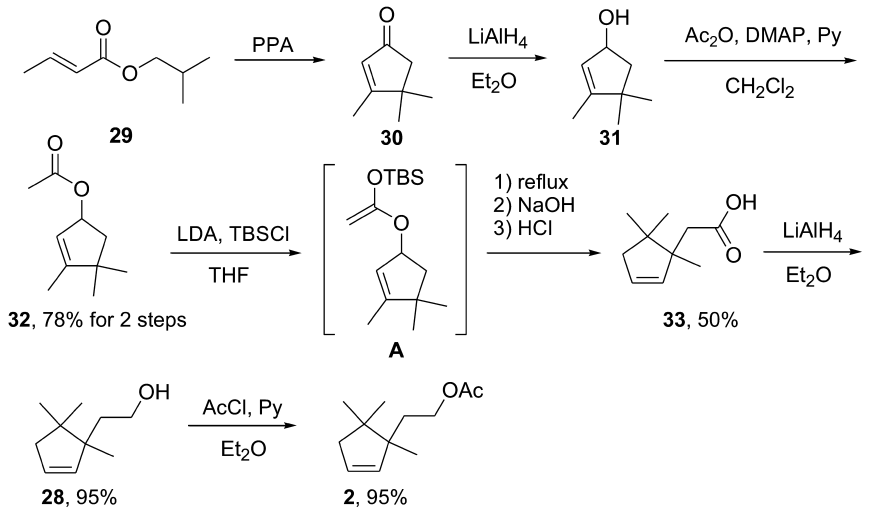
Sex Pheromone of the Longtailed Mealybug

We identified the irregular monoterpenoid 2-(1,5,5-trimethylcyclopent-2-en-1-yl)ethyl acetate **2** as the female-produced pheromone of the longtailed mealybug, *Pseudococcus longispinus*, with microprobe NMR being crucial for the identification. To prove the structure, the racemic pheromone was initially synthesized using a 2,3-Wittig rearrangement as the key step, but with a low 4.2% overall yield (*21*). Our improved synthesis is shown in Scheme 3 (*22*). We recognized that **2** could be derived from a one-carbon homologation of tetrasubstituted cyclopentene **23**, which in turn could be prepared from ketoester **20** in several steps. To our gratification, the key intermediate **20** was generated in excellent yield by Rh-catalyzed intramolecular cyclization of α-diazo-β-ketoester **19**. The cyclization favored insertion into a methine C-H bond and formation of five-membered rings (*23*), both factors working in our favor. The intermediate **20** contained a strategically placed ketone carbonyl group which provided two key features for further advance. First, it served as an additional activating group to regiospecifically direct methylation of the ketoester, providing the second, vicinal quaternary center. Second, it represented a latent endocyclic alkene in the correct position. In the end, this straightforward and scalable synthesis more than tripled the yield of the previous synthesis (13.5% vs. 4.2%) and provided more than 5 grams of the pheromone, sufficient for more than 200,000 pheromone lures using a standard dose of 25 micrograms per lure.

One concern with the synthetic route shown in Scheme 3 was the length of the reaction sequence (12 consecutive steps). To further expedite commercial development of the pheromone, we developed a more efficient synthesis based on a much shorter 6-step sequence (Scheme 4) (*24*). An important feature of our design was the recognition that **2** could be derived from γ,δ-unsaturated carbonyl compounds, such as **33**, which should be accessible from cyclopentenol **31** through a Claisen-type rearrangement. This key intermediate was readily available in two straightforward steps consisting of cyclization of the very cheap starting material isobutyl crotonate in hot polyphosphoric acid (*7*), followed by 1,2 reduction of the resulting cyclopentenone **30**. With this key intermediate in hand, we set about optimizing the conditions for the Claisen rearrangement. Johnson-Claisen rearrangement was unsuccessful and Eschenmoser-Claisen rearrangement gave unacceptably low yields, but gratifyingly, Ireland-Claisen rearrangement proceeded smoothly through the silyl ketene acetal **A** to give **33** in respectable yield. The synthesis was then completed by the trivial steps of reduction of the carboxylic acid and acetylation of the resulting alcohol.



Scheme 3. Synthesis of the longtailed mealybug pheromone 2. (Reproduced from reference (22). Copyright 2009 American Chemical Society)



*Scheme 4. Improved synthesis of the longtailed mealybug pheromone **2**. (Reproduced with permission from reference (24). Copyright 2010 Thieme)*

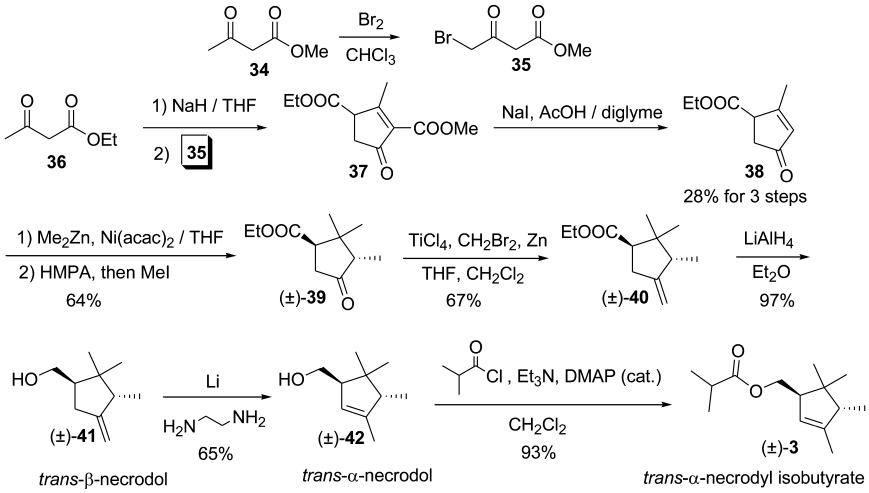
Although the absolute configuration of the pheromone has not yet been determined, the racemic pheromone has proven to be extremely attractive to male mealybugs in field tests and entirely sufficient for practical purposes.

Sex Pheromone of the Grape Mealybug

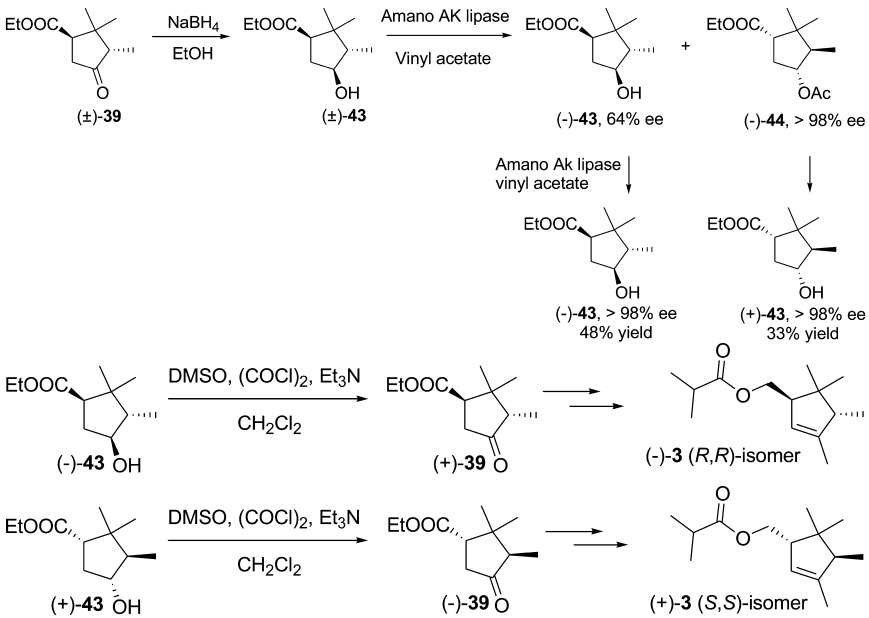
We identified the irregular monoterpenoid *trans*-α-necrodyl isobutyrate **3** as the female-produced pheromone of the grape mealybug, *Pseudococcus maritimus* (*25*). Interestingly, the core monoterpene alcohol structure of this pheromone was first identified from an unrelated insect, the carrion beetle *Necrodes surinamensis*, more than 20 years ago (*26*). The structure was confirmed by preparation of milligram quantities of one enantiomer via acylation of a sample of α-necrodol of uncertain stereochemistry obtained as a gift. Having neither the racemate nor the other enantiomer available for comparison, the absolute configuration of the pheromone could not be conclusively determined. To further study this pheromone and determine its possibilities for practical applications in management of the insect, an efficient synthesis capable of producing the racemic compound on gram scale and analytical amounts of both enantiomers for comparison purposes was required.

Scheme 5 summarizes our synthesis of **3** (*27*), which combined and optimized elements from several published syntheses of necrodol isomers, including a diastereoselective synthesis of β-necrodol **41** (*28*) and an efficient isomerization of β-necrodol to α-necrodol **42** (*29*), the core structure of the pheromone **3**. Thus, tandem alkylation of bromide **35** with ethyl acetoacetate followed by intramolecular Knoevenagel condensation of the intermediate diketodiester gave cyclopentenone **37**. Regioselective decarboxylation gave ketoester **38**. Interestingly, the exact compositions of the starting esters **34** and **36** and the resulting placement of the two esters in the Knoevenagel condensation product proved crucial: starting with methyl acetoacetate and the ethyl ester analog of bromide **35** yielded a diester product that gave only an intractable mixture of products in the decarboxylation step.

Conjugate addition of dimethylzinc to **38** with nickel catalysis, followed by trapping the resulting enolate with methyl iodide produced the *trans*-isomer **39** with complete diastereoselection (*28*). Methylenation of **39** under the neutral Takai/Lombardo conditions (*11*, *12*) gave **40**, with all the required carbons in place, which was then reduced to β-necrodol **41**. Timing of the subsequent isomerization of the *exo* double bond to the trisubstituted *endo* double bond of *trans*-α-necrodol **42** was crucial. Presumably, the orientation of the hydroxymethyl group shielded one of the two diastereotopic faces of **41** and induced the allylic deprotonation reaction to proceed across the more accessible face. Thus, upon brief exposure of **41** to lithium ethylenediamide (LEDA), the desired *trans*-α-necrodol **42** was obtained as the kinetic product. Prolongation of the LEDA treatment resulted in further transformation of **42** to the thermodynamically most stable isomer with a tetrasubstituted double bond. The synthesis was then completed by esterification with isobutyryl chloride.



*Scheme 5. Synthesis of racemic grape mealybug pheromone **3**. (Reproduced from reference (27). Copyright 2010 American Chemical Society)*

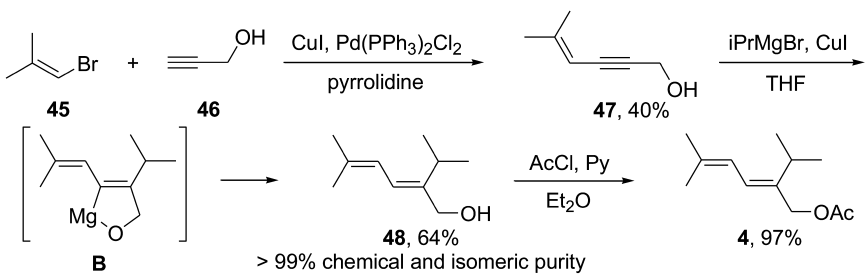


*Scheme 6. Synthesis of the (R,R)- and (S,S)-enantiomers of the grape mealybug pheromone **3**. (Adapted from reference (27). Copyright 2010 American Chemical Society)*

Our synthesis was readily adapted to production of both enantiomers of the pheromone via lipase-catalyzed kinetic resolution of the alcohol **43** generated by reduction of ketone **39** (Scheme 6), as previously described (*28*). Having resolved the enantiomers of **43**, they were then readily oxidized back to the enantiomers of ketone **39**, which were then independently carried through the rest of the synthesis to produce the enantiomers of **3** with known absolute configurations. With these standards in hand, we were able to determine, unexpectedly, that the insect-produced pheromone was actually a scalemic, 85:15 mixture of the (*R,R*)- and (*S,S*)-enantiomers. Nevertheless, in bioassays, the more easily made racemate was highly attractive to male mealybugs and satisfactory for detection and monitoring of this insect.

Sex Pheromone of the Passionvine Mealybug

The passionvine mealybug, *Planococcus minor* (Maskell), is a significant pest of major agricultural crops in Asia, including citrus, corn, grapes, and tree fruits. The possibility of its introduction into the continental United States is very high according to a U.S. Department of Agriculture risk assessment (*30*). The sex pheromone of this insect was recently identified by Ho and coworkers as the irregular monoterpenoid (*E*)-2-isopropyl-5-methyl-2,4-hexadienyl acetate **4** (*31*). To confirm the gross structure and determine the stereochemistry, a non-stereoselective synthesis based on Wittig reaction of a semi-stabilized allylic ylide was used to produce both isomers. The (*E*)-isomer was highly attractive to male mealybugs in laboratory bioassays, whereas the (*Z*)-isomer appeared to antagonize attraction (*31*). Consequently, this route was not suitable for production of the pheromone for practical use because the two stereoisomers can only be separated by HPLC in milligram amounts.



*Scheme 7. Synthesis of the passionvine mealybug pheromone **4**. Adapted with permission from reference (32) (Copyright 2007 Elsevier Ltd.).*

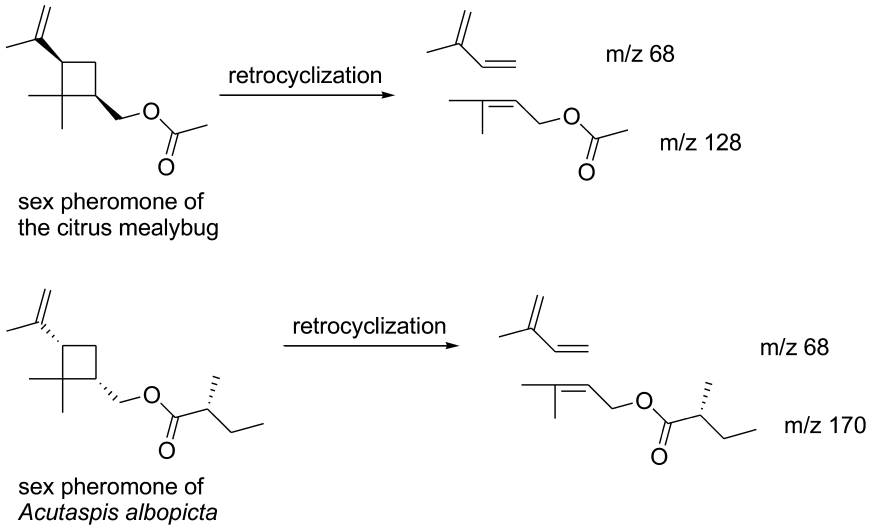
Our short and completely stereoselective synthesis of **4** is summarized in Scheme 7 (*32*). The substitution pattern of the trisubstituted alkene **48** was ideally set up for copper-catalyzed, regio- and stereoselective anti-addition of an isopropyl Grignard reagent to propargylic alcohol precursor **47**. The stereoselectivity was proposed to be the result of the formation of cyclic intermediate **B** (*33*). In the event, addition of isopropylmagnesium bromide to enynol **47** gave dienol **48** in 64% yield with >99% chemical and isomeric purity. Acetylation completed the 3-step synthesis.

With this efficient synthesis in hand, pheromone lures were provided to U.S. Department of Agriculture researchers in Florida, who were able to detect the first small, localized populations of passionvine mealybug in Florida in 2010 (*30*).

Sex Pheromone of the Invasive Scale *Acutaspis albopicta*

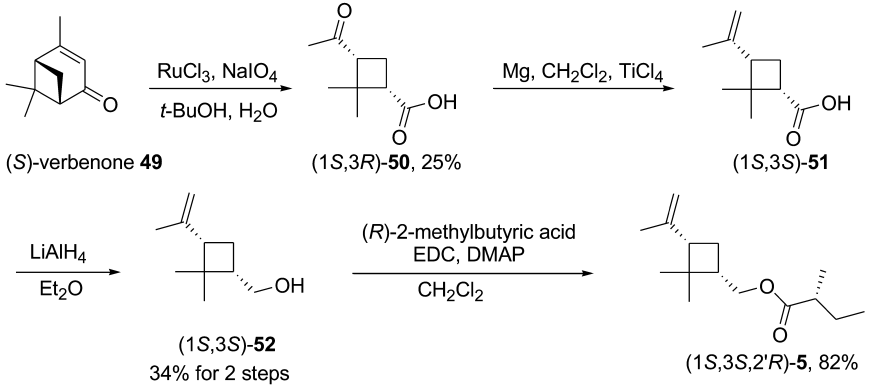
The annual crop value of California avocados is around $250 million. Historically, avocados in California were free of major insect pests, in large part because the U.S. Department of Agriculture prohibited shipment of fresh avocados from Central and South America and Mexico into the United States. However, under pressure from the North American Free Trade Agreement (NAFTA), since February 2007, fresh avocados from Mexico have been allowed to ship directly into all of the continental United States, including California (*34*). From the outset, shipments proved to be heavily infested with a number of exotic armored scale species that were not known to be present in California (*34*), and which may represent substantial threats to the California avocado industry should they become established. Consequently, we initiated a project to identify the female-produced sex pheromones of these insects, starting with *Acutaspis albopicta* (Hemiptera: Diaspididae) because it proved to be the easiest to rear under quarantine conditions.

The pheromone of *A. albopicta* was identified as the irregular monoterpenoid (1*S*,3*S*)-[2,2-dimethyl-3-(prop-1-en-2-yl)cyclo-butyl]methyl (*R*)-2-methylbutyrate **5** (*35*). Particularly useful to our structure elucidation were the prominent even-mass ions in the EI mass spectrum at *m/z* 68 (base peak) and *m/z* 170, suggesting a possible retrocyclization of a multiply substituted cyclobutane structure into a C_5H_8 isoprene fragment and a C5 ester of 3-methyl-2-buten-1-ol, analogous to the citrus mealybug pheromone, (1*R*,3*R*)-[2,2-dimethyl-3-(prop-1-en-2-yl)cyclobutyl]methyl acetate, whose mass spectrum has diagnostic rearrangement ions at *m/z* 68 and 128 (Scheme 8). These mass spectral data suggested that both pheromones had the same core monoterpene alcohol structure, with different ester side chains. These assumptions proved to be correct, with the additional twist that the monoterpene alcohols from the two species proved to have the opposite absolute configurations. Thus, full identification of the core structure was achieved with a relatively small amount of sample, i.e., without having to laboriously collect pheromone extracts from cohorts of virgin female scale for many months.



Scheme 8. The carbon skeleton of the A. albopicta sex pheromone suggested by its mass spectral fragmentation pattern

As summarized in Scheme 9, the key step of our synthesis was the previously known ruthenium catalyzed oxidative ring opening/decarboxylation of (*S*)-(-)-verbenone **49** to afford ketoacid **50** with the basic carbon skeleton in place (*36*).



*Scheme 9. Synthesis of the A. albopicta sex pheromone **5**. (Adapted with permission from reference (35) Copyright 2012 Entomological Society of America)*

In laboratory bioassays, the pheromone was highly attractive to male scales in microgram doses. The chirality of the monoterpene alcohol portion of the pheromone proved to be critically important, while the chirality of the acid portion was less so. It has not been possible to test the compound in field trials because the insect is not yet established in California. However, the pheromone has been used at selected sites in California to monitor for possible establishment.

Other Recently Identified Mealybug Pheromones

Over the past decade, pheromones have been identified for a number of other mealybug species. These include the pheromones of the pink hibiscus mealybug *Maconellicoccus hirsutus* **53** and **54** (*37*, *38*), the citriculus mealybug *Pseudococcus cryptus* **55** (*39*), the citrophilous mealybug *Pseudococcus calceolariae* **56** (*40*, *41*), the Japanese mealybug *Planococcus kraunhiae* **57** (*42*), the Madeira mealybug *Phenacoccus madeirensis* **58** (*43*, *44*), the Matsumoto mealybug *Crisococcus matsumotoi* (transferred from Pseudococcus) **59** (*45*), and the banana mealybug *Dysmicoccus grassii* **60** and **61** (*46*). The structures of these pheromones are shown in Figure 2.

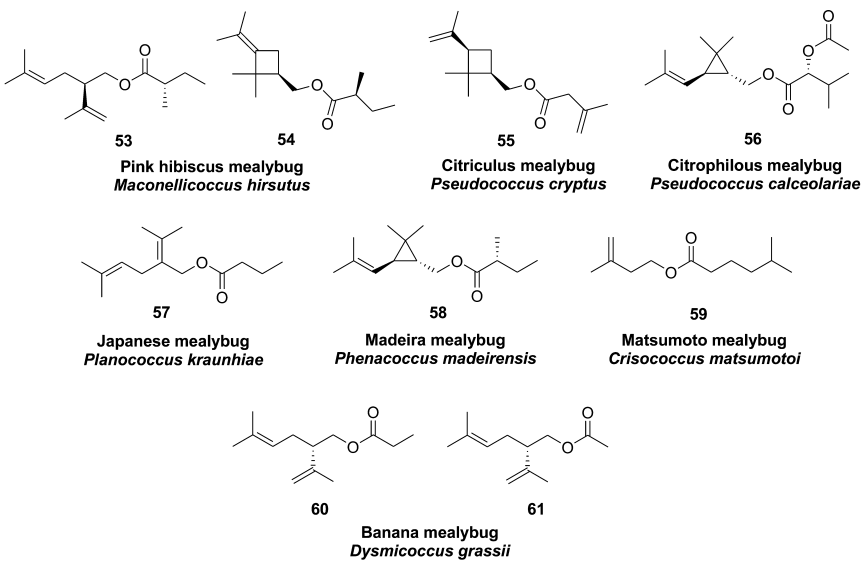


Figure 2. Additional mealybug pheromone structures identified over the past decade.

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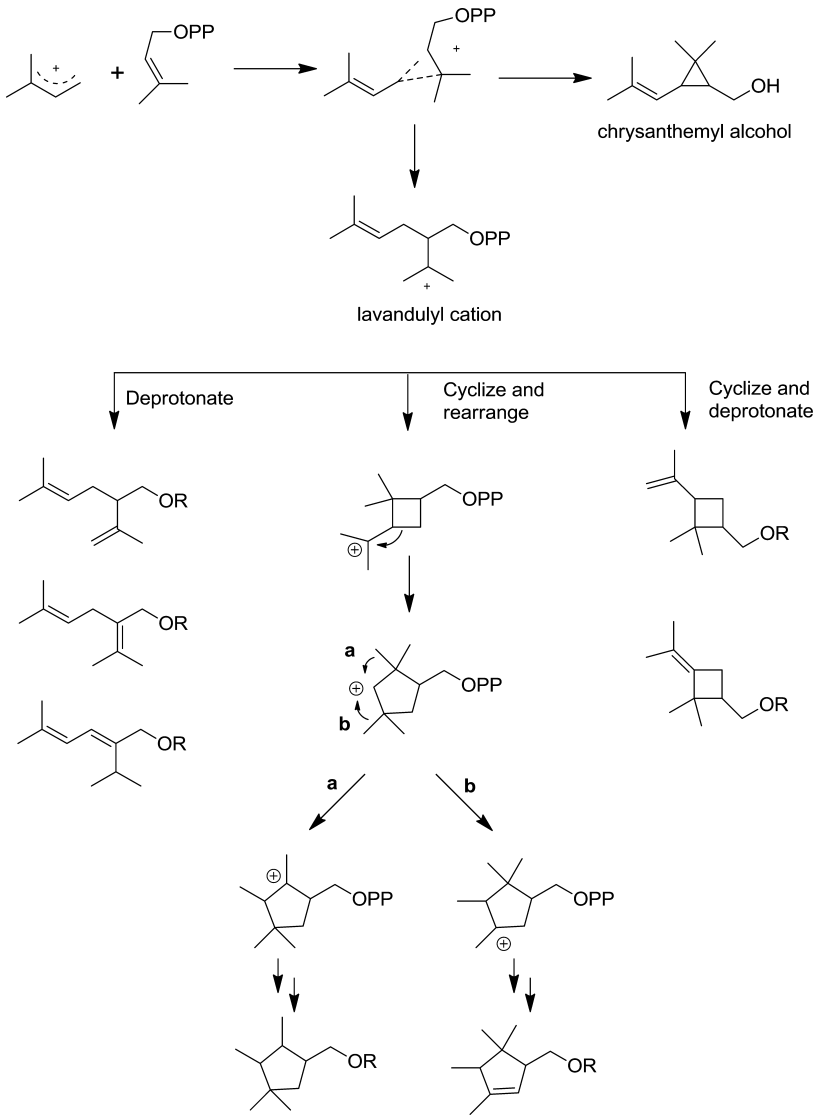

Scheme 10. Possible biosynthetic origins of mealybug pheromones based on lavandulyl, chrysanthemyl, cyclobutane, or cyclopentane type monoterpenoids

Possible Biosynthetic Origins of Mealybug Pheromones

The newly identified pheromones of mealybugs and the *Acutaspis* scale continue the trend of highly irregular terpenoid structures with unusual linkages between the isoprene units. None of the terpenoid skeletons of these pheromones have been reported from any of the insects' typical host plants, suggesting that

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these compounds are likely synthesized *de novo* by a clearly defined biosynthetic pathway, rather than being derived from closely related precursors from the host plants. It is also noteworthy that to date, each species from these large and diverse insect groups produces unique compounds. This is in contrast to the pheromones of many other insect families, in which species-specific pheromone signals are typically created from different subsets and ratios of a small group of compounds that are shared by some or all members of the group. The production of unique compounds may explain why as a general trend, mealybugs and scales are insensitive to stereoisomers or other analogs of their pheromones, i.e., there is no competition for a pheromone channel if the insect produces a unique compound. From a practical point of view, the insensitivity to stereoisomers or analogs is enormously useful, because it means that it is not necessary to develop stereospecific syntheses for production of the pheromone on a commercial scale. It also suggests that the pheromones of a number of species can be combined to make "generic" lures (see below). However, it must be noted that this is a general trend rather than a rule, because at least two species, the pink hibiscus mealybug (*38*) and the passionvine mealybug (*31*), have been shown to be inhibited by stereoisomers of their pheromones.

Examination of the compounds shown in Figures 1 and 2 reveals that in general, their structures are highly conserved across genera. Based on what is known about biosynthesis of irregular terpenoids (*47*), connection of an isoprene cation equivalent to carbons 2 and 3 of a dimethylallylpyrophosphate (DMAPP) unit produces the chrysanthemyl alcohol motif of the citrophilus and Madeira mealybugs (Scheme 10). Connection of only carbon 1 of the isoprenoid cation equivalent to carbon 2 of the DMAPP produces a lavandulyl cation, which can then deprotonate to give the lavandulol-type skeletons of the pheromones of vine, pink hibiscus, passionvine, and banana mealybugs. Alternatively, cyclization of the lavandulyl cation followed by deprotonation produces the cyclobutane structures of the citriculus and citrus mealybugs, and of the second component of the pink hibiscus mealybug (Scheme 10). Finally, as first suggested to us by Professor C. Dale Poulter, it is likely that the cyclopentane-based skeletons of the grape and obscure mealybugs arise from ring expansion of the cyclobutane followed by transfer of a methyl group from either one of the two geminal dimethyl groups flanking the resulting cation (Scheme 10).

Of all these structures, the only ones that do not fit this general scheme are the pheromones of the Matsumoto mealybug, whose structure suggests that it arises from esterification of a hemiterpene rather than a monoterpene, and the pheromone of the longtailed mealybug, whose connections suggest that it might arise from a geraniol type intermediate.

Practical Applications and Commercial Development of the Pheromones

Comprehensive descriptions of the known geographic and host ranges and the economic importance of scale and mealybug species can be found on the U.S. Department of Agriculture's Scalenet website (http://www.sel.barc.usda.gov/

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scalenet/valid.htm), and the information below is summarized from that site. Briefly, both obscure and longtailed mealybugs have a worldwide distribution and have been reported from a wide variety of plant families. Obscure mealybug is thought to have originated in South America or Australia. It has been reported as a pest of grapes, orchard crops, and tomatoes, and in temperate regions such as Great Britain, it can also be a pest in greenhouses. Similarly, the longtailed mealybug has a worldwide distribution in tropical and subtropical regions, as well as being a pest in glasshouses in temperate zones. In addition to grapes, it has been reported in various regions of the world as a pest of citrus, pome fruits, avocados, and indoor and outdoor plantings of ornamental plants. In contrast, grape mealybug, thought to be native to North America, has a more restricted geographic range, being found primarily in North and South America, with additional reports from Poland, Armenia, and Indonesia. In the United States, it infests grapes, pome fruit, apricots and pomegranates.

Passionvine mealybug is widely distributed throughout many tropical and subtropical regions of the world, including South and Central America, the Caribbean countries, and many parts of Asia. It has only recently been detected in low numbers in the mainland United States, in Florida (*30*). It has a very broad host range on a number of economically important plants, including cocoa, grapes, citrus, tree fruits, and corn.

The albopicta scale has been reported from several South and Central American countries, but it is not yet known to be established in the United States. It is known to infest citrus and some ornamental plants, and particularly, avocados. It is of concern as a potential threat to the California avocado industry because it is arriving in California in large numbers on shipments of fresh avocados from Mexico.

Following identification and development of syntheses for the pheromones of vine, obscure, longtailed, and grape mealybugs, numerous field trials have shown that these compounds are excellent additions to the integrated pest management (IPM) toolbox. These pheromones share several desirable characteristics for practical applications. First, they are all very powerful attractants for male mealybugs, so that even small populations can be detected. Second, standard rubber septum type pheromone lures loaded with only 25 micrograms of racemic pheromone remain attractive for several months under field conditions (*2*, *16*, *48*, *49*), minimizing the number of lure changes required throughout one season. Third, these very small effective doses will help to keep lure manufacturing costs down. These pheromones have now been used for detection and monitoring one or more of these mealybug species in North and South America, Europe, the Middle East, Australia, New Zealand, and South Africa. Field trials have also demonstrated that the pheromones of several species can be combined with minimal ill effects to provide lures for several species simultaneously (*16*). Such "generic" lures may find use in cropping systems such as nursery production of ornamental plants, where growers are primarily interested in whether or not they have mealybug infestations, rather than the particular species of mealybugs which are present, because control practices for all species are the same. In the case of invasive species that are not yet established in the U.S., such as the passionvine mealybug and the albopicta scale, the availability of pheromones will provide

regulatory agencies responsible for pest detection and exclusion with highly sensitive and selective methods of detecting incursions of these insect pests. Recent work using pheromone baited traps for detection and monitoring of the invasive passionvine mealybug in the Caribbean and Florida has demonstrated the value of these detection methods, with traps being used to detect the very low population densities characteristic of the beginning of a new infestation (*30*).

The testing and use of pheromones for monitoring mealybugs in California vineyards for more than a decade has revealed only very few downsides or problems with the use of this technology. In general, pheromone lures are highly sensitive and selective, easy to use, and stable for extended periods under field conditions. There have been anecdotal reports of possible cross-attraction of rye grass mealybug, *Phencoccus graminicola*, to vine mealybug pheromone, but these have not been confirmed with a proper study to determine whether they might have been random catches in areas with high populations of this species. The only other issue with using pheromone traps for detection and monitoring mealybugs is that they are almost too sensitive, i.e., it has been shown that male mealybugs can be caught in traps placed many meters from the nearest site of infestation (*2*), in part because the very small and fragile males can be carried some distance by the wind. Thus, trap catches can indicate the presence of mealybugs in the general area, but do not necessarily indicate the presence of an infestation in the vineyard in which the trap was placed. Follow-up visual surveys may be required to locate and determine the extent of infestations.

Pheromone lures for vine, obscure, longtailed, and grape mealybugs are now available from commercial suppliers (e.g. Suterra LLC, Bend, Oregon and Trécé Inc., Adair, Oklahoma), providing highly sensitive and selective methods for detection and monitoring of these major agricultural pests. Published studies detailing the use of these pheromones for mealybug detection and monitoring in vineyards (*2*, *48*, *49*, *56*) and other crops (*16*) are now starting to appear in the literature. The ready availability of these sensitive monitoring methods could not have come at a better time, because these insects vector leafroll virus and other diseases that are rapidly increasing in importance in vineyards in the western U.S. and worldwide, particularly for high quality red wine grapes (*50–53*).

Pheromones of a number of insect species have also been developed for control of insects in various crops, using mating disruption or mass trapping (*54*). However, pheromone-based methods of control are contingent upon the availability of multikilo to metric ton quantities of the pheromones at an affordable cost. To date, the vine mealybug is the only species among mealybug and scale insects for which large-scale commercial pheromone syntheses are available. Field experiments demonstrated the potential for controlling vine mealybug in vineyards, particularly when starting with relatively low populations in spring (*55*, *56*). A commercial mating disruption product developed by Suterra LLC (Bend, Oregon) has been used on tens of thousands of acres of wine grapes in central California for the past several years, and the acreage treated every year has been growing (*53*).

Conclusions

Research on the pheromones of mealybug and scale species has proven fruitful in terms of both basic and applied science. From the basic science aspect, the fascinating irregular terpenoid structures of the pheromones have proven challenging to identify and synthesize. There are further possibilities for advancing basic science by working out the biochemistry and molecular biology of pheromone biosyntheses, and particularly, the identification of the enzymes involved in the formation of the novel cyclobutane and cylopentane ring structures. It is also certain that there are additional novel structures waiting to be discovered in species whose pheromone chemistry has not yet been examined. The development of shorter and cheaper syntheses also represents a substantial challenge for organic chemists.

From a practical viewpoint, the development of highly sensitive and selective pheromone-based methods for detection of invasive species, and simple and straightforward monitoring methods for endemic species, has provided valuable new tools for management of these insects and indirectly, the pathogens that they transmit.

Acknowledgments

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Chapter 10

Personalized Pesticides – A New Paradigm

Case Study: Volatilization of Individual Components of Botanical Insect Repellents from Human Skin

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The idea of using generic substances for blanket management of arthropod pests has been long pursued by pesticide manufacturers despite various factors that affect efficacy of chemical pesticides. Plant essential oil-based pesticides are generally considered as safer alternatives for conventional pesticides and represent a relatively new class of natural pesticides efficacious against a wide range of pests. Unlike conventional pesticides, botanical pesticides consist of several active and inactive components that can chemically synergize or suppress each other, as well as affect physical properties of botanical pesticides such as rate of volatilization. Results from preliminary human trials that explored volatilization of individual components of a botanical insect repellent from human skin will be presented. In addition, variable patterns of volatilization of individual components of botanical insect repellents over time will be discussed, as well as their possible relation to the subjects' gender, ethnicity and skin condition.

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For more than six decades pest management programs extensively relied on toxic synthetic chemical pesticides. Several classes of pesticides have evolved over this period, from the chlorinated hydrocarbons to the organophosphates, carbamates and pyrethroids and most recently to the neonicotinoids (*1*). These pesticides have been favored by growers for many years due to their strong efficacy against various pests in large-scale agricultural practices. However, overuse of these products and their resilience not only rendered many of them ineffective – due to target resistance – but also imposed dire long lasting environmental and health risks to non-target organisms (*2*). Concerns over their negative impacts have lead to increasingly restrictive regulation of synthetic chemical pesticides and a new era of exploration for safer alternatives (*3*). Natural products, specifically botanical pesticides based on plant essential oils, have been at the center of attention as safer alternatives for synthetic chemical pesticides.

Plant essential oils are complex mixtures of monoterpenes, sesquiterpenes, phenols and other compounds. Some constituents of essential oils are low molecular weight volatile compounds that account for the fragrances of the oil. Several studies have demonstrated contact and fumigant toxicity of various terpenoids found in plant essential oils against arthropod pests (*4*). It has been shown that presence or absence of certain constituents in a mixture could significantly affect the efficacy of essential oil-based botanicals as contact pesticides (*5*). When rosemary oil was tested as a repellent against two-spotted spider mite (Figure 1), it was discovered that major constituents do not evaporate at the same rate and the composition of volatiles in the air, evolve over time (*6*). In the case of rosemary oil (Figure 1), some constituents were only present in the headspace after the level of other constituents decreased (i.e., d-limonene and camphene versus 1,8-cineole and camphor).

Volatilization of Botanical Insect Repellents From Human Skin

To analyze volatilization of selected essential oils from human skin, a zNose, an ultrafast portable gas chromatograph, was used (*7*). The zNose system was tuned with an *n*-alkane solution and calibrated with neat reagents prior to its application in each experiment. The zNose inlet, valve and initial column temperature were set at 200 °C, 165 °C, and 40 °C, respectively. During analyses, the column temperature was increased at 10 °C/sec to 200 °C. The surface acoustic wave (SAW) sensor was kept at 60 °C and the trap was kept at 250 °C. The helium flow during the 10 sec sampling period was set at 3.00 mL/min. The sampling period was set for 10 sec at a sample flow of 20 mL/min, after which the system switched to 20 sec of data acquisition. Thereafter, the sensor was heated to 150 °C for 30 sec, and parameters (see above) were reset. 0.25 mL of rosemary essential oil, as insect repellent, was applied on forearm of two human subjects and volatilizations of constituents were measured for 120 minutes (Figure 2).

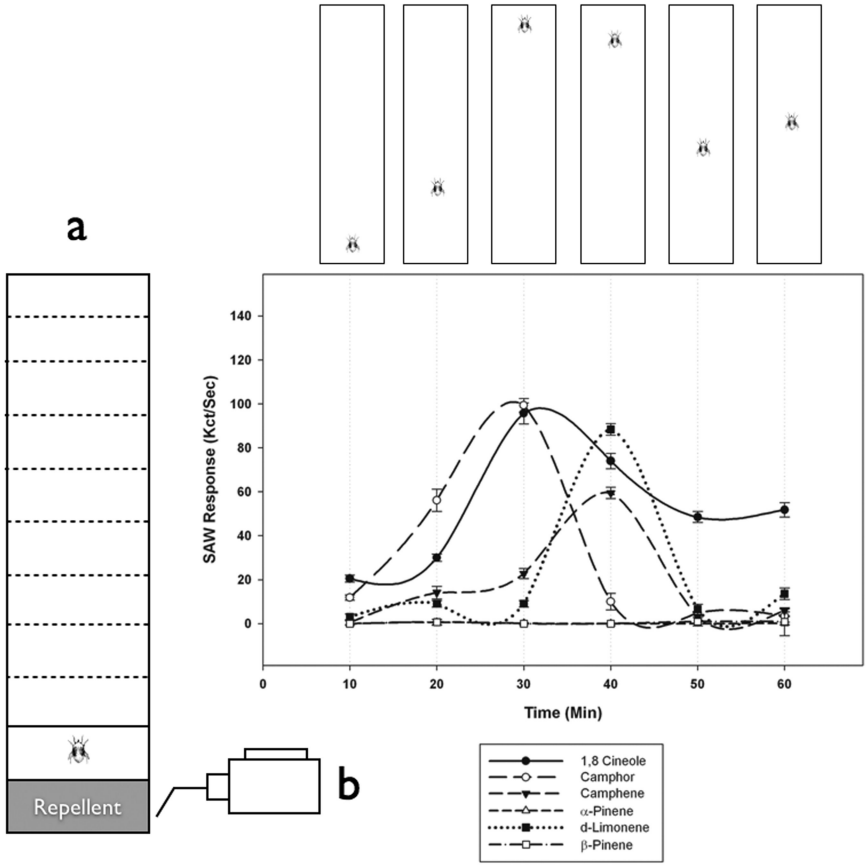

Figure 1. Comparing volatilization pattern of rosemary oil major constituents with position of naïve spider mites in a test arena over 60 minutes. (a) test arena, (b) zNose. Error bars represent mean ± SE.

Results of this pilot study supported previous observations. Major constituents did not evaporate at the same rate and the composition of volatiles in the air changed over time. However, another interesting phenomenon was also observed as a result of this pilot study. There was a significant difference in the overall volatilization pattern of the rosemary oil from the skin of the male subject compare to the female subject used in the pilot study. To better understand this phenomenon, a human trial was conducted with subjects of different gender, ethnic background (Caucasian, Indian, Asian, Middle Eastern), age (25-35) and skin condition (normal, dry, hairy) (n=18). Specific demographic details of

subjects are not presented here following the guidelines of UBC ethics board. Experiments were conduced in a laboratory under controlled conditions. Prior to each test, subjects washed their arms with unscented soap and lukewarm water and sat down for 30 minutes to bring down their heart rate to resting level. Each subject then received a single dose (0.25 mL) of a commercial botanical insect repellent (EcoSMART Insect Repellent) on his or her forearm (Figure 3).

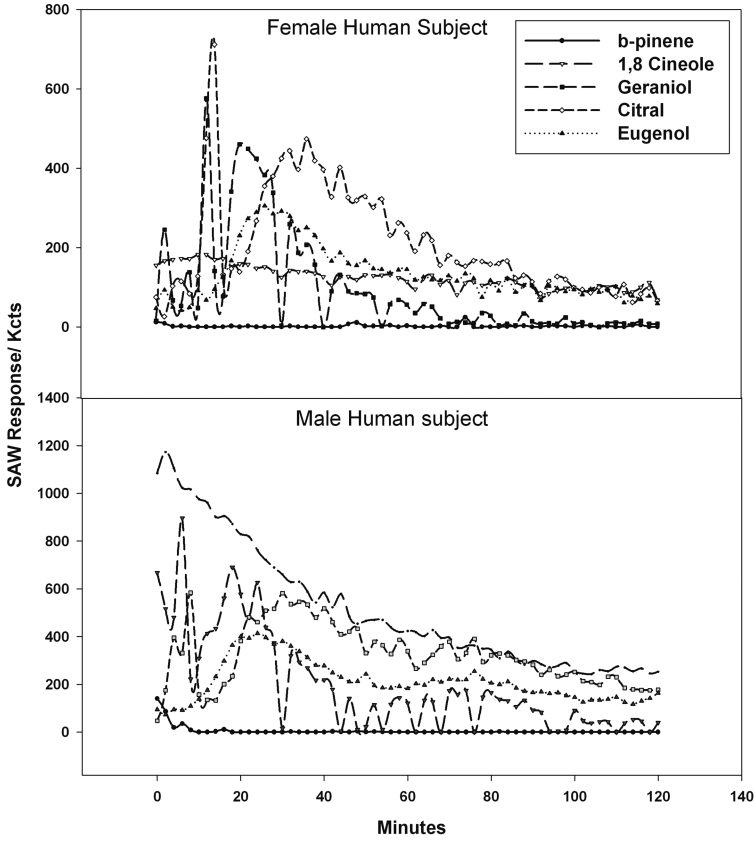

Figure 2. Volatilization of rosemary oil major constituents from human skin.

Subjects' arms were then placed on a clean laboratory desk. The zNose was positioned to collect volatiles at 10 cm above the skin of the subject every 3 minutes for one hour with the same parameters used in the pilot study. Subjects' skin surface temperature and pH were measured by a thermal scanner and pH meter. Changes in the patterns of volatilization were analyzed by generalized

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estimated equations (GEE) regression using R statistical analysis software (www.r-project.org). Various volatilization patterns were observed in relation to the subjects' gender, ethnicity and skin color and condition (Figure 4).

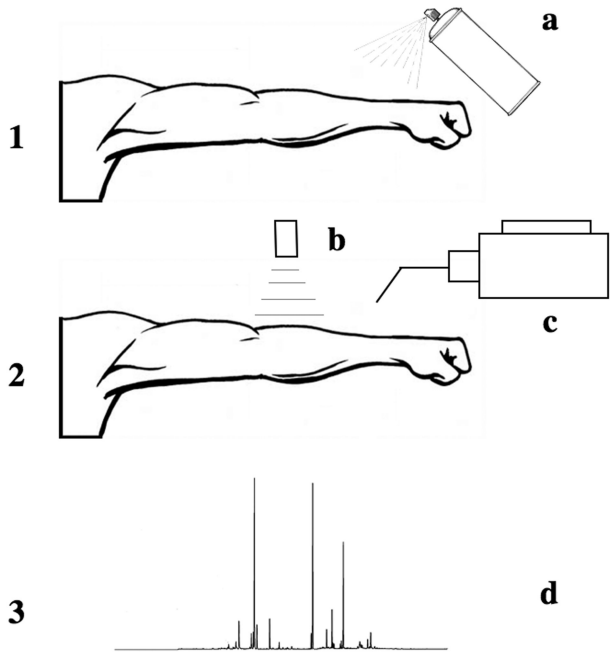

Figure 3. Volatilization of a commercial insect repellent from human skin. (a) insect repellent, (b) thermal scanner, (c) zNose, (d) chromatogram.

The results of this preliminary human trial indicated differences existed in the volatilization patterns of an individual product used on subjects of different genders, ethnic backgrounds, and skin conditions. However, because of the small sample size and great variation that existed among subjects, the results of this initial trial are not statistically powerful enough to make a strong correlation between either of those factors and different volatilization patterns. Larger standardized clinical trials (minimum of 1,000 subjects as it is customary in trials of this nature) with greater control over variables such as diet and exercise regime, sleep, drug intake, age group, etc. are necessary to fulfill experimental requirements for more definitive results.

Despite the statistical shortcomings of this trial, the observed differences can perhaps encourage a different approach towards production of complex botanical pesticides. The results also suggest that a "one size fits all" approach is no longer a viable option and that generic formulations cannot provide the blanket management of all target pests under all conditions – particularly in the case of insect repellents. What these results imply in practical terms is that a new

method of botanical pesticide manufacturing is needed to cater the needs of end users with specific biological features, hence the introduction of "personalized pesticides" as a new paradigm (Figure 5).

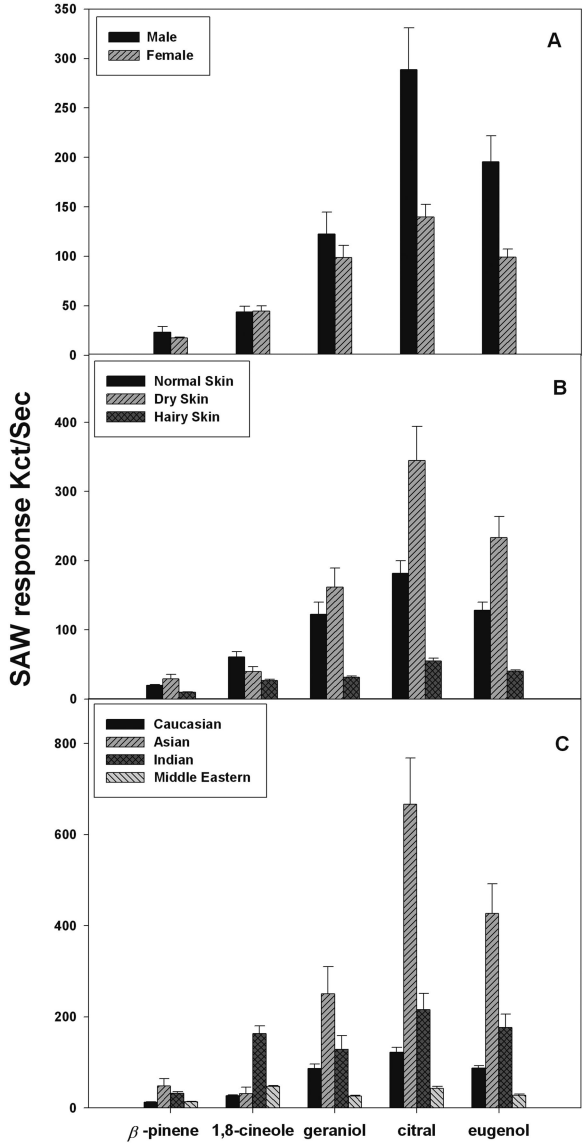

Figure 4. Various volatilization patterns of major constituents of insect repellent relative to the subjects' gender (a), skin condition (b), and ethnic background (c). Error bars represent mean ± SE (n=18).

Our understanding of how end users' biological features could affect volatilization of botanical insect repellents is limited at the moment. In the case of botanical pesticides, it is entirely possible that few features play a major role in efficacy of the botanical pesticides and thus enable manufacturers to develop specific types of pesticides that best match the needs of specific users. Such modifications and customizations have long been performed for cosmetics and hygiene products. In the era of cloud-based computation, it should be possible for end users to order and customize their specific personalized insect repellent via their smartphone by entering few key biological features that help the manufacturers to formulate them the best possible formulation that match their unique biological attributes.

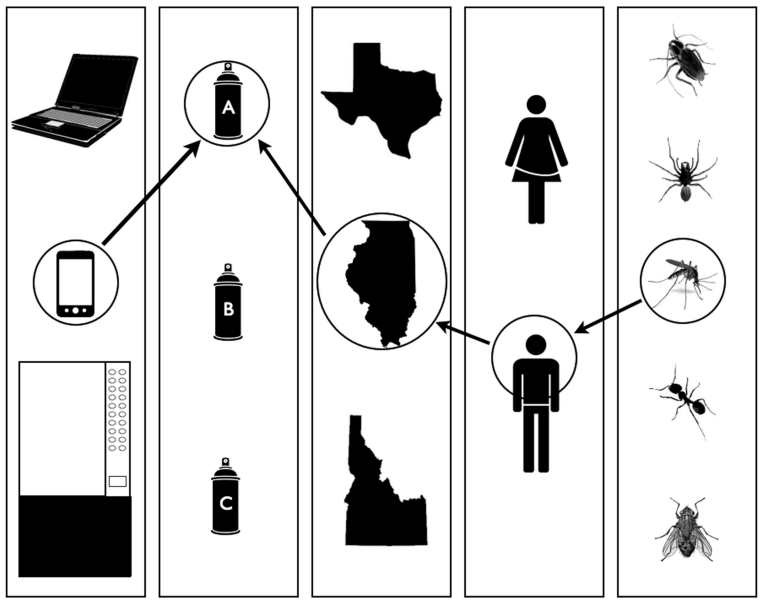

Figure 5. Proposed model of "personalized pesticide" production.

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Chapter 11

Microbial and Plant Metabolites as Potential Herbicides for the Control of Parasitic Plants

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Broomrapes (*Orobanche* and *Phelipanche* spp.) and dodder (*Cuscuta* spp.) attack strategic food crops. Their continuous spread limits the choice of rotational crops often forcing farmers to give up growing the most profitable host crops. In 2004 the annual food crop losses due to broomrape infestation were reported at 1.3-2.6 billion dollars. Difficulties in the control of parasitic plants arise from their physiological traits and life cycle. Environmentally friendly strategies for their control are based on the use of phytopathogenic fungi and/or the phytotoxic metabolites produced by these microbes. Plant phytotoxins may also contribute to competition and invasiveness of these parasite plants by suppressing the growth of neighbouring plant species. An alternative green solution is the suicidal germination of parasitic plant seed caused by fungal or plant root exudate metabolites.

Introduction

Broomrapes (*Orobanche* and *Phelipanche* spp.) and dodder (*Cuscuta* spp.) attack strategic food crops, and threaten the livelihood of many nations. The continuous spread of broomrapes and dodder limits the choice of rotational crops and often forces farmers to give up growing the most profitable host crops. As a result of broomrape infestation annual food crop losses in the Mediterranean area in 2004 were estimated at about $1.3 to 2.6 billion (*1*). Difficulties in the control

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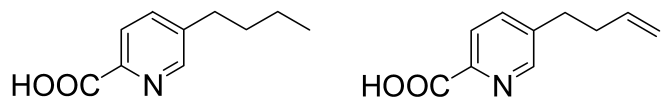
of parasitic plants arise from their physiological traits and life cycle. Traditional methods used to control parasitic plants have included crop rotation; delay in the sowing date; seed germination stimulants or inhibitors; plant breeding; use of catch and trap crops; soil solarisation; soil amendments; and chemical control by herbicides. However, effective management of parasitic weeds is very difficult (*2*, *3*).

Considering that seed germination is a key phase for parasitic plant development and infestation, a further approach for the management of these weeds has been proposed to use natural metabolites produced by some microorganisms (*4*) and by plant species as seed germination inhibitors. Indeed, plants may compete by suppressing the growth of neighbouring plants by the production and release of allelopathic compounds (*5*). Many phytotoxins of plant-origin are reported as allelochemicals, and for some of them the mode of action has also been studied (*6*). Environmentally friendly strategies are based on biological control using phytopathogenic fungi and/or the phytotoxic metabolites produced (*7*, *8*). An alternative ecologically-friendly solution is the suicidal germination of seeds of parasitic plants caused by application of fungal or plant root exudate metabolites that stimulate germination in the absence of the host plant.

Fungal and Plant Metabolites as Potential Herbicides

Identification of Phytotoxins Produced by *Phelipanche ramosa* Fungal Pathogens

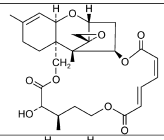
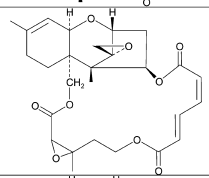
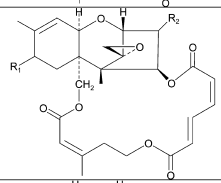
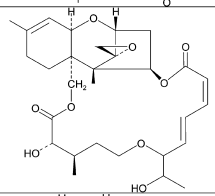
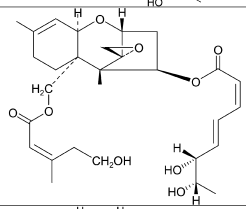
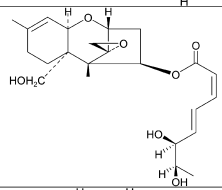
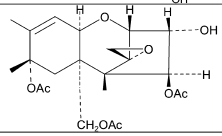
In a study (*9*) conducted in 2004, nine fungal strains (out of fifty three) mainly *Fusarium*, obtained from diseased *Phelipanche ramosa* L. samples were highly virulent, and 18 strains were positive for the production of fusaric and dehydrofusaric acids (**1** and **2**, Figure 1) at concentrations from 4 to 165, and from 9 to 204 mg L^{-1} in fermentation culture, respectively. Five extracts from liquid cultures caused total inhibition of seed germination, whereas fifteen extracts from the solid cultures of fungal strains did not cause high mortality when 0.5 ml for each organic extract was assayed on brine shrimp at a concentration of 1% in artificial sea water. In the same study, some of the screened strains were considered as new bio-control agents, but their virulence was not always positively correlated with the production of the two known phytotoxic compounds (*9*). Hence, they probably produce different phytotoxic metabolites.



Fusaric acid, **1** Dehydrofusaric acid, **2**

*Figure 1. Stuctures of fusaric and 9,10-dehydrofusaric acids (**1** and **2**).*

Table I. Trichothecenes produced by *Myrotecium verrucaria* and *Fusarium compactum* in liquid culture and their phytotoxic and zootoxic activity, assayed on *Phelipanche ramosa* seeds and *Artemia salina* brine shrimp

Name	Compound[c]	Activity					
		P[a]	Z[b]	P	Z	P	Z
		10 μg		1 μg		0.1 μg	
verrucarin A (**3**)		100	100	73	84	nt[d]	68
verrucarin B (**4**)		100	nt	59	nt	nt	nt
verrucarin L acetate R_1=OAc, R_2=H (**5**)		100	100	88	100	24	81
verrucarin M R_1=H, R_2=OH (**6**)		100	100	10	100	3	50
Roridin A (**7**)		100	100	100	76	nt	21
Isotrichoverrin B (**8**)		66	67	0	15	0	8
Trichoverrol B (**9**)		19	15	15	5	0	5
neosolaniol monoacetate (**10**)		100	100	100	41	0	8

[a]P = phytotoxicity, expressed as inhibition (%) of *P. ramosa* seed germination, [b]Z = zootoxicity, expressed as mortality (%) of *A. salina* larvae, [c]Neosolaniol monoacetate is produced only by *F. compactum*, all the other metabolites are produced only by *M. verrucaria*. [d]nt = not tested.

Two interesting fungi, *Myrothecium verrucaria* (Alb. & Schwein) Ditmar and *F. compactum* Gordon, were selected from the previous study and their biologically-active natural compounds were investigated (*10*). When grown in liquid culture, these two strains were able to produce metabolites that inhibit the germination of *P. ramosa* seeds. Eight metabolites were isolated from the organic extracts of *M. verrucaria* culture filtrates. The main metabolite was identified as verrucarin E, which is a disubstituted pyrrole. Seven other compounds were also identified by spectroscopic methods as macrocyclic trichothecenes, namely, verrucarins A, B, L acetate, M, and, roridin A, isotrichoverrin B and trichoverrol B (**3**-**9**, Table I). Verrucarins A, B, M, and L, isotrichoverin, and trichoverrol B were produced in very low amounts (between 0.8 and 3.10 mM) from *M. verrucaria* together with roridin A and verrucarin E (around 16 and 474 mM, respectively) (*10*). Because reference samples were not available, the chemical identification of verrucarins A, B, L acetate, M, and E, roridin A, isotrichoverin B, and trichoverrol B was essentially performed using spectroscopic methods and comparing the data obtained with compounds already reported in previous literature (*10*). In the same study (*10*), the main metabolite (also a trichothecene) produced in liquid culture of *F. compactum* was isolated and characterized as neosolaniol monoacetate (**10**, Table I).

All the metabolites assayed at 100 µM caused a total inhibition of the P. *ramosa* seed germination. At 10 µM, all the trichothecenes were still highly active, causing total inhibition of seed germination, except for isotrichoverrin B, which was slightly less active, and trichoverrol B, which was almost inactive (Table I). The phytoxicity of neosolaniol monoacetate and roridin A was particularly noteworthy at 1 µM, as they caused the total inhibition of seed germination. Verrucarin L acetate, verrucarin A, and verrucarin B were still quite active, all causing more than 50% inhibition of germination (Table I). In the zootoxicity test on brine shrimp (Table I), all the metabolites, except verrucarine E, caused 100% mortality of larvae at 100 µM. Trichoverrol B was almost inactive when assayed at 10 µM, whereas isotrichoverrin B proved to be slightly less toxic compared to the other metabolites (except verrucarin E) (*10*).

Metabolites with Herbicidal Activity Isolated from Aerial Parts of *Inula viscosa*

In a recent attempt to find novel bioactive metabolites for use as natural and safe herbicides for parasitic weed management, the organic extracts of 10 Mediterranean plants were evaluated for the inhibitory effects to seeds of field dodder (*Cuscuta campestris* Yuncker) and crenate broomrape (*Orobanche crenata* Forsk.) (*11*). Among them, the extract obtained from *Inula viscosa* (L.) Aiton was one of the most active. Further investigations were carried out in order to purify and identify the bioactive compound(s).

I. viscosa (syn. *Cupularia viscosa* G. et G., *Dittrichia viscosa* Greuter, family Asteraceae) commonly called sticky fleabane is a perennial weed native of the Mediterranean basin (*11*). This plant was previously reported as a source of a fungicidal preparation against foliar diseases of some important crops, including cucumber, tomato, potato, wheat and sunflower (*12*). *I. viscosa* is also used

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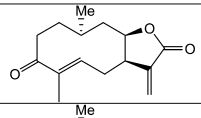
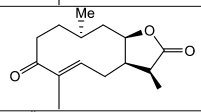
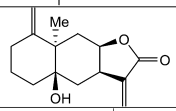
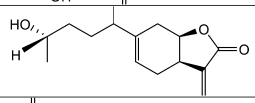
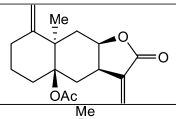
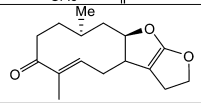
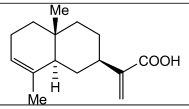
in folk medicine in the Mediterranean area, and is well known as a source of pharmacologically active metabolites (*13*).

Four new phytotoxic bi- and tri-cyclic sesquiterpenes, named inulinoxins A-D, were isolated together with the already known one (α-costic acid) from the plant aerial parts (*14*).

Table II. Metabolites from *Inula viscosa* and derivatives and their effect on seeds of parasitic weeds[a]

Name	Compound	Phytotoxic activity		
		Cuscuta campestris	*Orobanche crenata*	
		Seed germination (%)[b]	Seed germination (%)[b]	Germ tube elongation (μm)[b]
inuloxin A (**11**)		0 d	0 d	0 d
inuloxin B (**12**)		34 b	40 c	43 b
inuloxin C (**13**)		2 d	0 d	0 d
inuloxin D (**14**)		0 d	0 d	0 d
5-*O*-acetyl inuloxin C (**15**)		15 c	24 c	24 c
diazo derivative inuloxin A (**16**)		55 a	40 b	33 b
α-costic acid (**17**)		15 c	57 a	77 a
control (water)		55 a	34 bc	82 a
Control (water + methanol)[c]		62 a	36 bc	n.t.

[a]Inuloxin A-D and costic acid were assayed at $1.6x10^{-3}$ M and $1.7x10^{-3}$ and at $4.0x10^{-4}$ M and $4.3x10^{-4}$ M on *C. campestris* and *O. crenata*, respectively.

[b]Values within each column followed by different letters are significantly different (Duncan-test at $p \leq 0.05$). Mean of 3 replicates.

[c] In the case of assays on *O. crenata*, controls contained also the synthetic stimulant GR24 (see literature below, *24*).

The structures of inulinoxins A-D (Table II, **11**-**14**) were established by spectroscopic and chemical methods and determined to be: (4*E*,7*R**,8*R**,10*S**)-3-oxo-germacra-4,11(13)-dien-8β-12-olide (A), its 11,13-dihydro analogue (B), (5*R**,7*R**,8*R**,10*R**)-1,15-methylene-5β-hydroxy-eudesm-1(15),11(13)-dien-8β-12-olide (C), and (7R*,8*R**)-1,4-dimethyl-4-hydroxy-secoeudesm-5(10),11(13)-dien-8β-12-olide (D). By applying an advanced Mosher's method, the *S* absolute stereochemistry at C-5 of 5-hydroxyhexan-2-yl side chain of inuloxin D was assigned (*14*).

The phytotoxic activity of inuloxins A-D, that of the diazo and monoacetyl derivatives of inuloxin A and C, respectively (**15** and **16**, Table II), as well as that of α-costic acid (**17**, Table II) was evaluated against crenate broomrape (*O. crenata*) and field dodder (*C. campestris*) (Table II).

Inuloxins A, C and D were the most active on both parasitic plants and achieved up to 100% inhibition of the seed germination. Inuloxin B showed less activity on *Cuscuta* and was completely inactive against *Orobanche* (Table II). The main metabolite α-costic acid showed a suppressive effect on the dodder seed germination but had a stimulating activity on broomrape seed germination. On *Cuscuta*, the acetyl derivative of inuloxin C (**16**) showed strong germination inhibition, while significantly reducing the germination of crenate broomrape. Finally, the diazo derivative of inuloxin A (**16**) was completely inactive when tested on both parasitic plants.

These results led to hypothesize that, among the inuloxin group, the tetrasubstituted furanone ring is a structural feature with importance for the compound's activity, whereas the other different structures linked to this ring seem to be much less associated with their biological activity. The presence of the exocyclic methylene group in the furanone ring plays a particular role for the activity, because when it was saturated, as for inuloxin B, it resulted in the complete loss of activity. A role was also played by the tertiary hydroxy group at C-5 of inuloxin C as its acetylation, a reversible modification, induced a partial loss of activity (*14*).

Fungal and Plant Exudate Metabolites Inducing Suicidal Seed Germination of Parasitic Plants

Considering that the seed germination of parasitic plants depends on the presence of stimulating exudates produced by the roots of the host plant, an alternative approach for the management of parasitic host plants is the so called "suicidal germination". The latter approach comprises the induction of seed germination through the application of a germination stimulant into the soil, causing seeds to germinate in the absence of the host. Resulting seedling will then die within few days as they run out of stored nutrient, resulting in a reduction of the seed bank in soil.

Much attention has therefore been focused on the isolation and identification of germination stimulants from root exudates of host and non-host plants (*15*) as well as fungal metabolites that showed this peculiar acticvity. Among several fungal metabolites tested with the aim of finding new natural stimulants, Yoneama

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and co-authors (*16*) reported in 1998 that cotylenins and fusicoccins, two groups of closely related glucosylated diterpenoides, induced high seed germination (>50%) of *Striga hermonthica* (Del.) Benth and *O. minor* Smith at concentrations as low as 10^{-5} M.

Stimulation of Seed Germination of *Orobanche* Species by Ophiobolin A and Fusicoccins Derivatives

Fusicoccin A (**18**, FC, Figure 2) (*4*) stimulates seed germination. FC is the major α-glucoside of a carbotricyclic phytotoxic diterpenoid produced by *Fusicoccum amygdali* Delacr., the causative fungal agent of peach and almond canker, isolated in 1962 and structurally described in 1968. Since then, many studies have been carried out on the chemical, biosynthetic, and biological properties of this toxin as well as on structure-activity relationships (*4*).

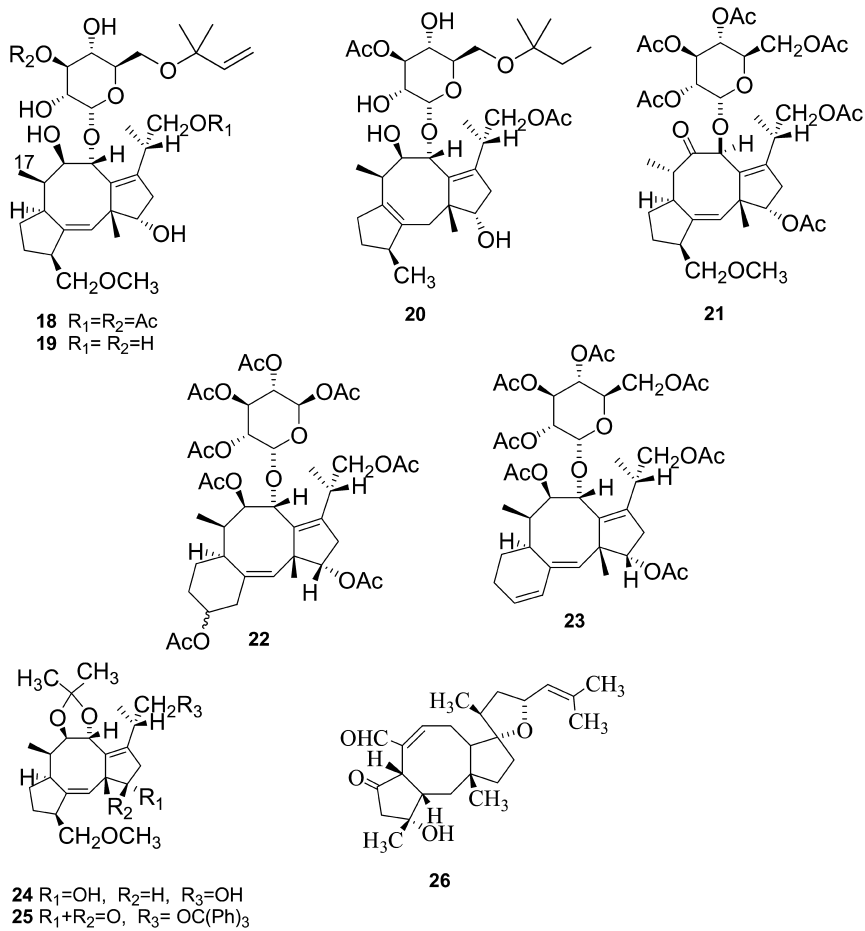


*Figure 2. Structures of fusicoccin, its deacetyl aglycone and some their derivatives (**18-25**) and ophiobolin A (**26**).*

Ophiobolins are sesterterpenoid phytotoxins closely related to fusicoccins and cotylenins and are produced by the pathogenic *Bipolaris* species, which usually infect rice, maize and sorghum. (*17*). Recently, ophiobolins were also isolated from a strain of *Drechslera gigantea* Heald & Wolf proposed for the control of *Digitalia sanguinalis* Scop. (*18*, *19*).

Considering the availability of several derivatives and natural analogues of fusicoccin and its aglycone, as well as of cotylenol, the aglycone of all cotylenins, a structure-activity study was carried out using the seeds of another parasitic plant species, *O. ramosa*, which proved to be useful in a preliminary screening. Some of the compounds, tested at concentration of 10^{-4} and 10^{-5} M, proved to be highly active, such as the 8,9-isopropylidene of fusicoccin deacetyl aglycone and the dideacetyl derivative of FC. In both groups of glucosides and aglycones (including cotylenol), the most important structural feature for activity appears to be related to the hydroxyl group at C-19 (*20*).

Considering these results and that of the FC ability in stimulating seed germination of parasitic plant, such activity could be species dependent, a further study was carried out to test the effect of some FC derivatives (**19**-**25**, Figure 2) and ophiobolin A (**26**, Figure 2) on seed germination of different *Orobanche* species namely *Phelipanche aegyptiaca* (Pers.) Pomel (syn. *O. aegyptiaca* Pers.), *P. ramosa*, *O. crenata*, *Orobanche cumana* Wallr., *Orobanche densiflora Salzm.*, *Orobanche foetida* Poir, *Orobanche gracilis* Sm., *Orobanche hederae* Duly, and *Orobanche minor* Sm. (*21*).

The results obtained showed that the stimulation of seed germination is species dependent and it is also affected by the stimulant concentration. The highest stimulatory effect was observed for ophiobolin A and the hexacetyl and pentacetyl isomers of 16-*O*-demethyl-de-*tert.*-pentenylfusicoccin (**22** and **23**, Figure 2), and the most sensitive plant species appeared to be *P.. aegyptica*, *O. cumana*, *O. minor*, and at lesser extent, *P. ramosa* (21).

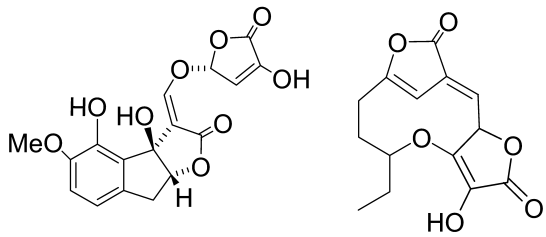
The fusicoccin derivatives **22** and **23** and ophiobolin A (**26**) could be considered potential herbicides in view of their practical application in agriculture for the biocontrol of parasitic *Orobanche* and *Phelipanche* species.

Stimulant from Root Exudates of *Orobanche* Host Plant

Among the metabolites isolated from root exudates of host and non-host plants more attention was directed to three different classes of plant secondary metabolites, dihydrosorgoleone, sesquiterpene lactones, and strigolactones (*22*) which are known to induce seed germination of these parasitic weeds, with strigolactones showing the strongest activity. Different strigolactones were isolated from *Orobanche*, *Phelipanche* and *Striga* host and non-host plants (*23*). Sorgomol was isolated from root exudates of sorghum (*Sorghum bicolor* L.), and fabacyl acetate from root exudates of pea (*Pisum sativum* L.) (*23*). In the root exudates of the same plant, well known strigolactones, namely didehydro-orobanchol, orobanchol, orobanchyl acetate and 5-deoxy-strigol were also identified (*22*).

Metabolites from Pea Root Exudates

More recently, two new strigolactone-like metabolites, named peagol and peagoldione (**27** and **28**, Figure 3), were isolated from the pea (*P. sativum* L.) root exudates of the same plant grown in Spain (*23*). Their structures were determined using spectroscopic methods and the two metabolites (**27** and **28**) characterized as 3a,4-dihydroxy-3-(4-hydroxy-5-oxo-2,5-dihydro-furan-2-yloxymethylene)-5-methoxy-3,3a,8,8a-tetrahydro-1-oxa-cyclopenta[a]inden-2-one and as 9-ethyl-6-hydroxy-4,8,13-trioxa-tricyclo[10.2.1.1.0*3,7*]pentadeca-1,6,12(15)-triene-5,14-dione, respectively. Furthermore, a significant nuclear Overhauser effect NOE effect observed between H_2-10 with H-2 suggested, in agreement with an inspection of Drieding model of **28**, a bended conformation of the A ring with the CH_2-10 pointed towards C-2 (*24*).



27 **28**

*Figure 3. Structures of peagol (**27**) and peagoldione (**28**).*

Peagol induced *P. aegyptiaca* and *O. foetida* seed germination when tested at a concentration of $5x10^{-4}$ M but low activity was observed on *O. crenata* and *O. minor*. Peagoldione tested at $2x10^{-3}$ M induced *P. aegyptiaca* seed germination only, with no activity on *O. crenata* or *O. minor*, and a very little one on *O. foetida*. The synthetic strogolactone GR24 stimulatory effect generally assumed for all broomrape species (*25*) is ineffective on some broomrape species such as *O. foetida*. Activity of peagol on *O. foetida* seed germination is characteristically relevant as no germination stimulant for this species was known. Specificity of the activity of peagol and pegoldione is in agreement with specificity on host recognition by *Orobanche* and *Pheliphanche* (*26*).

The specific activity of these two new strigolactone-like metabolites (**27** and **28**), which showed a lower and specific activity on different *Orobanche* and *Phelipanche* species, is probably due to the very unusual structures of **27** and **28** that differentiate them from the well known and highly active strigolactones previously isolated from the same plant.

Successively, five other metabolites were isolated as homogeneous amorphous oil and solids (**29**-**33**, Figure 4) (*27*). Three of them were new polyphenols and were named peapolyphenols A-C (**30**-**32**). The other two (**29** and **33**) appeared to be a polyphenol and a chalchone which have previously been reported (*28*, *29*). The first one (**29**) was identified as

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the 1-(2,4-dihydroxyphenyl)-3-hydroxy-3(4-hydroxyphenyl)-1-propanone (β-hydroxy-DHP). It was previously isolated from the licorice root (*Glycyrrhiza glabra* L.), licorice is a herb commonly used in cancer treatment, together with other polyphenols and chalchones and some of their glycosides (*28*). The chalchone (**33**) was identified as the 2′,4′-dihydroxy-4-methoxychalchone, previously isolated together with some flavonoids from *Oxytropis falcata* Bunge, a wild growing Leguminosae plant mainly distributed in the Qinghai-Tibet Plateau, China (*29*).

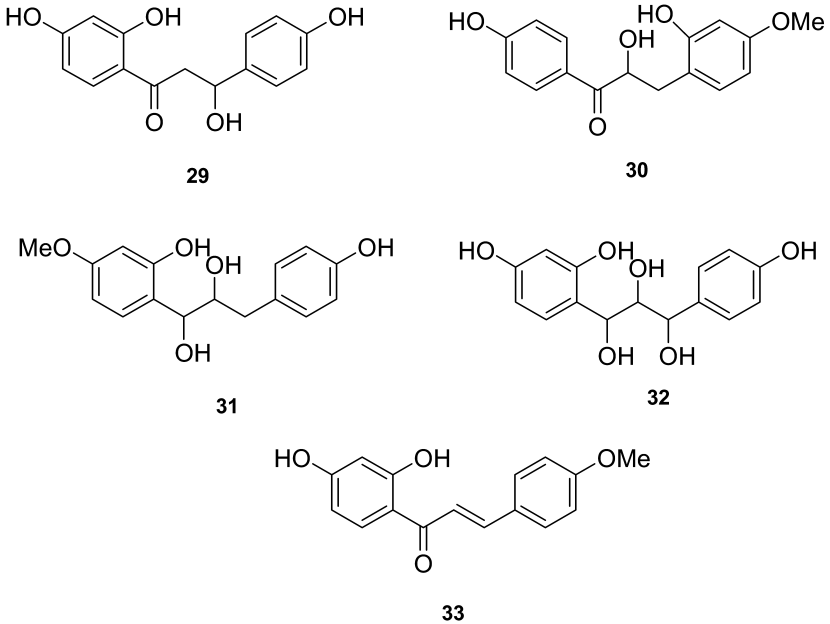

*Figure 4. Structures of peapolyphenols A-C (**30-32**), and 1-(2,4-dihydroxyphenyl)-3-hydroxy-3-(4-hydroxyphenyl)-1-propanone and 1-(2,4-dihydroxyphenyl)-3-(4-methoxyphenyl)-propenone (**29** and **33**).*

Pea root exudates can potentially induce the germination of all species tested similar to its crude ethyl acetate extract (*26*). Pea crops are highly damaged by *O. crenata* infestation, however it is little or not at all infested by *O. foetida*, *O. minor* and *P. aegyptiaca*. This observation suggested that its root exudates could induce the suicidal germination of these three important parasitic weeds (*30*). Polyphenols **29**, **30** and chalchone **33** induced seed germination of *O. foetida* when tested at 10^{-3} M with reduced activity at 0.5×10^{-3} M and inactive at lower concentrations. No activity was observed on *P. aegyptiaca*, *O. crenata* or *O. minor*. Polyphenols **31** and **32** did not show any activity on any of the *Orobanche and Phelipanche* species tested. The activity of **29**, **30** and **33** on *O. foetida* seed germination seems to be

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very important because as reported above, no germination stimulants, including the synthetic strigolactone GR24 (*25*), for this species were known, apart from the recently isolated peagol and peagoldione (*24*).

Metabolites from *Vicia sativa* Root Exudates

Common vetch (*Vicia sativa* L.) is a legume crop widely used as green manure, pasture, silage, hay and for livestock feed grain. Its major areas of cultivation coincide with areas of heavy infestation by broomrapes to which vetch is a host, mainly *O. crena* and *P. aegyptiaca*, *O. foetida* and *O. minor* .

V. sativa root exudates showed a high stimulatory activity on seed germination of the four broomrape species studied, namely *P. aegyptiaca* (75.3% seed germination), *O. crenata* (30.2%), *O. foetida* (35.6%) and *O. minor* (50.2 %) (*31*). The organic extract of *V. sativa* root exudates also showed a high stimulatory activity on seed germination of the four species, *P. aegyptiaca* (65%), *O. crenata* (76.5%), *O. foetida* (46.4%) and *O. minor* (91%) (*31*). Purification of the organic extract allowed the isolation of two homogenous compounds (**34** and **35**, Figure 5), both obtained as homogeneous oil with the most polar (**34**) slowly crystallizing. These compounds were identified as soyasapogenol B [olean-12-ene-3,22,24-triol(3β,4β,22β)] ((*32*); Figure 4) and *trans*-22-dehydrocampesterol 1-[(ergosta-5,22-dien-3-ol, (3b,22*E*,24*S*)], ((*33–35*); Figure 4 and Figure 5).

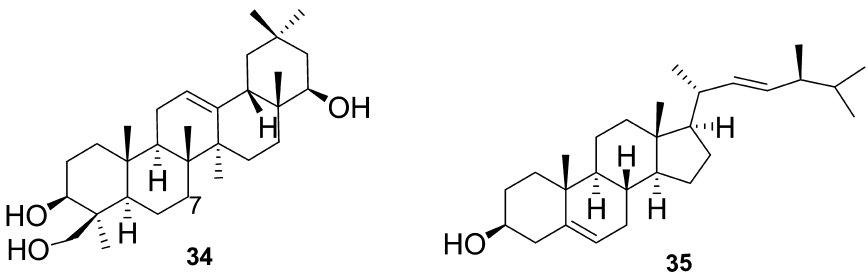

*Figure 5. Structures of soyasapogenol B and trans-22-dehydrocamposterol (**34** and **35**).*

Soyasapogenol B induced the germination of *O. minor* seeds in a differential manner, not stimulating any other *Orobanche* or *Phelipanche* species studied. A dose response study (Table III) confirmed high stimulatory activity on *O. minor* seed germination of soyasapogenol B isolated from *V. sativa* root exudates when tested at 10^{-3} (71.0%) and 0.5×10^{-3} M (64.1%). The stimulatory activity was still significant, but markedly reduced at 10^{-4} M (10.2%) but not significantly different (at $p<0.05$) from the negative control at a concentration of 10^{-5} M (5.1%). Soyasapogenol B commercial source was significantly active at 10^{-4} M (35.2%) only (*31*).

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V. sativa can be infested by a number of broomrape species (*36*), its root exudates induced the germination of all species tested. This is also achieved by *trans*-22-dehydrocampesterol (**35**) which stimulated *P. aegyptiaca*, *O. crenata*, *O. foetida* and *O. minor*. These results are in agreement with previous results (*37*) on soyasapogenol B (**34**) which was very specific in stimulating the germination on *O. minor* seeds only (Table III) and this reinforced its potential utility for broomrape management through suicidal germination. Before this approach can be recommended to farmers, further studies are needed on proper application, incorporation methods and on its stability in the soil.

Table III. Dose response of two sources of soyasapogenol B on germination of *Orobanche minor*[a]

Treatments	*Concentration*	*O. minor seed germination (%)*
Soyasapogenol B from *Vicia sativa* root exudates	10^{-3} M	71.0 b
	$0.5x10^{-3}$ M	64.1 b
	10^{-4} M	10.2 d
	10^{-5} M	5.1 de
Soyasapogenol B from *Glycine max*	10^{-3} M	0 e
	$0.5x10^{-3}$ M	0 e
	10^{-4} M	35.2 c
	10^{-5} M	0 e
GR24 (positive control)	10^{-5} M	85 a
Distilled water (negative control)		0 e

[a] Methanol (0.7%) was added to all treatments in order to dissolve the residual oil. Analysis of variance was applied to replicate data on each treatment. Treatment means marked with the same letter are not significantly different at $p<0.05$ (Duncan multiple range test).

In conclusion, the specific stimulation activity of peagol (**27**) polyphenols (**29** and **30**), chalchone (**33**) and *trans*-22-dehydrocampesterol (**35**) on *O. foetida* seed germination, is most relevant as no germination stimulants for this species have been known before, including the synthetic strigolactone GR24 (*25*). This specific activity is in agreement with specialization on host recognition by *Orobanche* and *Phelipanche* spp. supporting that this specialization could be mediated by unique combinations between type and amount of metabolites exuded by each host plant (*26*).

Conclusions

Natural products produced by plant and fungi appear to have potential for practical application in agriculture as herbicides against different weeds including parasitic plants. This could allow development of eco-friendly products with reduced or null risk for animal and human health.

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Chapter 12

Guaianolides for Multipurpose Molecular Design

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Guaianolides constitute a large and diverse group of biologically active sesquiterpenes from plants belong to the Compositae and Umbelliferae families. These compounds show a widely highlighted phytotoxic activity and also antifungal, bactericidal, anticancer and anti-inflammatory properties. In this sense, dehydrocostuslactone (DHC) has shown high activity in bioassays for phytotoxicity. DHC also has cytotoxic, fungicidal and antiviral activities. Consequently, the same compound has potential uses for the inhibition of plant growth and as a pharmaceutical. DHC could be used as a germination stimulator, with a clear agronomic use for parasitic weeds. A collection of new compounds, guaianestrigolactones, were synthesized and tested using this natural guaianolide as starting material.

Sesquiterpene Lactones

Sesquiterpene lactones (SLs) are among the most abundant natural products, with more than 8000 structures reported with a broad structural and functional diversity. The main characteristic of these sesquiterpenes is the presence of at least one lactone group, generally a γ-lactone, in positions 6,7 or 7,8 (*cis* or *trans*). There are several structural types and germacranolides, guaianolides and eudesmanolides are the most common (Figure 1).

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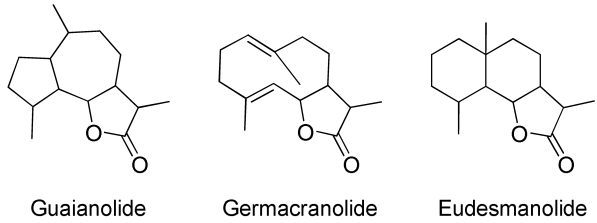

Figure 1. Typical structures of sesquiterpene lactones.

SLs have been frequently reported as allelopathic agents responsible for activity in plants belonging to the family Compositae (*1*, *2*), as well as in other families of plants such as Umbelliferae (*3*) and Targioniaceae (*4*). These compounds are mainly isolated from the aerial part of plants in these families, although they can also be found in the roots.

A significant number of the most aggressive weeds contain sesquiterpene lactones, such as the genus *Centaurea*, which contains the guaianolide centaurepensin (**1**) and the germacranolide cnicin (**2**) (*5*), or the genus *Parthenium* for which the pseudoguaianolide parthenin (**3**) has been reported (*6*) (Figure 2).

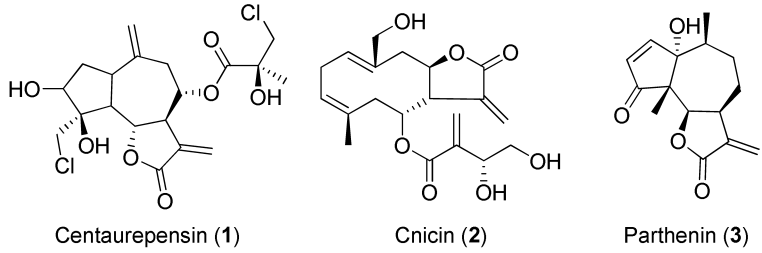

Figure 2. Examples of sesquiterpene lactones in weeds.

The aforementioned compounds have a defensive role in many of these plants and they have a wide spectrum of biological activities. In the last decade different authors have described various properties of these compounds, including fungicidal (*7*, *8*), antibacterial (*9*), antiviral (*10*, *11*) and anti-feedant (*12*) properties amongst others. It is expected that a single compound will have more than one role in a plant by the principle of economy of resources: plants synthesize compounds with different functions to save energy and carbon sources (*13*).

SL guaianolides can induce apoptosis in cancer cells (*14*) and they also have anti-inflamatory properties (*15*, *16*), albeit with low selectivity. As a result, the modes of action of guaianolides in animal cells have been intensively studied. However, little is known about the modes of action in plants, although it is commonly accepted that, in a similar way to animal cells, the inhibition of growth is associated with the presence of the fragment α-methylen-γ-lactone (*7*) along with other functional groups that increase or decrease the activity (*17*).

However, the high lipophilicity of the compounds reduces their solubility in aqueous media and makes them unsuitable for some applications. Lipophilicity can be changed and modulated by the addition of side chains and functional groups supported by quantitative structure-activity relationship (QSAR) and structure-activity relationship (SAR) studies. On the one hand, a low lipophilicity may prevent cell membrane crossing to reach the target and this may lead to a marked drop in activity. On the other hand, inadequate water solubility may affect the transport phenomena. Isozaluzanin C (*18*) will be discussed as a case study in the section entitled *Active guaianolides*.

The guaianolides represent an interesting family of sesquiterpene lactones. Of the wide range of sesquiterpene lactones discovered in the last decade, around 31% are guaianolides and compounds with related skeletons such as pseudoguaianolides and *seco*-guaianolides ((*19*), and previous from the same source) (Figure 3).

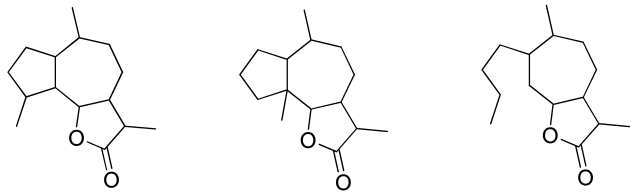

Figure 3. Skeletons of guaianolides, pseudoguaianolides and seco-guaianolides.

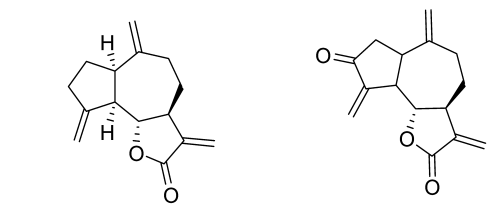

Figure 4. Structures of dehydrocostuslactone and dehydrozaluzanin C.

The allelopathic activity of guaianolides makes them interesting as leads for new agrochemicals. These compounds possess the advantages typically assigned to natural products when compared with classical agrochemicals, such as new modes of action, high activity at low concentrations and biodegradability, amongst others. Additional studies have pointed out a whole range of unexpected yet interesting biological activities in different organisms. The most attractive point

is that a single compound may act as a natural herbicide for specific plants and, at the same time, be active against specific cancer cell lines or have the ability to kill specific viruses. Moreover, appropriate modifications could lead to new improved compounds with multiple applications. An example of a strategy for multipurpose molecular design in guaianolides is shown below, where dehydrozaluzanin C (**5**) and oxetanelactones from DHC (**4**) are selected as lead compounds for Precise Control at Low Doses (PC-LD) (Figures 4 and 5).

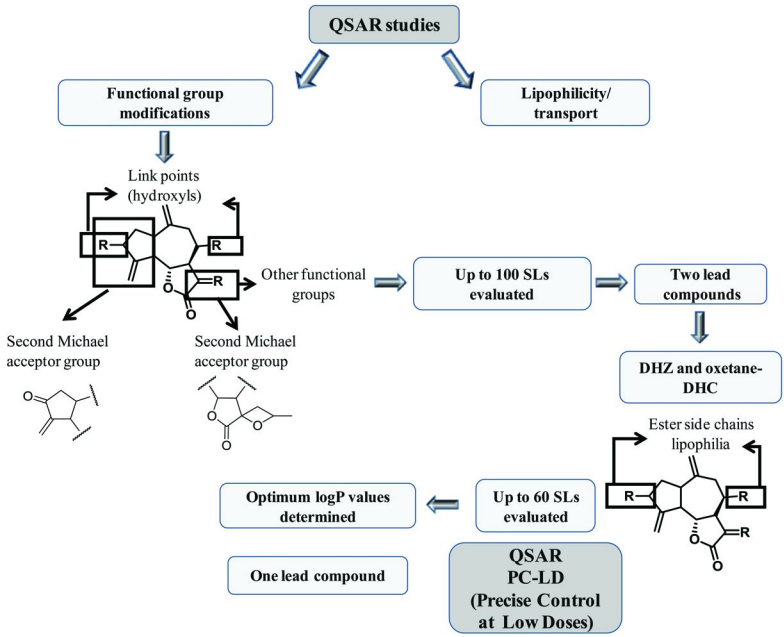

Figure 5. Development of new multipurpose structures.

Several examples of guaianolides and derivatives are discussed in the following sections.

Active Guaianolides

Numerous plants of the genus *Centaurea*, such as Russian knapweed (*C. repens* L.) and yellow starthistle (*C. solstitialis* L.), contain phytotoxic guaianolides. The guaianolides acroptilin (**6**), repin (**7**), solstitiolide (**8**) (Figure 6) and centaurepensin (**1**) inhibit root elongation in lettuce at concentrations above 10 ppm (*20*). Two of these compounds (**1** and **7**) have additional biological activities of interest (*21*).

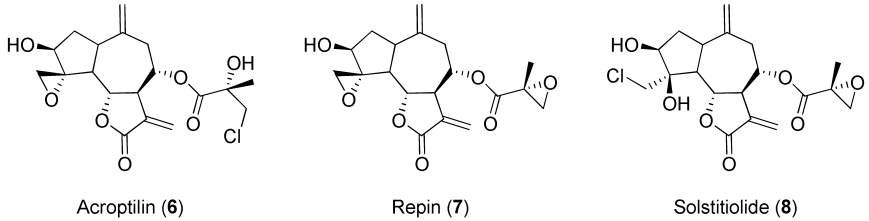

Figure 6. Some guaianolides with phytotoxic activity isolated from the genus Centaurea.

Regarding the bioactivity of microorganisms, antibacterial, antifungal and antiviral activities have been reported for **1** (*22*). The antibacterial activity was tested on *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Staphylococcus aureus*, as well as antifungal activity was studied on *Candida albicans* and *C. parapsilosis* with moderate activity.

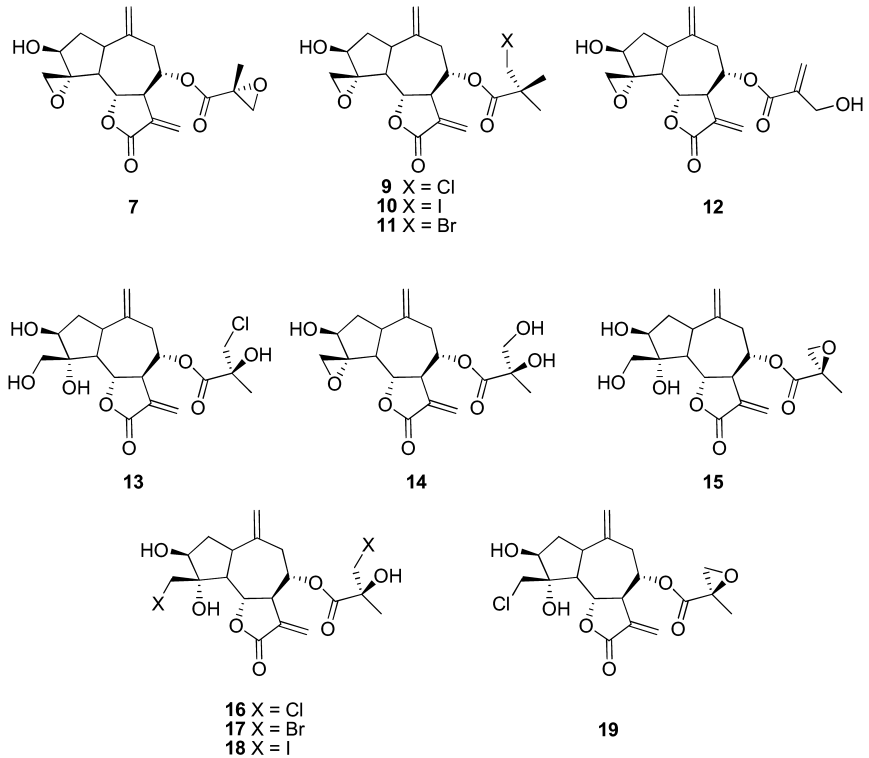

Figure 7. Repin (7) and analogs synthesized.

Compound **1** has shown potent antiviral activity and it might accelerate the healing process of labial and genital herpes lesions (*22*). Compound **1** can also act on the DNA of the virus HSV-1 (*23*).

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With respect to cytotoxicity, compound **7** was reported to have significant activity against seven cell lines (A549, lung cancer; MCF-7, breast cancer; 1A9, ovarian cancer; KB, nasopharyngeal cancer; KB-VIN, KB drug-resistant variant; HCT-8, ileocecal cancer; and SK-MEL-2, melanoma) (*24*). In light of these results, a SAR study of the guaianolide **7** and several derivatives (Figure 7) was carried out in order to obtain more effective compounds against tumor cells.

SAR studies revealed that compounds with a diol (**13**, **15**) at the side chain (**5**), and compounds with an allylhydroxy group (**12**) lose their activity. Nevertheless, repin epoxides (**9**–**11**) or halohydrins (**16**–**18**) are cytotoxic.

Sunflower (*Helianthus annuus* L.) is another remarkable source of guaianolides. A complete new family of guaianolides named annuols (*25*) (Figure 8) have been obtained from this source. These compounds are of interest as leads for new agrochemicals and pharmaceuticals. Phytotoxic bioassays on *Lactuca sativa* L. showed that **23** presents higher inhibition values for shoot and root growth than **21** and **22** (*26*). The same type of assay was carried out for **24** and this showed higher inhibition values than **22** but lower values than **23** (*27*). In another study compounds **20** and **26** were evaluated for inhibitor activity in the etiolated wheat coleoptile bioassay. In this case **20** and **26** had similar activity profiles, although **20** had a lower IC_{50} value (0.31 vs. 0.37 mM) (*28*).

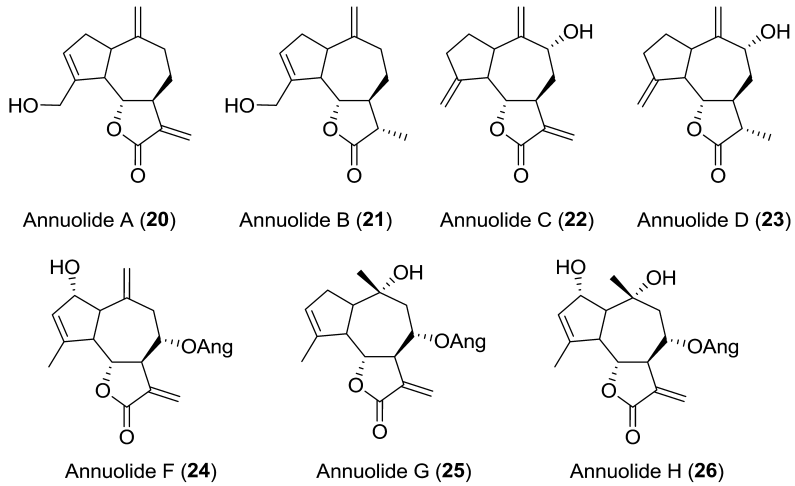

Figure 8. Annuols isolated from sunflower.

Dehydrocostuslactone: A Case Study

Compound **4** was isolated for the first time by Ukita (1939) and later by Crabalona (1948) from *Saussurea lappa* (*29*). As previously hypothesized by Macías et al. (*17*), the results of a recent study (*30*) have shown that **4** is exuded by sunflower roots and stimulates germination of the root parasite *O. cumana.* These results ruled out the role of strigolactones as the only natural witchweed germination stimulant. Bioassays with *O. cumana* showed 90% stimulation at 10

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μM of **4**. The effect of **4** is specific as the germination of other parasitic weeds such as *O. crenata*, *P. aegyptiaca* or *O. ramosa* could not be triggered (*31*). The possible applications of these results will be discussed further in the section entitled *Active* seco-*guaianolides*.

Compound **4** also inhibits the growth of etiolated wheat coleoptiles and weakly inhibits root and shoot growth of cress (*Lepidium sativum* L.) (*32*).

The lipophilicity of guaianolides can affect transport phenomena and reduce bioactivity. SAR studies were carried out on a series of hydroxylated derivatives of **4** with the aim of identifying a possible relationship between the number of hydroxyl groups and the phytotoxic activity. Seventeen sesquiterpene lactones were synthesized from **4** and tested in monocot and dicot species' as targets, with a commercial herbicide containing triasulfuron (0.6%) and terbutrine (59.4%) used as an internal reference (I.R.). The introduction of an increasing number of hydroxyl groups led to the loss of activity as a high polarity hampers transport across the cell membrane, although in a certain range the activity was enhanced (*32*).

In another study, different ester derivatives (**29** and **30**) of mono- (**27**) and di-hydroxylated (**28**) dehydrocostuslactone respectively (Figure 9) were evaluated using the wheat coleoptile growth test. In general, esters were more active than hydroxylated compounds (*18*). The most active compounds were those that contained alkylic side chains. However, aromatic and unsaturated side chains were even more active than the parent hydroxylated ones. These results are consistent with Hansch's nonlinear model and show that the changes introduced affected the transport phenomena.

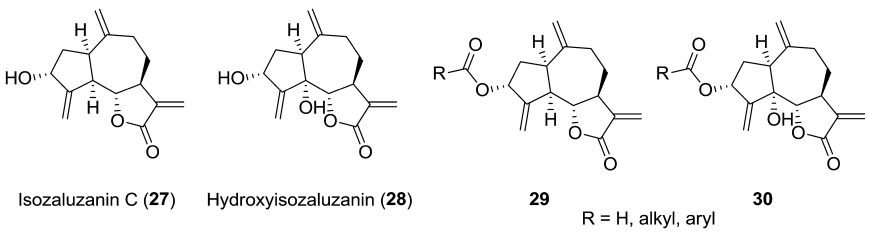

*Figure 9. Structures of hydroxylated and ester derivatives of **4**.*

In a similar way to other guaianolides, **4** exhibits multiple biological activities – fungicidal (*7*), antiviral (*33*), anti-inflammatory (*34*) and cytotoxicity against cancer cells (*35*) on human ovarian cells SK-OV-3 and a potent anticancer activity *in vivo* thus making it an excellent candidate for further studies *in vivo* (*36*).

Oxetaneguaianolides

Guaianolides with oxetane (oxacyclobutane) rings have been reported (*37*) to show a wide range of biological activities (platelets aggregant, anticancer, antibiotic) (*38*). The oxetane ring seems to be essential for the bioactivity. To date, very few oxetane ring-containing compounds have been isolated as natural

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products. Five natural oxetane guaianolides were isolated and characterized from *Centaurea clementei* Boiss (*39*, *40*) and *Cleirolophus canariensis* Brouss (previously *Centaurea canariensis*) (*41*) (Figure 10).

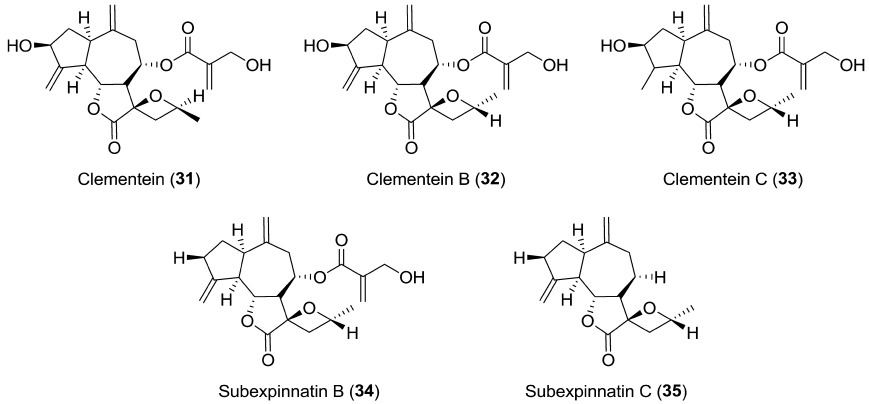

Figure 10. Structures of natural oxetane lactones.

The hemi-synthesis of several 11,16-oxetane lactones from **4** has also been reported (*42*) with the aim of performing conformational analyses. It is well established that conformational aspects are key factors in the biological behavior. A comparative study of the resultant geometries between these oxetane lactones and the starting material concluded that guaianolides with the α-methylene-γ-lactone moiety and non-bulky substituents adopt a twisted-chair conformation at the cycloheptane ring, while a chair conformation is preferred in the absence of the α-methyl-ene-γ-lactone group.

Dehydrozaluzanin C (5)

Another interesting source of guaianolides is the genus *Frullania* (in the Jabulaceae family). A number of guaianolides have been reported in the literature to have inhibitory activity against the germination and growth of rice in the husk (*43*), as shown in Figure 11 and Table I.

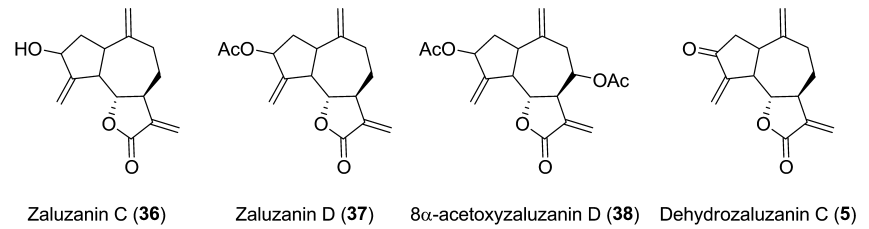

Figure 11. Natural guaianolides present in Frullania species.

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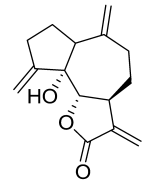
Table I. Inhibitory activity on germination and growth of rice in husk (*43*)

Guaianolide	*Total inhibition of germination at (ppm)*	*Total inhibition of root growth at (ppm)*
36	100	50
37	100	50
38	200	50
5	100	50

Compounds **36** and **37** have also shown antifungal activity (*44*). The antifungal activity of zaluzanin D has been studied in six crop-damaging plant-pathogenic fungi: *Botrytis cinerea* (a necrotrophic fungus mainly found in wine grapes), *Curvularia lunata* (a facultative pathogen of many plant species and of the soil), *Colletotrichum lindemuthianum* (causes anthracnose of the common bean), *Fusarium oxysporum* (affects a range of gymnosperm and angiosperm plants), *Fusarium equisetti* (a plant pathogen) and *Rhizoctonia solani* (a widespread, destructive and versatile plant pathogen that causes several plant diseases such as seed decay, damping off, foliage diseases, etc.) (*45*).

Compound **37** showed 100% inhibition of the mycelia growth of *Rhizoctonia solani*, 75% inhibition in both *Curvularia lunata* and *Botrytis cinerea* at 200 ppm. At lower concentrations, compound **37** showed only fungistatic activity and this was concentration-dependent.



5α-hydroxy-DHC (**39**)

Figure 12. Structure of 5α-hydroxyDHC.

An SAR study of compounds with different sesquiterpene lactone backbones against phytopathogenic fungi was also carried out and involved **4**, **5**, **36** and 5α-hydroxydehydrocostuslactone (**39**) (Figure 12). Compound **36** is a special case because the inhibition of mycelia growth was greater than that shown by the rest of the guaianolides and sesquiterpene lactones tested (*7*). Compound **5** was nearly as effective as the commercial fungicide used as a positive control against *Colletotrichum fragariae* and *Colletotrichum gloesporioides*. These fungi are considered to be major plant pathogens worldwide and failure to control these fungi may result in serious economic losses. The search for new chemical structures with new modes of action to ensure crop management is a very important area of research.

Compound **5** was isolated from different weeds of the Compositae family and it is one of the main guaianolides studied for its allelopathic potential (*46–48*). This compound can also be obtained by semi-synthesis from **4**, via the formation of **40** (Figure 13).

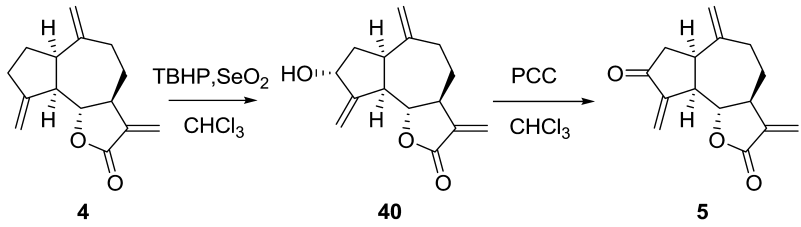


Figure 13. Semi-synthesis of DHZ from dehydrocostuslactone.

The potential of **5** as a plant growth regulator has been evaluated and it showed levels of activity similar to those of the commercial herbicide, which was used as an internal reference. Moreover, **5** is active on species such as carrot and cress, on which the internal standard did not show any activity (*49*).

Cynaropicrin (41)

Cynaropicrin (**41**) is one of the major constituents in artichoke (*Cynara scolimus* L.) and a wide range of biological activities have been reported. This guaianolide has proven to be active on *Lactuca sativa*, *Lolium rigidum* and *E. crus-galli* (*50*).

Compound **41** is cytotoxic against human cancer lines (*51–53*), is a deterrent to herbivores (*54*, *55*) and is able to display anti-aging activity by inhibiting the NF-κB activation pathway (*56*). A correlation has been proposed between the anti-feedant role of **41** in the artichoke and its bitter taste. A sensorial study was performed with fourteen compounds obtained by chemical modification of **41** in an effort to find structure-taste relationships. Bitterness was proposed to be strongly dependent on the presence of oxygenated polar groups and decreases when the lactone ring is opened (Figure 14).

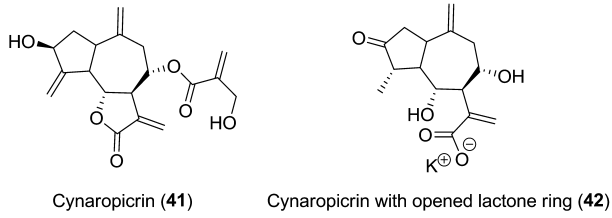


Figure 14. Guaianolides tested in a sensorial study.

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Diversolides

Diversolides have been isolated from *Ferula diversivittata* (Umbelliferae family) and tested for in *vitro* antibacterial and antifungal activity on *S. aureus*, *E. coli*, *A. niger* and *C. albicans* using gentamycin and fluconazole as positive controls. Diversolides (Figure 15) did not show any significant antifungal activity on *C. albicans* in the range 1.25–160 μg/mL. On the other hand, **46**, **48** and **49** showed antimicrobial activity against *A. niger* (*57*), with **49** identified as the most active compound in the series. Finally, all activities were far below the levels obtained with gentamycin and fluconazole.

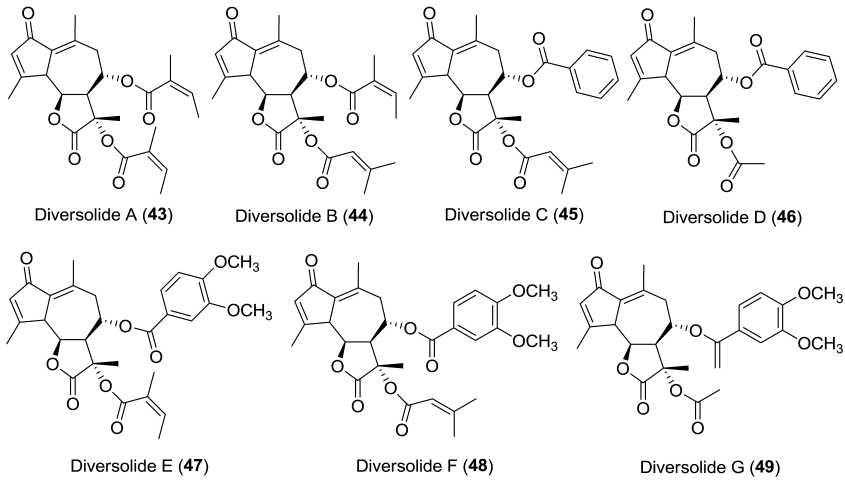

Figure 15. Structures of diversolides with antimicrobial activity.

Moreover, *Ferula* species might be a good source of cancer chemo-preventive agents as some of its constituents, e.g. **43**, **46**, **48**, and **49**, have shown anticancer activity. These compounds were studied on Epstein–Barr virus early antigen activation induced by 12-*O*-tetradecanoylphorbol-13-acetate and showed the following IC_{50} values: 8.7 nM (**43**), 10.7 nM (**46**), 9.0 nM (**48**), 9.1 nM (**49**) (*58*).

Active Pseudoguaianolides

Pseudoguaianolides have also shown some interesting properties. Some selected pseudoguaianolides with more than one type of biological activity are shown in Figure 16.

Compound **3** is the main compound from *Parthenium hysterophorus* L. (Compositae family) and antimalarial (*59*), antimoebic (*60*) and allelopathic properties (*61*) have been reported. Compound **3** inhibits growth of *Avena fatua* and *Bidens pilosa* by almost 50% at a concentration of *ca*. 0.2 mM (*62*).

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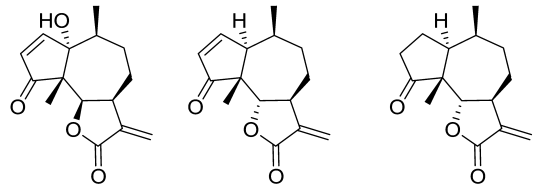

Figure 16. Some pseudoguaianolides with biological activity.

In another study, Batish et al. investigated the phytotoxicity of **3** against four weed species (*Amaranthus viridis*, *Cassia occidentalis*, *Echinochloa crus-galli* and *Phalaris minor*) in laboratory and greenhouse conditions (*63*). The strongest effects were observed for *P. minor* and *A. viridis* and the inhibitory effect was higher on root than on shoot growth (Figure 17).

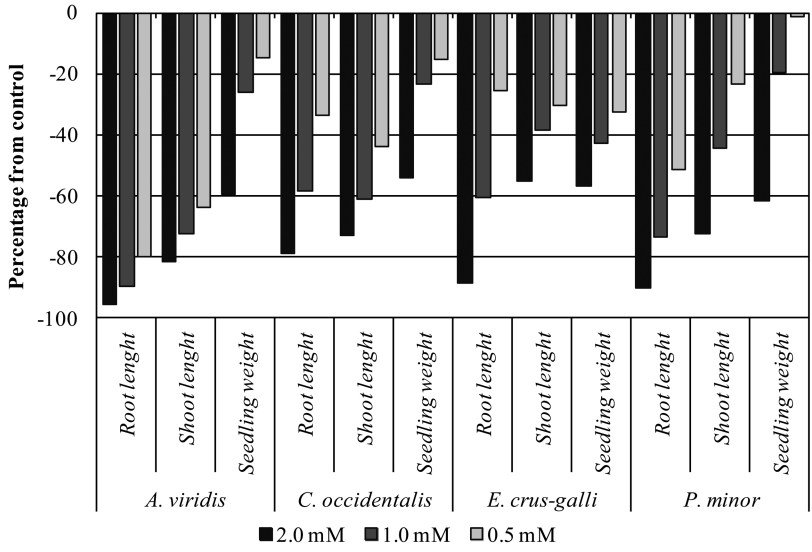

Figure 17. Effect of parthenin on several weeds.(63).

The activity of **3** and analogs against gram-positive (*Bacillus subtilis*, *Bacillus spharicus* and *Staphylococcus aureus*) and gram-negative (*Klebsiella aerogenes* and *Chromobacterium violaceum*) bacteria have been evaluated (Figure 18).

Compound **3** showed the highest activity. Compounds **3** and **52**–**54** exhibited moderate activity against all microorganisms tested. The derivatives **55** and **57**–**59** did not show any activity. Once again, the presence of the α-methylene-γ-butyrolactone moiety (ring C), an acceptor for a Michael addition, seems to be a prerequisite for activity.

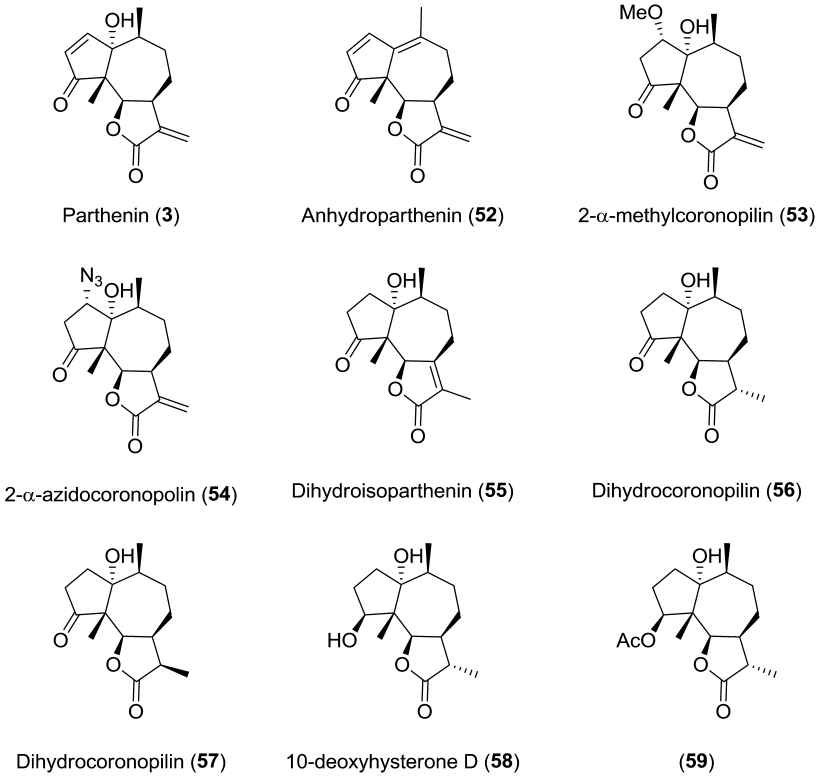

Figure 18. Structures of parthenin and analogs synthesized.

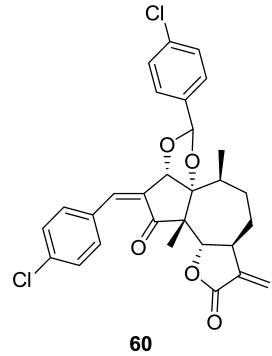

Figure 19. Parthenin analog with anticancer activity.

Moreover, **3** is reported to produce cytotoxic effects against tumor cell lines both *in vitro* and *in vivo*, with positive results obtained in terms of tumor size reduction (*64*). An analog of **3** named [2′-(4-chlorophenyl)-3-(4-

chlorophenylidene)-5,10-dimethyl-12-methylene-decahydro-1,2-(1′,3′-dioxo-cyclopenta[c])azuleno (4,5-*b*)furan-4,11-dione] (**60**) (Figure 19) has recently been prepared. This SL possesses strong anti-cancer activity in leukemia HL-60 cells and inhibits tumor growth in mice (*65*).

Compounds **50** and **51** have shown high inhibitory effect on wild oat, *Avena fatua* L., with EC_{50} values of 0.22 and 0.24 mM, respectively, and a total inhibition of root growth at 2 mM (*66*).

Compounds **50** and **51** have shown pronounced antifungal activity against the plant pathogenic fungi *B. cinerea* (EC_{50} values 413.9 and 332.5 mg/L, respectively) and *F. oxysporum* (EC_{50} values 246.6 and 230.2 mg/L, respectively) (*67*). These SLs have been proposed as lead compounds for the development of new antifungal agents.

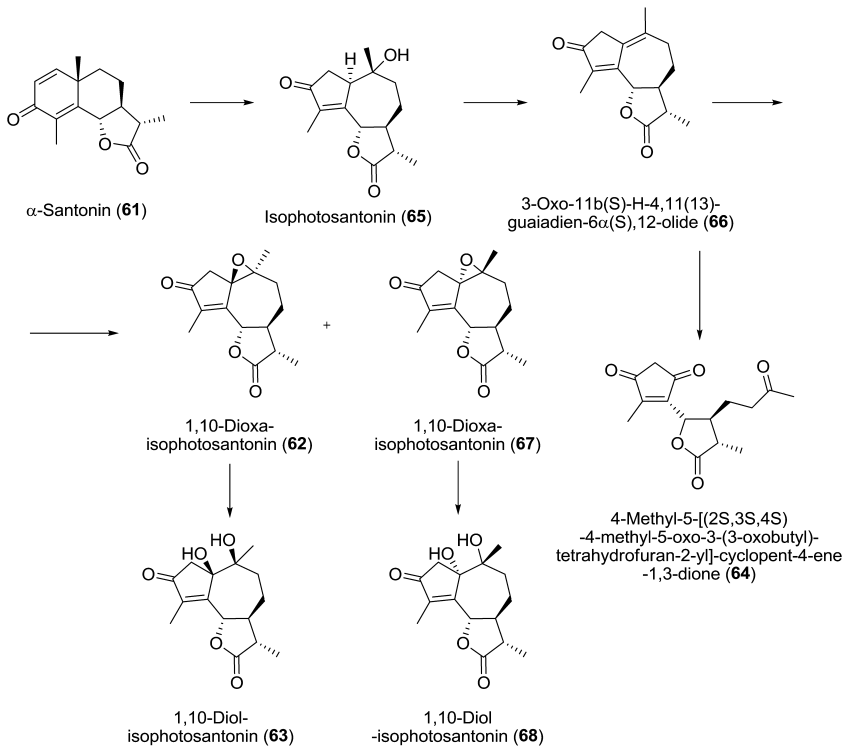

Figure 20. Guaianolides tested in the first activity level (coleoptile bioassay).

Active *seco*-guaianolides

Seco-guaianolides have the least common skeletons among guaianolides and related compounds. *Seco*-guaianolides constitute less than 5% of the total number of these compounds studied in the last decade (*19*). The backbone is similar to that of other guaianolides but with a C–C single bond broken.

The preparation and biological evaluation of a bioactive *seco*-guaianolide **64** from *Artemisia gorgonum* (Figure 20) has been reported. Eudesmadolide **61** was used as the starting material and photo-rearrangement led to the guaianolide backbone with suitable functionalization to perform the ring opening to give the target compound. The phytotoxicity of all intermediates was tested in the wheat coleoptile assay (*68*) (Table II).

Table II. EC_{50} values for compounds described (*68*)

Compound	*IC_{50} (μM)*	*Compound*	*IC_{50} (μM)*
61	345.8	**65**	487.2
62	819.4	**66**	317.9
63	>1000	**67**	394.6
64	43.8	**68**	>1000

The results show that *seco*-guaianolide **64** is the most active compound and is one order of magnitude more potent than the other guaianolides. This *seco*-guaianolide lacks the unsaturated α-methylene system in the lactone ring. In this case, the presence of a highly reactive cyclopentenedione ring should be responsible for the high activity observed, but further structure-activity studies have to be done.

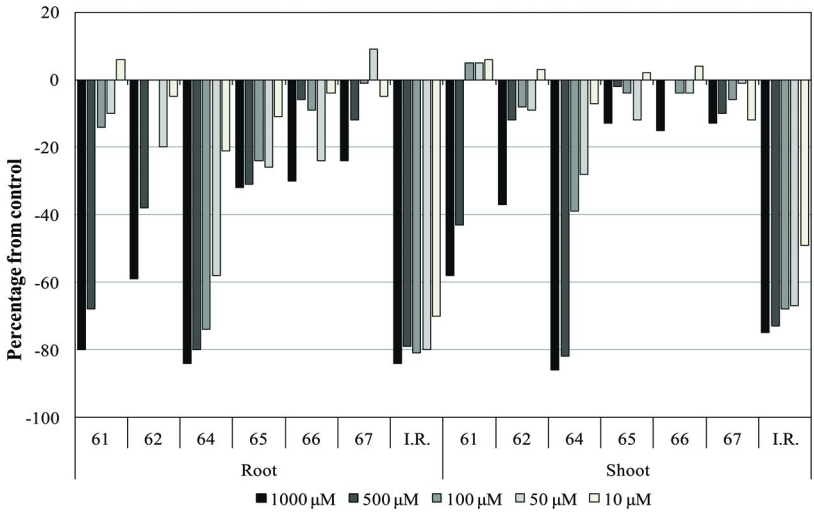

*Figure 21. Phytotoxicity of compounds **61**, **62**, **64-67** and the I.R. on onion (68).*

Compound **64** has a cyclopentenedione ring, which is uncommon in natural products, so this compound is a promising candidate for further bioactivity studies. In the seedling bioassay the *seco*-guaianolide showed excellent activities on root and shoot growth of cress, onion and tomato, and no significant activity on germination except for tomato (Figures 21 and 22). These results make it a promising candidate as a lead for the development of new agrochemicals (*69*).

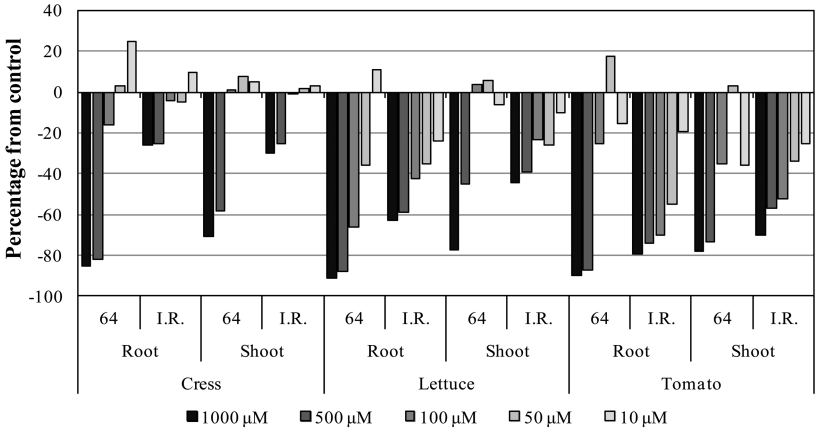

*Figure 22. Phytotoxicity of compound **64** and the I.R. on cress, lettuce and tomato (68).*

Germination Inducers for Parasitic Plants

Parasitic weeds constitute a serious threat to many economically important crops. The biology of such weeds is intriguing and extremely interesting in terms of adaptation. Half of their life cycle is host-dependent and the recognition mechanisms are chemical-based. To start with, germination is triggered by specific compounds exuded from the roots of their hosts. Depending on the weed species, other phases of their development also depend on certain chemicals exuded by their hosts. The main family of signal compounds that trigger the germination of the genera *Orobanche* and *Striga* is named strigolactones.

The first strigolactone was isolated in 1966 from the root exudates of the non-host *Gossypium hirsutum* L. (cotton) (*70*). These compounds are relatively few in terms of number in the lactone family and they originate from the carotenoid pathway. Consequently, they are not sesquiterpenes despite the fact that the number of carbons in the main backbone appears to indicate otherwise. These compounds also seem to be relatively widespread, as they have been isolated and characterized from different plant families (*71*). More recently, strigolactones have been identified as phytohormones responsible for shoot branching (*72*, *73*). They can also inhibit root branching and induce hyphal branching in arbuscular

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mycorrhizal fungi. A total of nine strigolactones have been characterized to date: strigol, strigyl acetate, 5-deoxystrigol, orobanchol, orobanchyl acetate, sorgolactone, 2-epirobanchol, solanacol and sorgumol (Figure 23) (*74*).

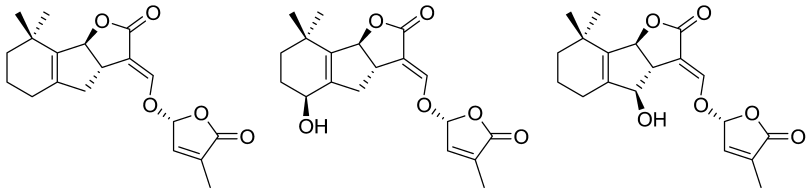


Figure 23. Examples of natural strigolactones.

Exudation of strigolactones from roots stimulates the production of fungal signals in arbuscular mycorrhizal fungi, but they also act as germination stimulants of parasitic plants of the genera *Striga*, *Orobanche* and *Phelipanche*. These parasites can cause great damage to commercial crops, such as sorghum, many leguminosae, tomatoes, lentils and carrots, amongst others; The parasitic plants attach to plant roots to obtain the nutrients they need from the host and prevent its normal growth.

In view of their germination-promoting activity, various strategies have been proposed to use strigolactones to control parasitic weeds. The so-called 'suicidal germination' approach (*75*, *76*) involves the application of a germination inducer in the soil as a pre-emergence herbicide. Premature germination of parasitic plants would kill them due to the lack of water and nutrition supplied by the host plant. However, this strategy is not considered to be viable because of the low stability and short half-life of strigolactones in soils. The synthesis of more stable analogs with a longer half-life has been an active research line for many groups (*77*, *78*).

An alternative approach has recently been proposed starting from sunflower. *Orobanche cumana* is the specific parasitic weed of sunflower. In this case, the chemical signal is the guaianolide **4**, which is exuded from the roots. Other studies have shown that SLs (mainly guaianolides, eudesmanolides and germacranolides) specifically induce the germination of *O. cumana* and *not Orobanche* (broomrape) or *Striga* (witchweed) species (*31*, *79*).

However, closer inspection of the structure of both backbones shows structural similarities with the SL backbone. For example, both backbones have a fused tricyclic system with one of the rings – usually denoted as C – being a lactone ring with an α-methylene-γ-lactone system. Strigolactones have an additional unsaturated lactone ring to form a γ-lactone-enol-γ-lactone system (rings C–D) (Figure 24). This enol-bridge is easily hydrolyzed and is the cause of the low soil stability of strigolactones. The different strigolactones all retain their C–D moiety and structural variations between strigolactones concern the A–B rings.

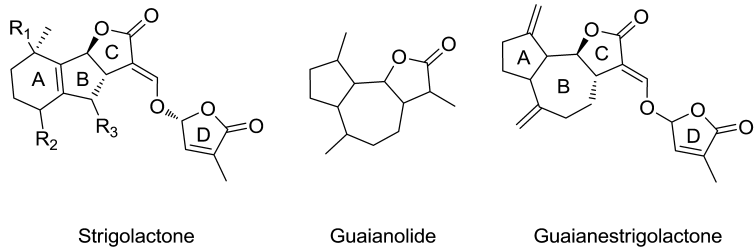
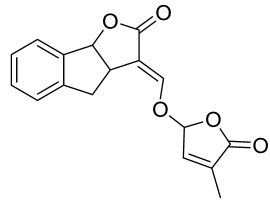


Figure 24. Differences between SLs, strigolactones and guaianestrigolactones.

Recently, the synthesis of mixed guaianolide- and strigolactone-based compounds named guaianestrigolactones has been reported. These new compounds are based on a guaiane-type structure but also have the characteristic lactone-enol-lactone moiety of strigolactones (*17*). Guaianestrigolactones are more potent germination inductors than guaianolides or the strigolactone GR-24 (**72**) (Figure 25) used as the internal standard. Moreover, the selectivity against *O. cumana* disappears and the new compounds are able to induce germination of other *Orobanche* species such as *O. ramosa*.



GR-24 (**72**)

Figure 25. Structure of GR-24.

Conclusions

Natural products are a promising alternative for weed control. This approach might reduce some of the hazards of traditional, synthetic pesticides and may offer new modes of action. For this purpose, one of the most remarkable groups of compounds are the sesquiterpene lactones.

Among the sesquiterpene lactones, guaianolides have a wide structural variety and there are a large number of compounds for which multiple activities have been reported, not only for guaianolides but also for related compounds (pseudo- and *seco*-guaianolides).

Thus, guaianolides, pseudoguaianolides and *seco*-guaianolides could be used for weed control and for the preparation of fungicides as well as applications in medicine, *etc*. Future research into guaianolides may afford promising tools for weed management and also provide new lead compounds for the development of pharmaceuticals.

Some natural guaianolides present high activities at low doses and different SAR and QSAR studies should allow this activity to be tailored to obtain Precise Control at Low Doses 'a la carte'. This is an example what natural products could offer for weed control: novel structures, new modes of action and lower active doses than commercial pesticides.

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Chapter 13

The Response of *Arabidopsis* to Co-cultivation with Clover

Investigating Plant−Plant Interactions with Metabolomics

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Allelopathy contributes to interspecific interference in plants beyond competition for nutrients and sunlight and is of interest in agriculture due to its potential use in weed suppression. In order to study allelopathic effects in a model system, *Arabidopsis thaliana* and *Trifolium repens* were co-cultivated on nutrient medium in sterile containers for two weeks and then harvested, extracted and analyzed by GC-TOF-MS. 163 metabolites were identified using the automated database BinBase. Comparing metabolite peak areas in co-cultivated and control seedlings revealed an altered metabolic profile for both species in terms of several metabolite groups including amino acids, phenolics, carbohydrates and lipids. 87 *A. thaliana* and 53 *T. repens* metabolites were significantly affected. In *A. thaliana* 34 metabolites increased relative to the control upon co-cultivation while 54 decreased; in *T. repens* 20 increased while 33 decreased. To our knowledge, this is the first plant−plant interaction study making use of untargeted library-based metabolomics. The observed

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decreases in *A. thaliana* of aromatic and branched-chain amino acids suggest a suppressant effect of *T. repens* on *A. thaliana* which may resemble that of herbicides, as the synthesis of these two groups of amino acids is inhibited by glyphosate and sulfonylurea herbicides respectively. While the results are not conclusive, they illustrate the power of untargeted library-based metabolomics in descriptive studies and in generating hypotheses for further study.

Introduction

The term allelopathy was invented by the Czech-Austrian botanist Hans Molisch who published a book in 1937 entitled "Der Einfluss einer Pflanze auf die andere: Allelopathie" (The influence of one plant upon another: Allelopathy) (*1*). The term was formed from the greek allelon (other) and pathos (suffering) meaning the suffering of the other (plant). In the original sense it referred to the negative influence one plant could have upon another by chemical means.

The textbook example of allelopathy is the black walnut tree (*Juglans nigra*) which produces the phytochemical juglone (5-hydroxynaphthoquinone). Juglone occurs in several tissues of the black walnut and is released into the soil where it causes the well known inhibition of growth of many plants within a radius of several meters of the mature black walnut tree (*2*).

The definition of allelopathy has gradually widened to refer in a more general sense to both positive and negative chemical interactions between species. Allelochemicals are thus semiochemicals, or signaling molecules, that act between species as opposed to pheromones, which act within species.

Examples of allelochemicals include flavonoids (*3*) and phenolic acids (*4*) which are widespread in the plant kingdom, benzoxazinoids (*5*) in rye, wheat and maize, and glucosinolates (*6*) in the brassicales.

Allelopathy is of interest to sustainable agriculture because the selection of crops for allelopathic potential towards weeds and herbivores may help decrease reliance on synthetic pesticides (*7*). Historically, natural products have accounted for a large proportion of novel insecticides and fungicides, but not herbicides (*8*) although allelopathy has been exploited in weed management (*9*).

Varieties of clover produce isoflavones in varying amounts, and among these is biochanin A (*10*). Its degradation in soil has been shown to be too rapid for it to be responsible for long term phytotoxic effects in the soil (*11*), but its degradation products are biologically active (*12*, *13*). Biochanin A itself also has an inhibitory effect on the growth of certain weed species (*14*).

Although it has gradually gained acceptance, allelopathy has been a controversial field of research throughout its history due to its simultaneous acknowledgement as important in plant−plant interactions (*15*) and criticism of many of the studies intending to shed light on it (*16*, *17*).

Investigating Allelopathy

Investigators of allelopathy face a variety of difficulties. The simplest mechanism of allelopathy is that an allelochemical is released by a plant into the environment from where it is taken up by the plant in which it has its effect. The investigation of this phenomenon is not straightforward: Measurement of apparent growth inhibition does not necessarily imply allelopathy, but may rather be due to competition for nutrients or light. A greater degree of certainty is achieved by chemical measurements, but even supposing an allelochemical interaction, there are complications in elucidating it. The compound released into the environment and taken up may not be the same, as its form (*18*) and availability (*19*) may have been altered by degradation or microbial transformation in the environment. These microbes, e.g. rhizobacteria or mycorrhiza, may also be the target organism of the allelochemical. The compound may be metabolized by the target plant such that a targeted chemical analysis will fail to detect its uptake. The effect may also be due to the action of several allelochemicals. Finally, the effect may be due to complicated and delayed chemical transformations in the soil, such as prunasin in the case of the peach replant problem, where prunasin persists for years in the soil until broken down into phytotoxic components by microorganisms from young peach tree roots (*20*).

A variety of laboratory techniques have been used to investigate allelopathy. An elaborate method developed by Fujii et al. (*21*) was the plant-box method used to measure the allelopathic effect of a plant as a function of distance from the plant. In this method a plant is cultivated in its own growth medium for a determined period of time before being transplanted along with a cylinder of growth medium around its root system into another container of growth medium. Seedlings are then cultivated in an array such that series of seedlings are various distances from the subject plant. A simpler method was the relay seeding technique was used by Navarez and Olofsdotter (*22*) to screen two hundred varieties of rice for allelopathy toward barnyard grass. The method relied on planting seeds of the allelopathic donor and acceptor in alternating rows, planting the donor seeds seven days prior to the acceptor and the ten-day co-cultivation period.

Frequently, allelopathy is investigated by treating a target plant with an extract obtained from a putative allelopathic plant. This approach suffers from the disadvantage of being unspecific, but permits the targeting of purification efforts toward suspected allelochemicals if the appropriate analytical techniques are available. J. L. Harper (*16*) criticized this approach as the addition of organic material to the soil or growth medium of the assay could provoke a microbial growth spurt which robbed the assay plant of nitrogen, producing the observed decrease in growth.

In the present work we have used an adaptation of the equal compartment agar method developed by Wu et al. for investigating the allelopathic potential of wheat varieties (*23*).

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Materials and Methods

Experimental Materials

Phytatray II cultivation vessels, phytagel, sucrose, citric acid, tribasic sodium citrate and Murashige and Skoog (MS) salts were purchased from Sigma-Aldrich (Schnelldorf, Germany). Acetic acid was purchased from J. T. Baker (Deventer, the Netherlands). HPLC grade solvents were purchased from Rathburn (Walkerburn, Scotland). Water was obtained from a Dionex (Hvidovre, Denmark) MilliQ purifier. Seeds of *T. repens* var. Rivendel were obtained from Dr. Birte Boelt of the Department of Agroecology at Aarhus University. Seeds of *A. thaliana* var. Columbia were purchased from Lehle Seeds (Round Rock, TX).

Cultivation of Seedlings

Growth medium was prepared containing 0.3% phytagel, 0.5% sucrose and 0.22% MS salts. Seeds of *T. repens* were surface-sterilized by agitation first for 10 min in 5% sodium hypochlorite and then for 10 min in 70% ethanol before being rinsed three times with sterile water. Seeds of *A. thaliana* were surface-sterilized by agitation for 5 min in 5% sodium hypochlorite before being rinsed three times with sterile water. Seeds were then sown on Petri dishes containing 20 ml growth medium. *T. repens* seeds were sown individually using a Pasteur pipette, whereas *A. thaliana* seeds were suspended in sterile water and pipetted into a Petri dish en masse. The Petri dishes containing either *T. repens* or *A. thaliana* seeds were then left under a light source with a photoperiod of 16 h light and 8 h dark for 2 days to allow germination to occur. Germinated seedlings were then transplanted in a regular manner into Phytatray II cultivation vessels containing 40 ml growth medium. This produced a regular 6 × 3 array of seedlings positioned at one or both ends of the rectangular vessel. Control vessels contained either *T. repens* or *A. thaliana* seedlings growing only in 6 × 3 arrays with conspecifics, and the experimental vessels contained both an array of *T. repens* seedlings and an array of *A. thaliana* seedlings. *T. repens* seedlings were planted 5 days prior to their *A. thaliana* counterparts, and white cardboard dividers were inserted into the growth vessels at 1 cm above the growth medium to avoid competition for light between the species. The cultivation vessels were placed directly under the light source to avoid the divider casting a shadow. After planting of *A. thaliana* seedlings, cultivation was continued for a period of 15 days. Both germination and cultivation took place at room temperature. Three replicates were used for each of the four experimental treatments.

Harvesting and Extraction of Seedlings

After cultivation, the sterile container was opened and the growth medium partially dissolved with 60 ml sodium citrate buffer (10 mM, pH 6). Seedlings were then extracted from the medium using tweezers. Extracted seedlings were washed with 10 ml citrate buffer and each array was transferred to a centrifuge

tube. All individuals within each array were pooled and the total fresh weight of each seedling array recorded. Samples were then frozen at -18 °C for storage before lyophilization. After lyophilization, plant samples were extracted first with dichloromethane and then methanol. To each centrifuge tube was added 5 g chemically inert Ottawa sand and 20 ml solvent. The centrifuge tubes were then agitated on an Elmi Intelli-Mixer (Riga, Latvia). Each sample was extracted twice overnight first with dichloromethane and then with methanol to obtain 40 ml non-polar extract and 40 ml polar extract. Extracts were stored at -18 °C until analysis.

GC-MS Analysis

Plant extracts were derivatized using *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) according to the following protocol: For each sample, 4 ml extract was transferred to a 4 ml vial and evaporated under nitrogen. After addition of 50 µl methoxyamine solution (40 µg/ml in pyridine) the samples were shaken for 1.5 hours at 30 °C. 350 µl MSTFA containing 1% fatty acid methyl ester (FAME) marker mixture were then added to the samples before shaking at 37 °C for 30 min. The samples were then transferred to vials for GC-TOF-MS analysis. The derivatized samples were analyzed in duplicate on a Leco (St. Joseph, MI) Pegasus GC-TOF using an Agilent (Santa Clara, CA) 6890 gas chromatograph and a Gerstel (Muehlheim, Germany) autosampler with automatic liner exchange and cold injection. Separation was achieved on a Restek (Bellefonte, PA) Rtx-5Sil MS column of length 30 m and 0.25 mm i.d. and coated with 0.25 µm 95% dimethyl 5% diphenyl polysiloxane film. A 10 m integrated guard column was used. The GC oven temperature was held initially at 50 °C for 1 min and then ramped to 330 °C at 20 °C/min. The final temperature was held for 5 min. The ion source temperature was 250 °C and the transfer line temperature was 280 °C. Electron impact ionization was effected at 70 V. Spectra were collected in the range m/z 85-500 at 20 spectra/sec. FAMEs were used as retention index markers. The method corresponds to that used to record the FiehnLib mass spectral library (*24*). Two analytical replicates were used for each of the three replicates for each treatment, species and solvent, for a total of 48 injections and 24 samples.

Data Processing and Analysis

GC-TOF data were processed using Leco's ChromaTOF software with the FiehnLib mass spectral library to produce the data files needed for annotation of spectra using BinBase (*25*, *26*). BinBase annotates peaks in three ways according to confidence in analyte mass spectra and retention index as true positives, false negatives, and true negatives. The latter were included to provide noise values needed for undetected compounds in statistical analysis. GC-MS peaks were normalized according to the fresh weight of the seedling samples and principal component analysis (PCA) models were constructed for the known compounds in both the dichloromethane and methanol extracts. For those compounds occurring in both extracts the normalized peak areas were summed before

further data processing and analysis. The mean was taken over the two replicate injections performed on each biological replicate and a two-tailed t-test used to categorize changes in metabolite concentrations observed with co-cultivation as not significant or significant at the 10%, 5%, 1% or 0.1% levels for both species.

Results and Discussion

Seedling Growth

A. thaliana grown in the presence of *T. repens* was observed not to grow as tall during the two week period of the experiment as *A. thaliana* grown alone, and a decrease in mean fresh weight of *A. thaliana* per growth container from 1.8 g to 1.5 g was observed. In the case of *T. repens* there was no size difference immediately visible, but the mean fresh weight increased from 1.6 to 1.9 g upon co-cultivation. Table I illustrates the effect of cultivation on the fresh weight of *A. thaliana* and *T. repens*. While the spread of biomass values for co-cultivated *A. thaliana* was large, all values fell below the spread of the values of *A. thaliana* grown alone. The sum of the fresh weights of control samples of *A. thaliana* and *T. repens* was nearly identical to the sum of the fresh weights of co-cultivated *A. thaliana* and *T. repens* (3.4 g in each case), although the mass of *A. thaliana* decreased upon co-cultivation and the mass of *T. repens* increased. The unaltered total fresh weight suggested that nutrient limitation was not a factor in the experimental setup.

Table I. Fresh weight (g) of arrays of plants cultivated separately (control) or together (co-cultivated) for 15 days

Species	*Control*	*Co-cultivated*	*p*
A. thaliana	1.77	1.49	0.06
T. repens	1.62	1.92	0.01

The inclusion of plant arrays cultivated for other lengths of time, and not included in the chemical analysis, allowed an assessment of the significance of the co-cultivation effect over the entire period of cultivation. Figure 1 shows the growth curves using the Gompertz function (*27*) for both *A. thaliana* and *T. repens* cultivated separately as well as together. ANOVA revealed that inclusion of a term in the Gompertz function to differentiate co-cultivated and control plants in the growth curves was statistically significant for both *A. thaliana* ($p < 0.001$) and *T. repens* ($p < 0.05$).

Table II. Changes in aromatic and branched-chain amino acids, and selected phenolics, in co-cultivated *A. thaliana* and *T. repens* compared to controls

	A. thaliana		*T. repens*	
Metabolite	*% change*	*p*	*% change*	*p*
phenylalanine	-68	0.003	+17	0.508
tryptophan	-59	0.003	-11	0.697
tyrosine	-71	0.008	+7	0.913
isoleucine	-60	0.005	+7	0.834
leucine	-61	0.003	+28	0.471
valine	-44	0.009	+2	0.960
formononetin	+19	0.078	+50	0.128
cis-sinapate	+66	0.060	+10	0.222
α-tocopherol	+212	0.001	-7	0.203
γ-tocopherol	+335	0.012	-9	0.130
arbutin	+284	0.001	+28	0.085
coniferin	+19	0.046	+6	0.831
salicin	+42	0.099	+213	0.158

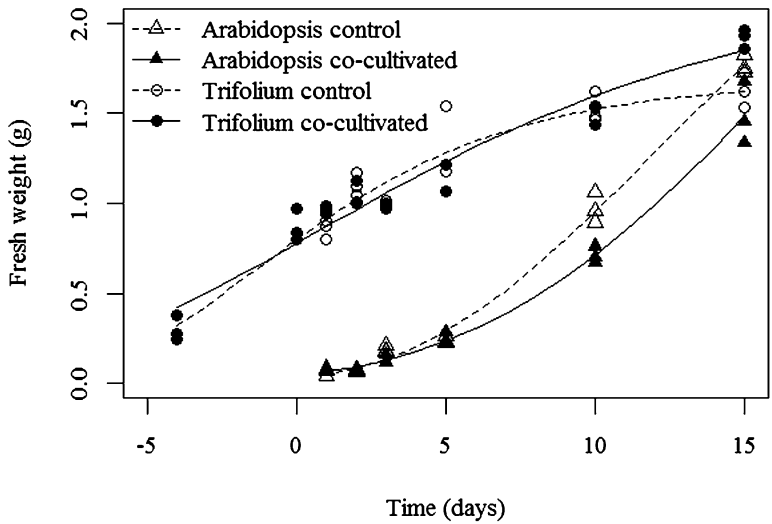

Figure 1. Gompertz growth curves for co-cultivation of Arabidopsis thaliana and Trifolium repens.

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Identification of Metabolites

Querying BinBase with the results of the GC-TOF-MS analysis yielded a total of 163 known compounds in *A. thaliana* and *T. repens*. In *A. thaliana* the concentrations of 87 of the 163 known compounds were significantly altered in the co-cultivation experiment. 34 were increased by co-cultivation and 53 were decreased. In *T. repens* only 53 metabolite concentrations were significantly altered, with 20 increasing and 33 decreasing with co-cultivation. Table II lists a selection of identified metabolites and the difference in GC-TOF-MS signal intensities per mg fresh weight between co-cultivated and control plants cultivated separately for both *A. thaliana* and *T. repens*. The difference is given as a percentage change relative to the control along with the associated p value. Both the number of metabolites affected and the significance of the change were greater in *A. thaliana* than *T. repens*, which suggested that *A. thaliana* was more affected by co-cultivation than *T. repens*.

Principal Component Analysis

Metabolic fingerprinting has been used to study plant−plant and plant−host interactions (*28*, *29*). However, because GC-MS furthermore allows identification of metabolites from libraries, the advent of library-based GC-MS metabolomics provides a useful tool for descriptive analysis of complex chemical interactions as in the case of allelopathy. The fact that allelopathic interactions are mediated by specific sets of secondary metabolites and potentially affect a large set of primary metabolites implies two strategies for analyzing the chemical effects: One strategy is the targeted and quantitative analysis of a relatively small set of allelochemicals and their transformation products while the other is the untargeted and only semi-quantitative analysis of a large group of primary metabolites. Given that there are hundreds of thousands of secondary metabolites (*30*), their analysis requires some knowledge of which type of compound is causing the effect in question (e.g. clover isoflavonoids) so that a targeted analytical method can be developed. In contrast, the number of metabolites in GC-MS libraries is large enough to cover much of the primary metabolism in an untargeted manner.

PCA showed a clear separation of *A. thaliana* samples into co-cultivated and control groups, whether the extraction solvent was dichloromethane or methanol. A similar separation was seen for clover, but it was less clear. Figure 2 shows the PCA score plot for the methanol extracts of *A. thaliana* seedlings cultivated alone and with *T. repens*.

Changes in Amino Acids and Phenolics in *A. thaliana*

Of the proteinogenic amino acids, only glutamine and glutamate increased significantly ($p < 0.01$) in *A. thaliana*. These amino acids may be produced as end products of protein degradation and arginine catabolism (*31*) and this raises the possibility, along with the observed increase in the concentration of urea, of an increased mobilization of nitrogen for transport in co-cultivated *A. thaliana*.

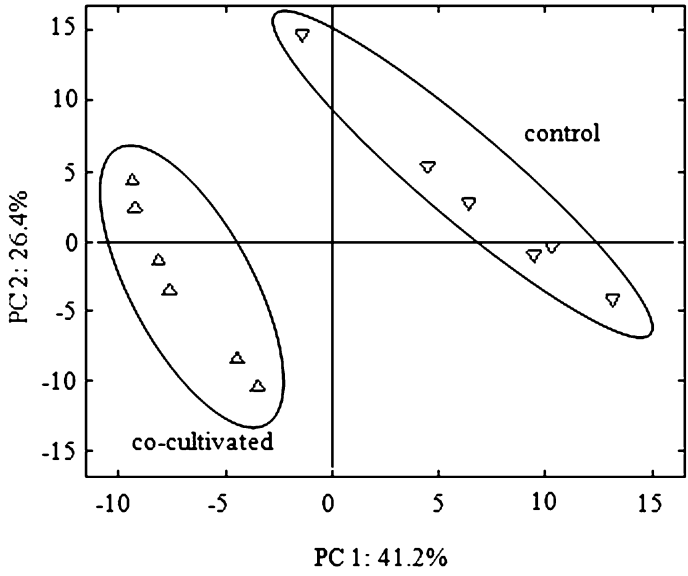

Figure 2. PCA score plot showing separation between A. thaliana cultivated alone (control) and with T. repens (co-cultivated). The model used 163 identified metabolites in the methanolic A. thaliana extract.

The observed increase in the concentration of phenolics in *A. thaliana* suggests that co-cultivation with clover caused stress to *A. thaliana* although the exact type of stress is unknown. Out of ten analyzed phenolic aglycones, the concentrations of four (formononetin, *cis*-sinapate, α- and γ-tocopherols) increased on co-cultivation while none declined. Of four phenolic glycosides, three increased (salicin, coniferin and arbutin) while only one decreased (4-hydroxyphenyl-2-ethylglucopyranoside). Although mostly primary metabolites were identified, the isoflavone formononetin was found in both *A. thaliana* and *T. repens*. It cannot be concluded that formononetin was passed from *T. repens* to *A. thaliana*, however, as *A. thaliana* can synthesize this compound despite its lack of isoflavone synthases (*32*).

Hypothesis Formation from the Data

Herbicide-Resembling Effects of Co-cultivation with *T. repens*

The concentration of aromatic amino acids in *A. thaliana* fell by 50% ($p < 0.01$ for phenylalanine, tyrosine, and tryptophan) upon co-cultivation. This is similar to inhibition of the synthesis of aromatic amino acids by the post-emergence herbicide glyphosate. No significant change in the shikimate concentration was, however, observed, although this would be expected from glyphosate treatment. Glyphosate treatment of a sensitive line of soybean lead to the appearance of a nitrogen-rich amino acid profile after the inhibition of photosynthesis (*33*). A similar effect is not contradicted here although only glutamine increased in

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concentration among the nitrogen-rich amino acids. The concentrations of lysine and tryptophan decreased, but the total content of nitrogen-rich proteinogenic amino acids increased by 27%. Although the effect is less than might be expected from a herbicide, this is not unexpected for an allelochemical effect, and its occurrence warrants further investigation.

Sulfonylurea herbicides inhibit 2-acetolactate synthase, causing a reduction in the synthesis of branched-chain amino acids (*34*). 2-acetolactate is an intermediate in the production of valine and leucine, while isoleucine may be produced by transamination of valine or derived from threonine via a multi-step process. While the synthesis of these amino acids was not suppressed entirely in our study, their concentrations did decrease by >50% in *A. thaliana*. These findings are consistent with studies reviewed by Zhou et al. for sulfonylurea herbicides (*35*). In addition, sucrose, fructose-6-phosphate and glucose-6-phosphate accumulated in co-cultivated *A. thaliana*. Zabalza et al. likewise reported an accumulation of those sugars along with a decrease in leucine and valine in pea roots in response to treatment with imazethapyr, an acetolactate synthase-inhibiting enzyme not of the sulfonylurea class (*36*). No such response was observed in *T. repens*.

Taken together with previous knowledge of *T. repens* secondary metabolites, the findings suggest that secondary metabolites released by *T. repens* and taken up by *A. thaliana* are responsible for the observed effects. While secondary metabolites were not analyzed in this study, the changes in the metabolite profile of *A. thaliana* suggests possible effects of phytochemicals passed from *T. repens* to *A. thaliana*. These secondary metabolites are now the object of a study in our laboratory in order to correlate them with the primary metabolite profiles.

Possible Role of Nutrient Limitation

Co-cultivation produced a decrease in proline concentration ($p < 0.1$), an increase in glutamine concentration ($p < 0.05$) and no change in the asparagine concentration ($p > 0.1$) in *A. thaliana*, and the total concentration of those three amino acids increased by 28%. The effect of nitrogen limitation on *A. thaliana* was studied by Lemaître et al., who observed changes in the amino acid profile: proline, asparagine and glutamine decreased (*37*). This implies that nitrogen limitation as a result of the co-cultivation is not the explanation for the altered metabolite profile or decreased growth.

Upon co-cultivation the content of simple sugars and oligosaccharides in *A. thaliana* increased by 23%, but organic acids decreased by nearly 50% and the content of proteinogenic amino acids remained unaltered although the content of non-proteinogenic amino acids increased by 62%. It has been reported that phosphorus limitation in *A. thaliana* led to an accumulation of carbohydrates, organic acids, and amino acids (*38*). These changes were not evidenced by the recorded metabolic profiles of our study. The lack of a significant change in phosphate concentration also indicated that phosphorus deficiency did not play a role in this study.

In our study the content of nitrogen-rich proteinogenic amino acids in *A. thaliana* increased by 27% and nitrogen-rich non-proteinogenic amino acids increased by 44% while cysteine and methionine decreased by 6% and 70%

respectively. The expected increase in tryptophan, however, was not observed: Tryptophan decreased by 59% upon co-cultivation. The content of phenolics increased by 30% and the content of tocopherols (α- and γ-) increased 269% but did not exceed 12% of the total phenolic signal intensity. The content of dehydroascorbate decreased, but not significantly.

The response of *A. thaliana* to sulfur deprivation has been shown to involve a reduction in cysteine biosynthesis and downstream metabolites as well as the accumulation of precursors in addition to an increase in the biosynthesis of nitrogen-rich amino acids and ureides as well as aromatics, specifically tryptophan (*39*). Glutathione levels have been shown to decrease in response to sulfur deficiency (*40*) and it can be speculated that this would cause an increase in dehydroascorbate through the glutathione-ascorbate cycle. The observed changes in our study were thus inconsistent with sulfur limitation in terms of the changes in tryptophan and cysteine levels.

Conclusion

Co-cultivation of *A. thaliana* and *T. repens* as hypothesized allelochemical acceptor and donor produced a decrease in biomass of *A. thaliana* and a similar increase in biomass of *T. repens*. GC-TOF-MS analysis revealed significant changes in the levels of 87 metabolites in *A. thaliana* and 53 metabolites in *T. repens* out of a total of 163 metabolites identified in the two species. The conservation of biomass and the inconsistency of the observed changes in metabolic profiles with nitrogen, phosphorus and sulfur limitation indicated that nutrient availability was not a limiting factor in growth and that the observed metabolic changes, therefore, had another cause. The similarity of the observed response in *A. thaliana* to changes associated with glyphosate or sulfonylurea herbicide treatment suggests that the changes may be induced by secondary metabolites with herbicide-like effects originating from *T. repens*. *Trifolium* species are used as cover crops for weed suppression and are known to contain phytotoxic secondary metabolites (*10*). This suggests that clover secondary metabolites were responsible for the observed effects, but further study is needed to confirm this. The use of GC-MS based metabolic profiling in allelopathic experiments is a significant improvement on more limited analyses relying on biometric data or analysis of specific metabolites. Beyond providing metabolomic fingerprints of the experimental classes, the technique provides rapid information on the chemical nature of the interaction and suggests further avenues of investigation. The present example involves clarifying whether clover secondary metabolites can produce effects similar to synthetic herbicides, and this is now an ongoing investigation in our laboratory. Provided GC-MS library-based metabolomics are applied to plant−plant interactions, as the number of reported metabolomic studies grows it will also become easier to select likely compounds for targeted analysis as well as to draw conclusions on the mode of action by comparing fingerprinting results from study to study.

Acknowledgments

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Chapter 14

Clues to New Herbicide Mechanisms of Action from Natural Sources

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The last commercial herbicides to introduce a new mechanism of action were the HPPD inhibitors launched more than 20 years ago. There is a growing need for new mechanisms of action because of the increasing evolution of target site-based herbicide resistance in weeds. Natural compounds have been and continue to be good sources of new herbicide molecular target sites. In the past, glufosinate and the triketone herbicides were derived from natural compounds and introduced important new mechanisms of action. In particular, plant pathogens are good sources of phytotoxins. The mechanisms of action of natural product phytotoxins, such as leptospermone, tentoxin, actinonin, hydantocidin, thaxtomin, coronatine, AAL-toxin, and other natural products or natural product derivatives are discussed. These examples provide proof that plants can be killed with compounds that inhibit enzymes that are not among the twenty molecular target sites of currently used herbicides.

Introduction

More than 20 years have passed since the last herbicide with a new mechanism of action (MOA) was introduced (*1*). Before this time, a new herbicide MOA was introduced about every 2.5 to 3 years (*2*), accumulating to the approximately 20 MOAs that are now available (*1*). During the past 20 years, the incidence of evolved, target-site-based herbicide resistance has more than doubled (*3*), with a growing incidence of multiple resistance to herbicides with different

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MOAs within the same weed species (*e.g.*, *Amaranthus tuberculatus* (*4*)). All strategies mitigating or delaying the evolution of resistance to herbicides rely on the utilization of complementing weed management practices, including rotation of herbicide MOAs or combining MOAs in tank mixes or sequential sprays in the same growing season (*e.g.*, (*5*)). As more weeds evolve resistance to the herbicides with the currently available MOAs, the need for herbicides with new MOAs to maintain MOA diversity becomes more critical.

Natural compounds offer a source of molecules that are phytotoxic through MOAs that are not exploited by current herbicides (*6*). This short chapter provides examples of natural compounds that kill plants by MOAs that are not shared by commercial herbicides and discusses the prospects for use of natural compounds both as tools for new MOA discovery and as templates for new herbicide chemistries that are affected at unexploited molecular target sites. The examples we provide are divided into phytochemicals and microbial phytotoxins.

Phytochemicals

One might expect that out of self preservation, plants would not synthesize potent phytotoxins because of the potential for autotoxicity problems. However, some plants do produce potent phytotoxins that are used in plant/plant warfare (allelopathy). The literature of allelopathy is replete with reports on mildly phytotoxic compounds with little evidence of their involvement in allelopathy (*7*), but there are compounds such as momilactones in rice (*8*) and sorgoleone in *Sorghum* species (*9*) that are clearly involved in allelopathy. There are other phytochemicals, such as the antimalarial drug artemisinin, that are very phytotoxic (*10*) but do not have a clear role in allelopathy. Unfortunately, the MOAs of most of the more active compounds involved in allelopathy (*e.g.*, the momilactones) have not been determined.

The last MOA introduced in commercial herbicides were those that inhibit *p*-hydroxyphenylpyruvate dioxygenase (HPPD). The first group of HPPD inhibitors marketed was the triketones. These compounds were discovered by working with leptospermone as a starting molecule (Figure 1) (*11*, *12*). Leptospermone and other natural triketones are produced by a number of genera of woody shrubs from Australia and New Zealand, such as *Callistemon* and *Leptospermum*. The essential oil of *L. scoparium* (called manuka oil) consists of about 18.4% triketones, with leptospermone making up about 73% of the triketone fraction (*13*). The triketone grandiflorone (Figure 1), found at less than 1% of the triketone fraction of manuka oil, is a much better HPPD inhibitor than leptospermone with activity approaching that of sulcotrione (Figure 1), a commercial triketone herbicide (Figure 2) (*13*). Manuka oil is effective as a soil-applied herbicide, and leptospermone is persistent enough in soil for good activity (*14*). This example demonstrates that phytotoxic secondary metabolites of plants can be useful in discovery of new herbicide target sites. Other natural products from plants, such as usnic acid from lichens and sorgoleone from *Sorghum* spp. are also HPPD inhibitors (*15*), albeit weaker than leptospermone and most of the other triketones from manuka oil.

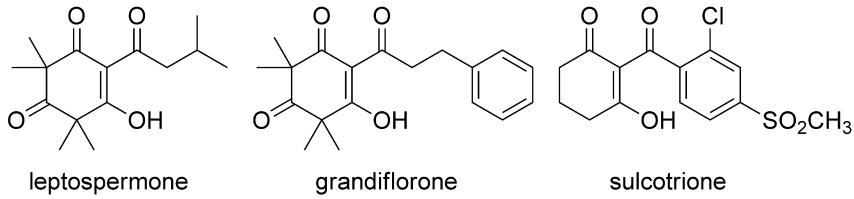

Figure 1. Structures of leptospermone, grandiflorone, and sulcotrione.

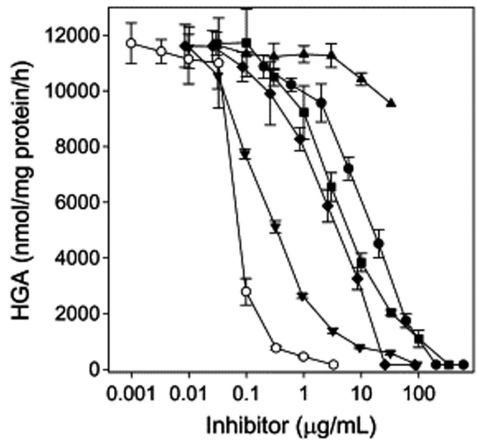

Figure 2. Inhibition of HPPD by manuka oil (•), the β-triketone-rich fraction (■), and its individual β-triketone components: leptospermone (♦), flavesone (▲), and grandiflorone (▼). The synthetic herbicide sulcotrione (○) was added as positive control. HGA = homogentisic acid. Reproduced with permission from reference (13). Copyright 2007 Elsevier.

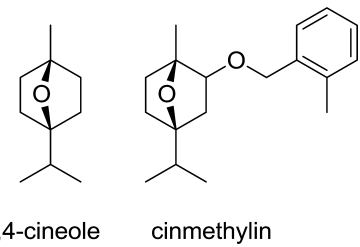

Figure 3. Structures of 1,4-cineole and cinmethylin.

The triketones are the only commercial herbicides with a clear molecular target site that was discovered through work with a phytochemical. It is possible that the once sold herbicide cinmethylin was derived from the plant monoterpene 1,4-cineole (Figure 3), although this is not documented. The molecular target site of cinmethylin was recently reported as tyrosine aminotransferase (*16*), which is not one of the twenty MOAs currently targeted by herbicides. Whether 1,4-cineole or a metabolite of cinmethylin also inhibits this enzyme has not been reported.

As mentioned earlier, the MOAs of few allelochemicals or other phytotoxic phytochemicals have been clearly determined. Sorgoleone, one of the better studied allelochemicals, apparently has multiple molecular target sites such as HPPD, the D-1 protein of photosystem II and root H^+-ATPase (*9*, *17*, *18*). Multiple target sites make evolution of target site-based resistance highly unlikely.

For the reasons discussed above, the production of potent phytotoxins by plants would seem unlikely. However, plants have highly phytotoxic metabolic intermediates (*e.g.,* protoporphyrin IX and sphingosine) that are kept by the plant at very low concentrations *in vivo*. To our knowledge, such compounds have not been studied as herbicide leads. Other highly phytotoxic compounds are produced and rapidly sequestered or excreted to avoid autotoxicity. In the case of artemisinin, the compound is only produced by the cells of glandular trichomes which secrete it into the space between the cell wall and cuticle of the gland, where it has no access to the cytoplasm of the producing plant (*19*). The molecular target sites of few such phytotoxins have been determined.

Microbial Phytotoxins

We know much more about the MOAs of phytotoxins of microbial origin than those from plants (*20*). Table I provides a summary of some of the phytotoxic microbial compounds with MOAs that are not shared by commercial herbicides. Of these, the number produced by plant pathogens and those by soil or saprophytic microbes are about the same. Following the example of the pharmaceutical industry, the pesticide discovery industry has focused most of their efforts on soil microbes and saprophytes, at least partly because transporting these organisms between countries and culturing them is easier than for plant pathogens. The MOAs of relatively few structurally identified microbially-produced phytotoxins are known, so the examples in Table I may be the tip of the iceberg. A few of the examples from Table I are discussed below.

MOAs from Soil and Saprophytic Microbes

The evolutionary significance of phytotoxins from soil and saprophytic microbes that are not plant pathogens is unclear. Nevertheless, these biota provide numerous examples of compounds with unique MOAs.

One of these compounds, L-phosphinothricin, from *Streptomyces* spp., is the active component of the highly successful herbicide glufosinate, a racemic mixture of the synthesized form of the molecule (*40*). D-Phosphinothricin is herbicidally inactive. L-Phosphinothricin is the only commercial herbicide that targets the

enzyme glutamine synthetase (GS). However, a small amount of a biosynthesized tripeptide (bialaphos), that is metabolized to L-phosphinothricin in the target weed, is marketed in Japan. There are several more microbial compounds that inhibit GS (reviewed in (*20*)), but none of these have been developed into commercial herbicides.

Table I. Some microbially-produced phytotoxins and their non-commercial herbicide molecular target sites

Compound	*Target site* [a]	*Ref.*
AAL-toxin (P) [b]	Ceramide synthase	(*21*)
Acivicin (S)	Glutamate synthase	(*22*)
Actinonin (S)	Plastid peptide deformylase	(*23*)
Anhydro-D-glucitol (P)	Fructose-1,6,-bisphosphate aldolase	(*24*)
Cornexistin (S)	Aminotransferase	(*25*)
Coronatine (P)	Jasmonate receptors	(*26*)
Cyperin (P)	Enoyl reductase	(*27*)
Fosmidomycin (S)	1-Deoxy-D-xylulose-5-phosphate reductoisomerase	(*28*)
Gabaculin (S)	Glutamate-1-semialdehyde aminotransferase	(*29*)
Gostatin (S)	Amino transferase	(*30*)
HC-toxin (P)	Histone deacetylases	(*31*)
Hydantocidin (S)	Adenylosuccinate synthase	(*32*)
Hymeglusin (P)	2-Hydroxy-3-methylglutaryl CoA synthase	(*33*)
Phaseolotoxin (P)	Ornithine transcarboxylase	(*34*)
Rhizobitoxin (P)	β-Cystathionase	(*35*)
Streptomycin (S)	Plastid 30S ribosomal subunits	(*36*)
Tagetitoxin (P)	Plastid RNA polymerase	(*37*)
Tentoxin (P)	CF_1 ATPase	(*38*)
Thaxtomin (P)	Cellulose synthase	(*39*)

[a] There is more than one example of natural phytotoxins for some of the target sites listed. [b] P = from a plant pathogen; S = from a soil or saprophytic microbe.

Actinonin (Figure 4) inhibits plastid peptide deformylase (PDEF). PDEF is necessary for *N*-terminal processing of plastid-encoded proteins (*23*, *41*). Overexpression of PDEF confers actinonin resistance (*42*) (Figure 5), proving unequivocally, that PDEF is the only target site of actinonin.

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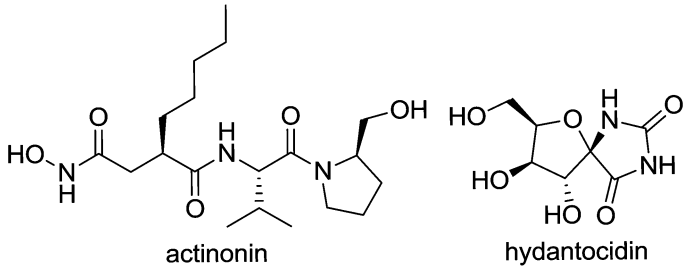

Figure 4. Structures of two phytotoxins from soil microbes discussed in text.

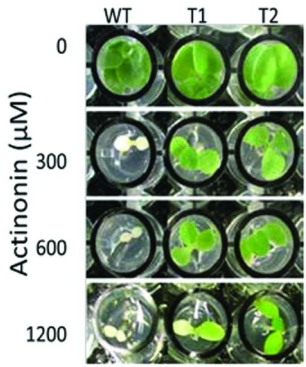

Figure 5. Resistance to actinonin imparted by overexpression of PDEF in the plastome of Arabidopsis thaliana. Two plastomic lines were used, T1 being more resistant than T2. Reproduced with permission from reference (42). Copyright 2011 Springer.

Hydantocidin (Figure 4) is a protoxin that must be metabolically converted to the active enzyme inhibitor. It is phosphorylated to 2α-phosphohydantocidin which is an analog of inosine monophosphate, the substrate for adenylosuccinate synthase (AdSS) (*32*, *43*, *44*). This phosphorylated metabolite is a competitive inhibitor (*43*, *45*) that acts as a broad spectrum herbicide. Several companies generated patents for herbicides based on hydantocidin analogues. Another microbial product, ribofuranosyl triazolone, is an inhibitor of AdSS (*46*).

MOAs from Plant Pathogens

Plant pathogens often kill host cells rapidly with phytotoxins to prevent the host from producing fungitoxic or bactericidal compounds. In most cases such pathogens are non-obligate parasites, because they can live on the dead parts of the plant that they infect as saprophytes. The evolutionary significance of the phytotoxin for such pathogens is obvious. The toxin alone often causes the same symptoms as the pathogen, and often the toxin tranlocates to kill parts of the plant where the living pathogen has not yet invaded.

AAL-toxin (Figure 6), a product of pathovars of *Alternaria alternata*, is highly toxic to a wide variety of plant species at doses lower than those required by most herbicides to have the same effect (*47*). It inhibits ceramide synthase, causing rapid and massive accumulations of the precursor for this enzyme (sphinganine), as well as the substrate derivative phytosphingosine (*21*) (Figure 7). Sphinganine and phytosphingosine are both highly phytotoxic, causing symptoms similar to AAL-toxin (*48*). The very rapid effects of these compounds on plasma membrane integrity, suggest that the killing effect of AAL-toxin is mainly through disruption of membranes by sphinganine and phytosphingosine, rather than by loss of ceramide and/or ceramide derivatives or by apoptosis, as has been suggested by some (*e. g.,*(*49*)). Stress caused by plasma membrane dysfunction at lower AAL-toxin concentrations might induce apoptosis. Unfortunately, AAL-toxin is an analogue of the fumonisins, a class of mycotoxins from *Fusarium* spp., which are highly toxic to mammals. A limited amount of work has been done to find analogues that are toxic to plants, but not to animals (*50*).

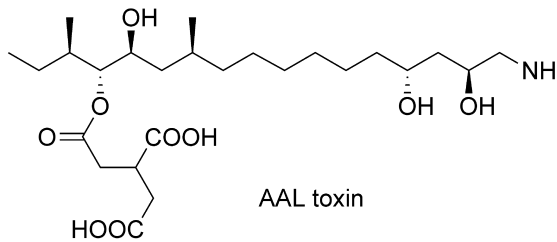

Figure 6. AAL-toxin, a potent phytotoxin from Alternaria alternata.

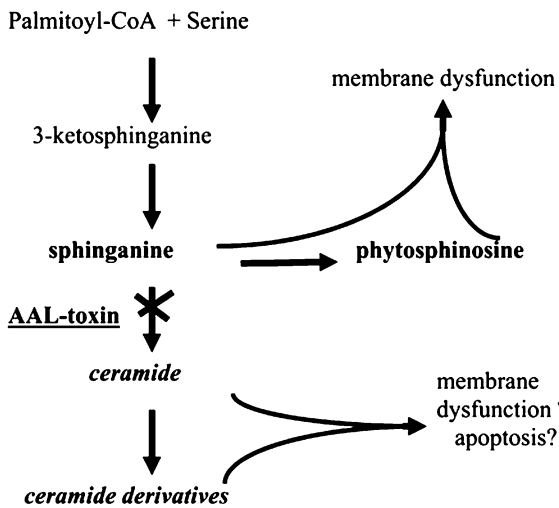

Figure 7. Mode of action of AAL-toxin on the ceramide synthesis pathway. Bold letters indicate increases and italics represents decreases in concentrations.

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Jasmonic acid (Figure 8) is heavily involved in signaling necessary for plant growth and development, as well as for responses to all types of plant stress. Several pathovars of the plant pathogen *Pseudomonas syringae* produce a jasmonate analog, coronatine (*51*), and it is believed to exert its phytotoxicity by mimicking jasmonate (*26*, *52*, *53*). Cinnacidin, from the fungal plant pathogen *Nectria* sp., is also a jasmonate analog that acts as a phytotoxin via the same MOA and with similar activity as coronatine (*52*).

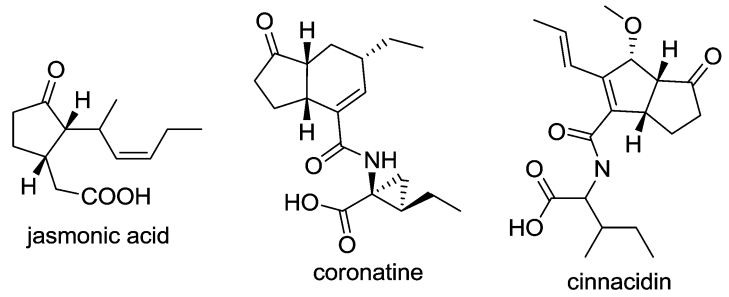

Figure 8. Structures of the plant hormone jasmonic acid and two phytotoxic analogues from microbes.

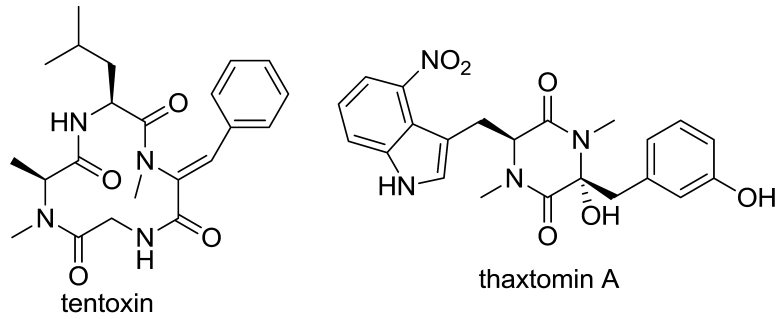

Figure 9. Structures of tentoxin and thaxtomin, two plant pathogen-produced phytotoxins.

Tentoxin (Figure 9) is a cyclic tetrapeptide produced by another pathovar of *A. alternata*. It is a potent inhibitor of energy transfer of CF1/CF0 ATPase of the thylakoids by binding to CF1 (*38*, *54*). It also stops the transport of the nuclear-coded protein polyphenol oxidase (PPO) into the plastid (*55*). This even happens in etioplasts which should have no CF1/CF0 ATPase activity. This activity is

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associated with thylakoids, and etioplasts do not have thylakoids. PPO is inactive as an enzyme until it is processed during incorporation into the plastid. The two mechanisms (blocked PPO processing and inhibited CF1/CF0 ATPase) appear to be linked, because both effects are seen in sensitive species and both are absent in insensitive species (*56*). A single amino acid substitution (Asp to Glu) resulting from variation at codon 83 of the β subunit of CF1produces resistant plants (*57*). How the β subunit of CF1 is linked to the PPO effect is yet to be explained. However, very little ATP is required for transport of nuclear-coded proteins into the plastid, and uninhibited CF1/CF0 ATPase has some ATP hydrolysis activity in the dark (*58*).

Thaxtomin A (Figure 9) is the most active analog of a group of phytotoxic dipeptides produced by *Streptomyces scabies*, which causes common potato scab and scab diseases in some taproot crops (*59*). It is apparently a cellulose synthase inhibitor (CESA) (*39*, *60*). Thaxtomin inhibits glucose incorporation into the cellulosic fraction of cell walls at nanomolar concentrations (Figure 10). Genes associated with primary and secondary cellulose synthesis, as well as other genes associated with cell wall formation are affected by this compound (*60*). Isoxaben, a synthetic herbicide that inhibits CESA had similar effects. However, the finding that the pattern of enhancement of lignification by thaxtomin A is different from that caused by isoxaben suggests that is may have a slightly different MOA. Thaxtomin has been approved by the USEPA as a bioherbicide for use in cereals, particularly rice and turf.

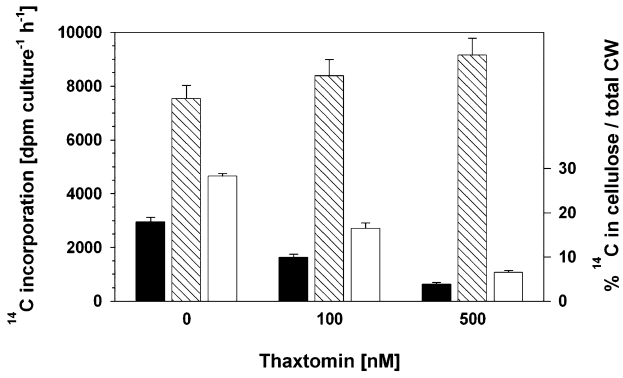

Figure 10. Thaxtomin inhibition of ^{14}C-glucose incorporation into the cellulosic cell wall fraction of dark-grown A. thaliana seedlings. Black and cross-hatched bars represent the amount of label incorporated into the acid-insoluble (cellulosic) cell wall (CW) fraction and the acid-soluble cell wall fraction, respectively. White bars indicate the percentage of label in the cellulose fraction relative to the amount of total label in the cell wall (right ordinate). Reproduced with permission from reference (39). Copyright 2003 American Society of Plant Biologists.

Caveats and Conclusions

In this short chapter, we have only been able to provide brief examples of natural phytotoxins with novel MOAs. Clearly, from the examples of Table I and those discussed in more detail, microbes provide a rich source of compounds that are effective in killing plants via MOAs that are not among the twenty MOA of commercial herbicides. As well, a few phytochemicals, such as certain plant-derived triketones, also served as good starting material to develop herbicides with novel MOAs. As pointed out by Gerwick (*2*), however, there are numerous reasons that these examples of good phytotoxins have not translated into good herbicides, including cost of production, mammalian toxicity, and improper physicochemical properties for uptake and/or translocation.

Most of the natural product discovery focus by the pesticide industry has been on soil microbes, for the reasons mentioned above and also perhaps because they have been a rich discovery source for pharmaceuticals. Despite this, there are slightly more new MOAs from compounds derived from plant pathogens than from soil microbes (Table I). This small sample may be insufficient for conclusions to be drawn, but almost certainly many more soil microbes than plant pathogens have been screened for phytotoxins. Most of the discoveries from plant pathogen have come from those that infect crops, the focus of the vast majority of plant pathologists. Pathogens of the huge number of non-crop and very minor crop plant species offer new vistas in phytotoxin discovery. A few scientists such as Antonio Evidente have recognized this opportunity and are discovering new phytotoxins from pathogens of these understudied plants that offer the promise of more new MOAs (*e.g.,* (*61*, *62*)). Recent work on one of these compounds, ascaulitoxin aglycone, indicates that is has a novel MOA related to amino acid transport or metabolism (*63*).

The question of how many good herbicide target sites exist is an important, unresolved question. Molecular biology has not been able to answer this question for reasons detailed elsewhere (*1*, *64*, *65*). The absolute proof that a particular target site is a good one is the killing of plants with low doses of a chemical that targets that site. This has been accomplished with natural phytotoxins for several target sites. As with the case of the highly successful ryanodine receptor insecticides, discovery that a particular target is viable with a natural product (*66*) can lead to the synthetic chemistry research to develop successful pesticides (*67*).

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Chapter 15

Induction of Cryptic Natural Product Fungicides from Actinomycetes

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Natural products have been a productive source of lead chemistry for the development of crop protection fungicides. Unfortunately, the past discovery of thousands of natural fungicidal compounds has complicated natural product discovery since rediscovery of known compounds is common. At Dow AgroSciences we believe that natural products can provide novel fungicidal chemistry for fungicide development; however, continued effective discovery will require creative approaches. Several such approaches are described in this review. First, it is known that microbial genomes encode many pathways for novel natural products that are unexpressed. Induction of these cryptic pathways represents tremendous potential for discovery of novel compounds. Second, chemical synthesis is a proven method for development of new commercial fungicides. Natural products both novel and known can be a productive source of novel bioactive scaffolds.

Introduction

There is a great need for new agrochemical fungicides. As the world population has grown beyond 7 billion people, demand for food has increased. This demand is potentiated by dietary changes (especially meat consumption), increased bio-energy use and a decrease in tillable land (*1*). This has created a

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situation where cropland must be optimized to produce maximal yield. One of the threats to maximal yield is disease caused by plant pathogenic fungi. Although many chemical fungicides for disease control exist, the availability of many of these chemistries is threatened. Some chemistries are no longer effective due to evolution of resistance by disease pathogens (*2*). Other fungicides have been or may soon be removed from the market due to unacceptable environmental or toxicity profiles or failure of re-registration. New chemical solutions for disease control are needed for continued high productivity in agriculture.

Natural products have been an underexploited source of novel chemistry for agriculture uses. While up to fifty percent of registered drugs were derived from natural sources in 2007 (*3*), only eleven percent of agrochemical active ingredients were natural product derived (*4*). The impact of natural product based chemistries on agriculture is increasing as 21.1% of agrochemical registrations since 1997 were natural product derived (*5*). Eleven natural products fermented from microbes have been used commercially as agrochemical fungicides (Figure 1) (*6*). A number of plant essential oils, terpenes, alkaloids and extracts have also been used for disease protection (*5*, *6*). In addition, although not fungicidal, several natural products (jasmonic acid, salicylic acid, harpin) and the natural product based acibenzolar-S-methyl (Figure 2) activate plant defenses for disease control (*5*, *7*). However, the biggest impact of natural products in the agrochemical fungicide market has been the inspiration for synthetic fungicides. The phenylpyrrole fungicides, fludioxonil and fenpiclonil (Figure 2), were derived from the natural product pyrrolnitrin first isolated from *Pseudomonas pyrrocina* (*8*). The target site for these phenylpyrrole fungicides has not been definitively determined but does involve the osmotic-sensing histidine kinase cascade (*9*).

The most significant natural product-based chemistry for the fungicide market is the strobilurin fungicides which include over a dozen registered fungicides. Strobilurins and oudemansins (Figure 3), which are produced by multiple fungal genera, were the starting point for the strobilurin fungicides (*10*, *11*). Myxothiazole (Figure 3) and cystothiazole, discovered from myxobacteria (*12*), act at the same active site as strobilurins and expand the chemical diversity around the strobilurin chemistry. Strobilurins are potent inhibitors of mitochondrial electron transport acting at the Q_O site of complex III (METIII-Q_O). However, the natural product strobilurins were unstable and ineffective in field applications (*10*). The development of commercial fungicides from the natural strobilurins (Figure 3) demonstrated the degree to which important field performance characteristics can be built into natural compounds using chemistry. Early methoxyacrylate stilbene derivatives of strobilurins stabilized the structure (UV stability) leading to improved activity under field conditions. Several synthetic compounds such as azoxystrobin and trifloxystrobin have excellent plant mobility or redistribution, which has contributed to good acceptance in specific applications in the fungicide market (*10*). Derivatives of the pharmacophore, the oximino esters (e.g., kresoxim-methyl), led to improved efficacy against certain diseases such as powdery mildew (*10*). Pyraclostrobin has improved potency and spectrum of activity resulting in improved crop safety and yield enhancement. This diversity of product attributes has resulted in extremely broad acceptance and utilization of the strobilurin fungicides in the agrochemical market.

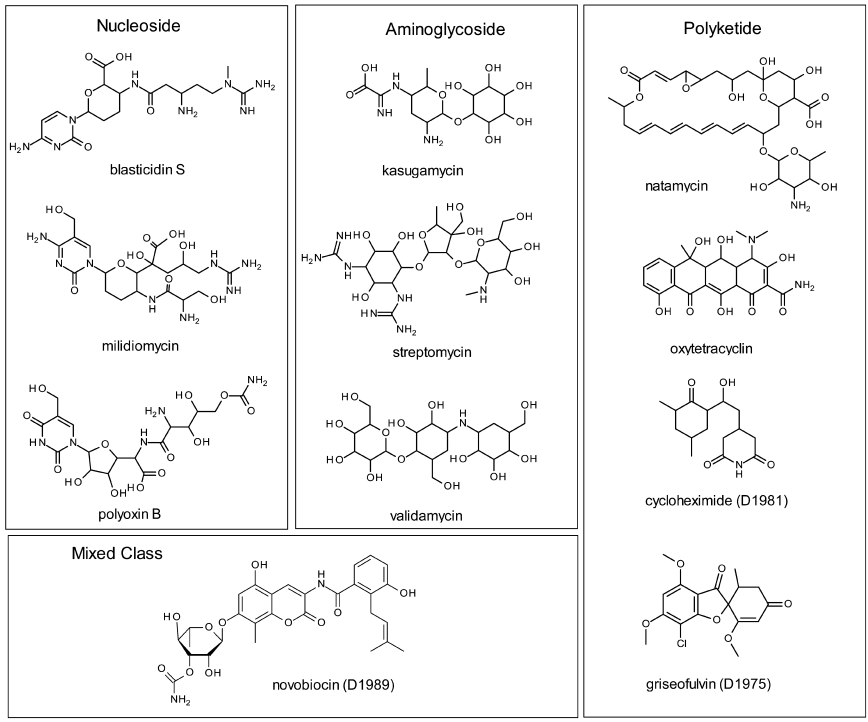

Figure 1. Natural product agrochemical fungicides. All compounds are produced by actinomycetes except griseofulvin, which is a fungal product; D = registration was discontinued in the year indicated.

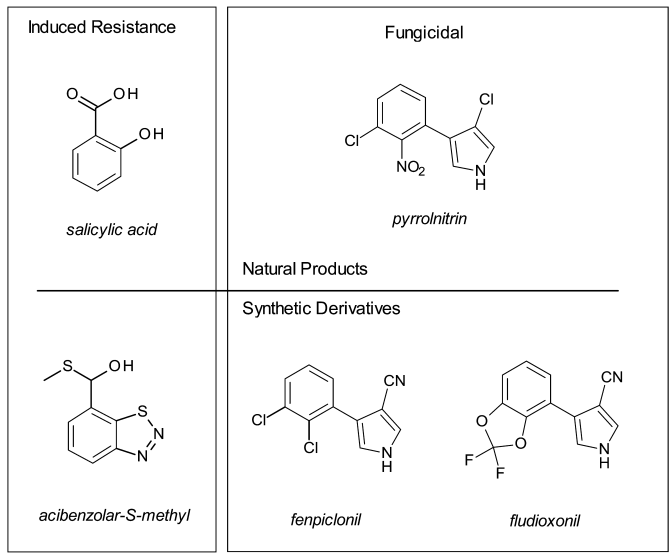

Figure 2. Natural product-based crop protection agents against plant pathogens.

This review will detail strategies used by Dow AgroSciences to utilize natural products for discovery of novel fungicides for crop protection. The first section discusses strategies for the induction of cryptic metabolites from actinomycetes followed by a survey of some recent strategies used for discovery of natural product fungicides from actinomycetes and other bacteria.

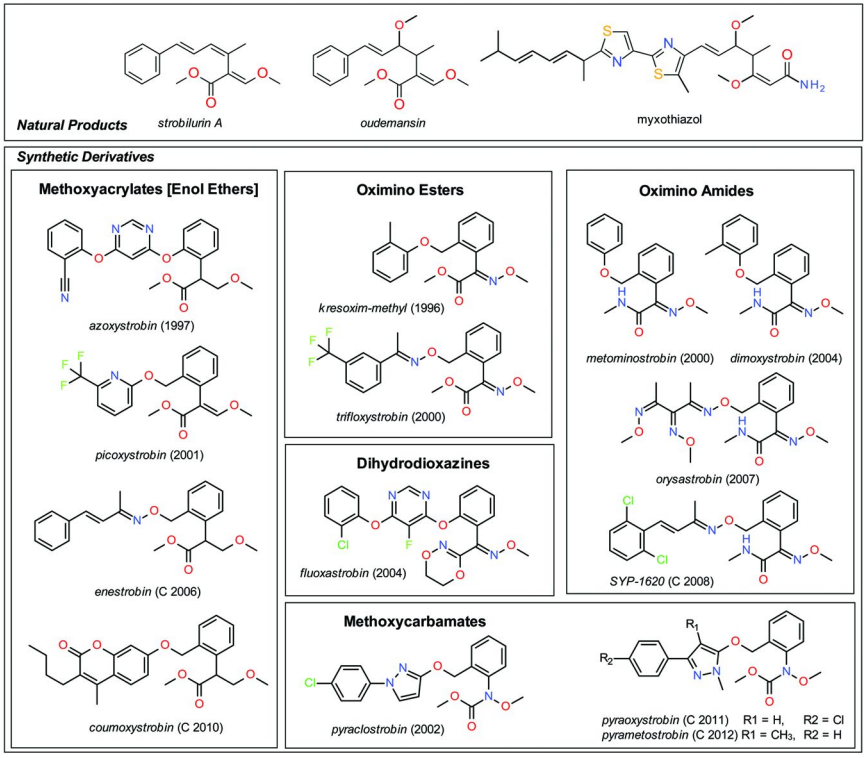

Figure 3. Strobilurin Based Synthetic Fungicides. Strobilurin fungicides categorized by chemical class of pharmacaphore. Year of registration in parenthesis; C = registered only in China.

Discovery of Antifungal Compounds from Actinomycetes

Actinomycetes have been a rich source of novel fungicidal chemistry (*6*, *13*). In over 60 years of antibiotic screening >2,000 antifungal compounds have been discovered from actinomycetes (*14*). Actinomycete derived fungicides which have been commercialized for drug use include the polyene antibiotics

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(amphotericin B, candicidin, filipin III, hamycin, natamycin, nystatin, rimocidin) and geldanamycin. Most natural fungicides used as commercial crop protection agents were also derived from actinomycetes (Figure 1). These include three nucleoside antibiotics (blasticiden S, milidiomycin, polyoxin B), three aminoglycoside antibiotics (kasugamycin, streptomycin, validamycin) and two polyketide antibiotics (natamycin, oxytetracycline). Two additional actinomycete antibiotics (cycloheximide, novobiocin) were used for many years, but their registration has been discontinued (*15*). Therefore, actinomycetes can be seen as a productive source of commercial fungicidal chemistry.

The Need for Dereplication in Natural Product Discovery

In an effort to discover novel leads to develop crop protection products, Dow AgroSciences screened actinomycetes for fungicidal activity over a 15-year period. The actinomycete sources used were varied, however, most extracts came from large high-throughput collections composed of random soil isolates. Most of these isolates were characterized phylogenetically and were fermented in multiple media (2-4 each). These extracts were screened for inhibition of multiple plant pathogenic fungi. The target fungi changed over the test period, but included one or more of the following fungi: *Phytophthora infestans* (Bayer code PHYTIN), *Plasmopara viticola* (PLASVI), *Saccharomyces cerevisiae* (SACCCE), *Leptosphaeria nodurum* (LEPTNO), *Septoria tritici* (SEPTTR) or *Ustilago maydis* (USTIMA).

The antifungal chemical diversity of actinomycetes also produces a challenge since rediscovery of known fungicidally active compounds is common. Therefore, efficient natural product discovery requires a robust dereplication process to avoid rediscovery of these known compounds. The importance of dereplication was evident on our experience in natural product screening. Over the course of screening >205,000 actinomycete extracts at Dow AgroSciences, over 6,800 fungicidal extracts were observed. Chemical evaluation (LC/MS dereplication and isolation) of these extracts revealed 176 different fungicidal compound classes. The majority of these compound classes (120 or 68%) were found only rarely: <0.1% of all fungicidal extracts (Figure 4A). Seventy-six compounds were found only once and nine compounds were novel, new-to-science structures. Although these rare structures were highly desirable, many of the antifungal compounds were found repeatedly. Fungicidal activity in half of active extracts (49.9%) was due to one of seven compounds. These seven compounds that were each present in at least 5% of the active extracts were labeled nuisance compounds (Figure 4A). In addition, eight common compounds (found in ≥2% of active extracts) and nine frequent compounds (≥1%) were observed repeatedly. These 24 frequently isolated compounds (sum of nuisance, common and frequent) were present in 84% of active extracts. Therefore, the ability to detect these 24 compounds enabled the primary discovery effort to concentrate on just 16% of the active extracts. This ability to focus on the extracts most likely to yield novel chemistry is the essence of dereplication (*16*).

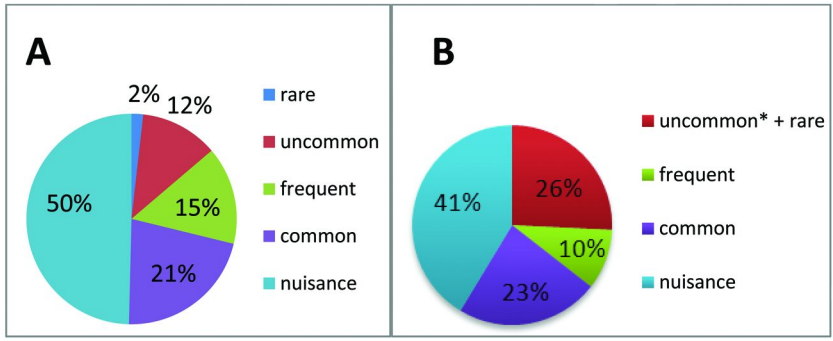

Figure 4. A) Repetitive observation of fungicidal compounds in screening actinomycete extracts from Dow AgroSciences (DAS); and, B) screening by Genilloud et al (17) for pharmaceuticals included antibacterial targets, however, no antibacterials were frequent (>1%).

Recently the results of antifungal screening and dereplication of a set of 28,000 actinomycete extracts (from 8,670 strains) was published (*17*). The concept of frequently isolated classes of chemistry (observed in ≥1% of active extracts) was similar in antifungal screening against pharmaceutically important fungi (Figure 4B). Several nuisance compounds (≥5% of active extracts) were observed: nocardamine, polyenes (n=5 or 7), actinomycin D and nigericin. In addition, nine common compounds (deferoxamine mesylate, polyene (n=4), blastmycin, bafilomycins, salbomycin, geldanamycins, azalomycins, polyketomycin) and six frequent compounds (griseorixin, rubromycin, mithramycin, griseolutin, oxo-polyene, chartreucin, oligomycin) were observed. Although different sets of actinomycetes were screened, the highly repetitive nature of antifungal compounds produced was similar (Figure 4). Interestingly, of the 19 compounds active against human pathogenic fungi which were observed frequently (≥1%) by Genilloud et al. (*17*), only 11 were observed in screening against plant pathogenic fungi at Dow AgroSciences. Therefore, the compounds targeted in dereplication were highly dependent on the target organisms used for bioassay.

Cryptic Natural Product Biosynthetic Pathways

The most frequently observed antifungal compound by Genilloud *et al* (*17*) was nocardamine. Nocardamine, also called deferoxamine E, is a hydroxamate siderophore which is important for iron scavenging in actinomycetes (*18*). Four genes required for biosyntheses of nocardamine (*sidA-D*) were discovered in *Streptomyces avermitilis* using genome mining (*19*). The *sidA* gene from the nocardamine biosynthetic gene was used to search for similar genes in published actinomycete genomes (*20*). Genes with high similarity to *sidA* were found in all published *Streptomyces* genomes queried (n=29), as well as at least 15 other actinomycete genera. Therefore, nocardamine biosynthetic genes appear to be very common in actinomycetes, particularly the genus *Streptomyces*. Although 64% of the 8,670 strains screened by Genilloud *et a*l (*17*) were *Streptomyces*

(5,530), nocardamine was only observed in about 8% of bioactive extracts. This suggests that very few of nocardamine biosynthetic pathways, perhaps less than 1%, were expressed. Unexpressed biosynthetic genes are called cryptic or silent pathways, therefore, the majority of nocardamine biosynthetic pathways appear to be cryptic under the conditions which the organisms were cultured.

The predominance of cryptic pathways in *Streptomyces* was also observed in uncommonly encountered compounds.Maltophilin is a known antifungal compound which was first isolated from actinomycetes at Dow AgroSciences (*21*). The biosynthetic genes for a close analog of maltophilin, the frontalamides (*ftdA-F*) were published recently (*22*). Based on the structural similarity of the frontalamides and maltophilin, it is likely that the biosynthetic genes are highly similar. Homology searches with *ftdA-F* revealed that 10 of 13 *Streptomyces* genomes queried contained gene clusters with striking similarity to the frontalamide gene cluster (*22*). Therefore, many *Streptomyces* spp. have the genes for maltophilin or frontalamide production. Maltophilin was observed infrequently (<1%) in the >6,000 active extracts in the Dow AgroSciences screening set detailed above. This indicated that the biosynthetic genes for maltophilin-type compounds in most *Streptomyces* spp. are cryptic.

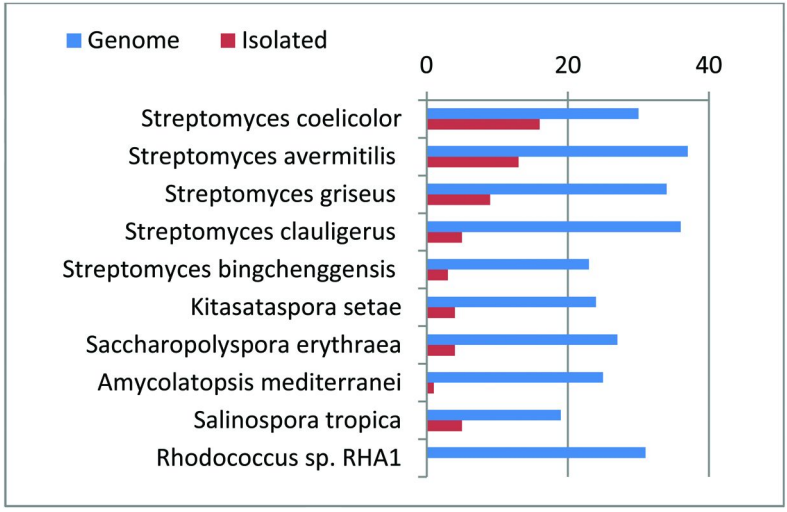

Figure 5. Cryptic Metabolites: Chemical and Genomic Evidence. Grey bars indicate the number of metabolites isolated from culture. Black bars indicate the number of secondary metabolite gene clusters predicted from the genomic sequence. Adapted from Nett et al. (24).

With the recent expansion of genomic data and genetic analysis it has been suggested that actinomycetes could be dereplicated using genetics (*23*). Genetic dereplication would use hybridization or genomic sequencing to eliminate strains containing genetic pathways of known compounds. Genetic analysis

could be done on early isolates and avoid the cost of fermentation and chemical dereplication. Although useful in principle, genetic dereplication is inadvisable due to the common occurrence of cryptic metabolites. Genetic dereplication of strains containing maltophilin or nocardamine genes would eliminate a large number of actinomycetes which do not actually produce these metabolites. This would result in lost opportunities for discovery of novel metabolites.

Although it is widely understood that actinomycetes have rich metabolic potential for antibiotic biosynthesis, genomic evidence indicates that the vast majority of natural product biosynthetic genes in actinomycetes are cryptic. In Figure 5 the number of natural product biosynthesis pathways discerned from genetic data is compared to the actual number of compounds isolated from select actinomycetes. In every case, the compounds associated with most biosynthetic pathways have not been observed. Even after coordinated effort on the most studied organisms, *Streptomyces coelicolor*, *Streptomyces avermitilis* (*24*) and *Streptomyces griseus* (*25*), fewer than 50% of the compounds associated with the coded pathways have been isolated. This indicates that cryptic is the normal condition for *Streptomyces* secondary metabolite biosynthetic genes. Unlocking these unexpressed genes represents a tremendous potential for discovery of novel natural product chemistry from actinomycetes.

Induction of Cryptic Biosynthetic Pathways

The process of natural products discovery from actinomycetes has generally been one of accessing the available natural compounds expressed by novel isolates. As a result, pharmaceutical companies and researchers throughout the world have amassed large collections of actinomycetes in the hope of finding a new producer of a novel metabolite. This was very successful in the "Golden Age" of antibiotic discovery where common metabolites, "low-hanging fruit," were discovered and commercialized (*26*). As natural product discovery progressed, the difficulty of discovering rarer metabolites, the "high-hanging fruit," led to rising costs and diminished returns. As a result, many large pharmaceutical companies abandoned natural product discovery. With our current understanding of natural products in actinomycetes as being predominately cryptic, we propose changing the game from finding available fruit to making fruit available. A number of schemes have been proposed to induce cryptic natural products using fermentation conditions, stressors and elicitors. Genetic methods to turn on expression of cryptic pathways *in vivo* or in heterologous hosts have also been demonstrated (*27–29*); however, genetic methods will not be covered in this review. We seek to devise conditions to induce actinomycetes to produce new metabolites from cryptic biosynthetic pathways.

One method to induce cryptic natural products is to vary the fermentation conditions. Several groups have demonstrated that subjecting each strain to multiple fermentation media and growth methods can induce cryptic metabolites (*17*, *30*, *31*). Unfortunately, this affects a few metabolites in a few strains and does not address the dozens of metabolites which are cryptic in each strain. For example, in an effort to discover novel metabolites, Genilloud *et al.* (*17*)

fermented a set of 400 *Streptomyces* strains in eight fermentation conditions. As illustrated in Figure 5, each strain may have the genetic capacity to produce 20-40 metabolites, therefore, this set of 400 strains should be capable of producing thousands of metabolites even if there is significant genetic redundancy between strains. The extracts produced from these 400 strains were screened for antifungal and antibacterial activity and the bioactive metabolites were determined. Almost 180 different bioactive metabolites were accumulated from the 8 media (*17*). The best media resulted in over 70 metabolites or 43% of the total. Each additional fermentation condition resulted in diminishing returns (i.e. metabolites which were primarily redundant with the first media plus a few additional metabolites). Ultimately the exercise resulted in less than one novel metabolite per 2 strains (180/400). This limited output leaves the majority of metabolites encoded in the genome inaccessible through multiple fermentation conditions.

It has been demonstrated that nutritional, genetic and physical factors applied to actinomycetes result in modest induction of new metabolites (*32*). Some actinomycetes produce stimulatory factors which can induce antibiotic production when strains are grown in close proximity (*33*). Of these factors, the γ-butyrolactones are widely spread in *Streptomyces* spp. and have been well characterized (*34*). Unfortunately, the γ-butyrolactones are diverse in structure and generally strain specific, and cannot be used to induce collections of strains. Actinorhodin production in *S. coelicolor* can be induced by histone deacetylase inhibitors (*35*) as well as growth in the presence of predatory *Myxococcus xanthus* (*36*). Some antibiotic resistant mutants over-express known compounds or produce new metabolites (*37–39*). Several studies indicated that ethanol added to fermentations can stimulate antibiotic production (*40*). Also, dimethyl sulfoxide (DMSO (*41*);), scandium chloride (*42*), *N*-acetyl-glucosamine (*43*) and heavy metals (*44*) can induce production of known and novel metabolites or new bioactivity in specific strains. Some of these methods can be applied broadly to impact metabolite production in collection of strains (*35*).

Antibiotics as Inducers of Cryptic Metabolites

Antibiotics from actinomycetes are toxic toward bacteria, so it has been widely believed that actinomycetes in nature use antibiotics to inhibit the growth of other bacteria and reduce competition for resources. However, since antibiotics rarely reach lethal concentrations in the environment, Julian Davies and others have suggested that the natural function of antibiotics is not toxicity, but rather potent modulation of gene expression and metabolic processes (*45*). Sub-inhibitory concentration of antibiotics, induce a variety of effects in *Escherichia coli, Staphylococcus aureus, Streptococcus pneumonia, Bacillus subtilus, Pseudomonas* spp. and *Salmonella* spp. (*45*, *46*). These effects include induction of a stress response, transport, cell wall biosynthesis, virulence genes, or depression of protein synthesis or protein transport. Some evidence suggests that antibiotics may directly modulate gene expression (*45*). Only two of the antibiotics from the studies above, tetracycline and bacitracin, have been tested in actinomycetes; both had the ability to induce cryptic metabolites in *Streptomyces*

(*47*). Polyether antibiotics were shown to induce antibiotic production in multiple actinomycete species (*48–50*). In addition, *S. coelicolor* could detect an external antibiotic and mounted a specific transcriptional response to it (*51*).

The question remains: are these isolated effects or can antibiotics be applied to induce cryptic metabolites in a wide range of actinomycete isolates? A study was initiated at Dow AgroSciences to evaluate whether antibiotics added in sub-inhibitory concentration to culture medium could induce production of cryptic pathways in actinomycetes. Several strains (*Streptomyces pactum* ATCC27456, *S. avermitilis* ATCC31267 and *Streptomyces* sp. CP1130) were fermented in the presence of sub-inhibitory concentrations of antibiotics confirmed to induce gene expression in non-actinomycetes in previous studies (*45*, *46*), as well as physical factors reported to induce metabolites (Table I).

Table I. Elicitors and Stressors Used to Influence Cryptic Metabolite Production

Compound	***Treatment***[a]	***Reference***
azaleucine	1 ppm in medium	(*45*)
bacitracin	1 ppm in medium	(*47*)
ciprofloxacin	1 ppm in medium	(*46*)
nigericin	1 ppm in medium	(*48*)
phleomycin	1 ppm in medium	(*46*)
puromycin	1 ppm in medium	(*46*)
tetracycline	1.05 ppm in medium	(*47*)
trimethoprim	11 ppm in medium	(*46*)
dimethylsulfoxide	1% in medium	(*41*)
N-acetyl glucosamine	100 μM	(*43*)
$ScCl_2$	70 μM	(*42*)
ethanol	flooded and air dried, d3	(*40*)
heat shock	45 °C, 30 min, day 3	

[a] Antibiotics were added at the indicated concentration to solid medium MM (*56*) in 24-well plates. Strains were patched onto MM and grown for 8 days at 30 °C. Ethanol and heat shock were treated as indicated on day 3. Extracts were made by the addition of 50:50 ethanol:water to mature culture wells, soaked for 1 hr and filtered.

Actinomycetes commonly produce pigments and it was observed that additions of various stressors and physical factors affected pigmentation. Some stressors induced while others repressed pigment formation in various strains. Fermentations were extracted and analyzed by LC-TOF for the presence of specific antibiotics.

Streptomyces pactum (ATCC 27456) is known to produce the metabolites pactamycin (*52*), piericidin (*53*), and actinopyrone (*54*). Two of the known metabolites, piericidin A1 and pactamycin were observed in the control extract (no antibiotic addition). Piericidin was induced about 2.5-fold by heat shock and pactimycin was induced by ciprofloxacin (~4.6-fold), nigericin and bacitracin (both ~2-fold). Conglobatin was produced in the control and was induced ~2 fold by nigericin. Conglobatin had not been observed previously in *S. pactam*, but has been detected in other strains producing piericidins (*55*). In addition, two cryptic metabolites were induced: tunicamycins (VII, VIII and IX) which were not produced in the control were induced by azaleucine and heat shock; and, ligomycin A was only observed at low levels under inducing conditions (heat shock and ethanol). Actinopyrone was not included in the LC-TOF library thus was not observed.

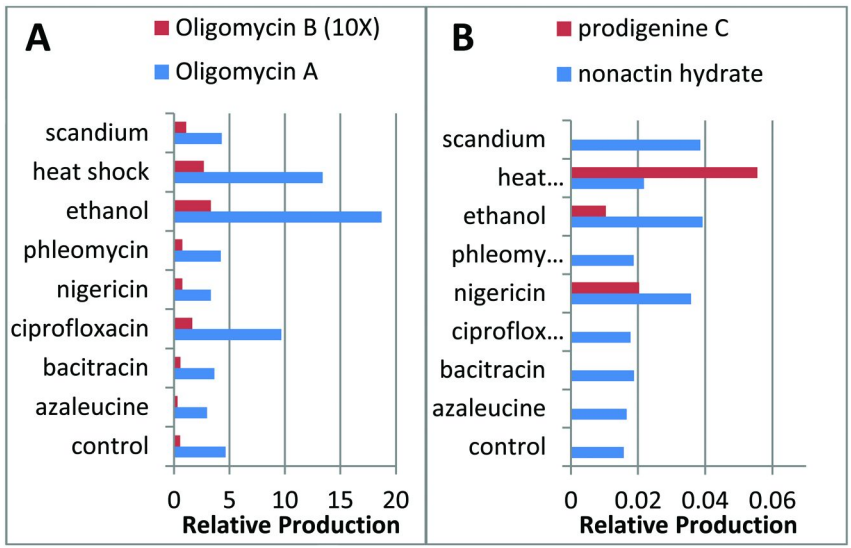

Figure 6. Induction of Metabolites from Streptomyces spp. Elicitors are listed on the Y axis; compounds observed are indicated by bars and legend. The X axis is relative to the internal standard, spinosyn D (1.0 = ~0.25ppm). A) Induction of oligomycins in S. avermitilis ATCC 31267. Relative production values for Oligomycin B is 10x scale to enable charting with oligomycin A. B) Induction of metabolites in Streptomyces sp. CP1130.

Streptomyces avermitilis (ATCC 31267) is best known for production of the agriculturally important antibiotic avermectin (Abamectin®). The genomic sequence of *S. avermitilis* ATCC 31267 has been determined and oligomycin A, filipin III and ten other compounds have been isolated from ATCC 31267 (*24*, *57*). In this study, neither avermectin B1a nor filipin III were observed in the control, but both were induced to low levels by ciprofloxacin. Avermectin B1a was also induced by heat shock and filipin III was induced by phleomycin.

Oligomycin A was produced at significant levels in the control (~1 ppm) and induced ~3-fold by heat shock and ciprofloxacin (Figure 6A); oligomycin B was induced by heat shock, ciprofloxacin and ethanol. No cryptic metabolites were observed in extracts from *S. avermitilis* ATCC 31267.

Streptomyces sp. CP1130 has been shown to produce three insecticidal compounds: prodigenine C, undecylprodigenine (prodigenine O) and tartrolon C (*58*). Tartrolon C was detected in the control (~0.5ppm), but was repressed under most inducing conditions. The prodigenines were also repressed under most inducing conditions. Prodigenine O was observed at very low levels in the control (<0.01ppm); prodigenine C was not detected in the control, but was produced at a very low level with nigericin (<0.01ppm) and induced three fold higher by heat shock (Figure 6B). In addition, two cryptic metabolites were detected: nonactin was detected in the control, and induced by nigericin, ethanol and scandium chloride; and, conglobatin was induced to low levels by heat shock.

These experiments showed that fermentation of actinomycete strains in the presence of antibiotics or stressors is clearly able to induce production of secondary metabolites. It was observed that: 1) the levels of known compounds could be induced significantly; 2) new compounds from cryptic pathways could be induced; and 3) cryptic metabolites can be induced at high frequency in high throughput fermentation of strains from culture collections.

From Discovery to Development

Although the discovery of antifungal novel natural metabolites is difficult, transformation of *in vitro* fungitoxicity into field efficacy is complex, as well. New active ingredients must satisfy complex commercial, technical and environmental hurdles (*10*). On the commercial side the crop protection market is mature so new products generally replace existing products and thus must have superior technical characteristics. These technical characteristics can be improved efficacy, physical properties or reduced environmental impact. Recently regulatory agencies have added new stringency in toxicological and environmental fate profiles which must be met. A few natural products such as spinosad (*59*, *60*) have strong technical characteristics and can be marketed directly. Other products like spinetoram or abamectin require minor chemical modification (i.e. semi-synthesis, (*6*), (*59*)). Finally other natural compounds, such as pyrrolnitrin or strobilurin, cannot meet these strict criteria but can serve as scaffolds for synthesis of effective crop protection products. Three examples of recent discoveries of fungicidal crop protection agents at Dow AgroSciences are presented below: one from an existing compound, one from an unexpected source;and, one from traditional screening of actinomycetes.

Discovery of Antifungals from Old Natural Products

Although natural product discovery programs typically seek new chemistry, occasionally known natural compounds can form the basis of discovery of novel crop protection agents. In 1949 the antibiotic antimycin was discovered from a soil isolate *Streptomyces antibioticus* (*61*). A fairly common metabolite,

antimycin can be produced by approximately 1 in 1,000 *Streptomyces* soil isolates (*57*). Antimycin has antifungal activity against a wide range of plant pathogenic fungi; however, it was unattractive as a crop protection agent due to general cytotoxicity. The mode of action of antimycin is inhibition of complex III of the mitochondrial electron transport (METIII). Antimycin was an attractive target for development of a crop protection fungicide since METIII is a validated target of a leading class of fungicides, the strobilurins. In addition, the antimycin binding site, METIII Qi, is distinct from the strobilurin binding site, METIII Qo, and antimycin is active against strobilurin insensitive METIII Qo mutants (*62*). For this reason Dow AgroSciences undertook an effort to develop synthetic antimycin-based chemistry that retained the antifungal mode of action but had a greater selectivity against non-target organisms.

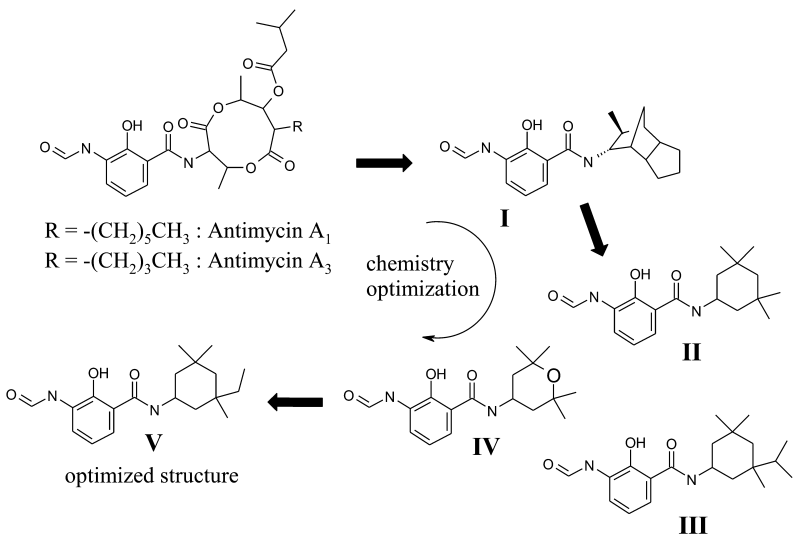

Figure 7. Synthetic milestones in the optimization of N-formyl aminosalicylamide (FSA) analogs based on antimycin. Reproduced with permission from Ref. (64). Copyright 2007 American Chemical Society.

It was known that the formyl salicylic acid (FSA) "head" of antimycin was required for binding at the METIII Qi site (*63*). A large number of natural antimycin analogs exist which vary in the substitution of the bis-lactone either at the R group (Figure 7) or the ester. Since these natural variants do not have the attributes desired for crop protection, modifications to the bis-lactone ring were investigated (*64*). A large number of FSA compounds with various aromatic, aliphatic (cyclic and non-cyclic), hetero or bicyclic ring systems were synthesized

and the norbornane derivative (**I**) demonstrated interesting activity (Figure 7). This was further improved through evaluation of target site and antifungal activity to arrive at the optimized 3-methylethyl, 5-gemdimethyl-cyclohexyl group (**V**; Figure 7). Target site inhibition by compound **V** was at least 10-fold better than azoxystrobin, depending on the source species (*64*) and activity against non-target species was reduced. Compound **V** also demonstrated superior *in vitro* control of *Plasmopora viticola* and *Phytophthora infestans* as well as improved potency against these phytopathogenic fungi in field testing.

Discovery of Antifungals from Unexpected Sources

A number of antifungal metabolites have been isolated from the entomopathogenic (insect pathogenic) bacterial genera *Xenorhabdus* and *Photorhabdus* (*65*). Entomopathogenic bacteria seem an odd place to search for antifungal compounds; however, antifungal metabolites play a vital role in the life cycle of these organisms. *Xenorhabdus* and *Photorhabdus* can be free living in the soil or can be symbionts of soil nematodes. As symbionts, they live in the upper gut of nematodes that prey on soil living insect larvae. When the nematodes invade an insect, they regurgitate their bacterial symbiont which begins to replicate. Insecticidal toxins produced by the *Xenorhabdus* or *Photorhabdus* symbiont kill the insect and antifungal and antibacterial metabolites produced by the symbionts help preserve the insect carcass as a food source for both the bacteria and the nematode. Since antifungal metabolites are important for the *in vivo* insect stage of the bacteria we postulated that growth *in vivo* could induce *Xenorhabdus* and *Photorhabdus* symbionts to produce antifungal metabolites.

A large number of *Xenorhabdus* and *Photorhabdus* from the Dow AgroSciences culture collection were grown in Trypticase™ Soy Broth (TSB (*66*);) and injected under the cuticle of larvae of *Spodopetera exigua* (beet armyworm; Bayer code LAPHEG). After two days, dead infected larvae were macerated, extracted into ethanol and screened for fungicidal activity. Fifteen strains had antifungal activity. Independently it was found that *Photorhabdus* spp. grown in TSB + proline (artificial hemolymph) were induced to produce new metabolites relative to growth in TSB alone (*67*). The *in vivo* induced antifungal DAS strains were grown in TSB + proline and the majority (11/15) were found to reproduce the *in vivo* antifungal activity. Therefore, much simpler *in vitro* growth in TSB + proline was used for scale-up of these antifungal cultures.

Using bioactivity-based fractionation, bio-active metabolites were isolated from five of the antifungal strains. Three strains of *Xenorhabdus* spp. (ILM68, ILM69 and ILM82) produced the known metabolites xenofuranone A and B (Figure 8). Both xenofuranones showed antifungal activity against plant pathogenic fungi. Two strains of *Photorhabdus* spp. (HDP57 and ILM62) produced a series of novel stilbenes which were fungicidal. *Photorhabdus* sp. ILM62 also produced purpurin-1-methylester which was weakly fungicidal. A novel pyrone, which was not fungicidally active, was also produced by *Photorhabdus* sp. HDP57.

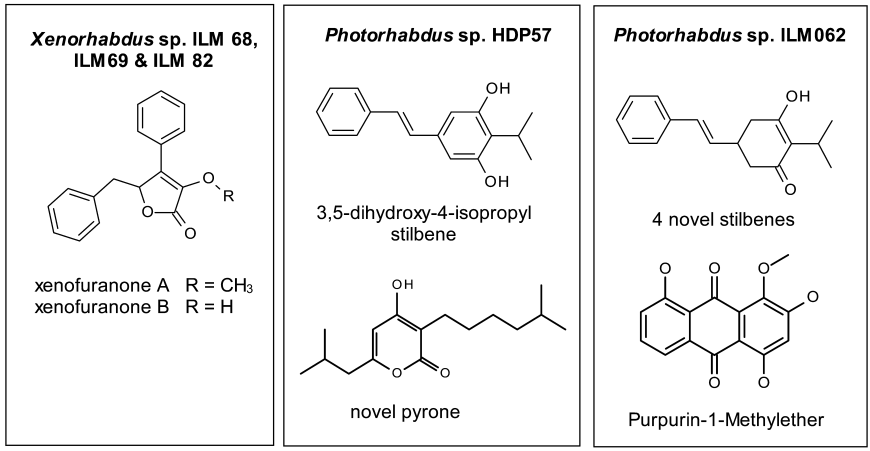

Figure 8. Antifungal metabolites isolated from Xenorhabdus spp. and Photorhabdus spp.

A New Antifungal from an Actinomycete

Large collections of actinomycetes that were randomly isolated from soil have been a highly productive resource in the discovery of novel fungicidal metabolites (*17*). In the course of screening actinomycete extracts from MerLion Pharmaceuticals, an extract with potent *in vitro* fungicidal activity was found. The crude extract from *Streptomyces* sp. MLA1839 was prioritized for further study based on its ability to control fungal disease on plants. Bioactivity-based fractionation led to the isolation of two bio-active metabolites (*68*). The analogs were new structures in the phoslactomycin family of antifungal metabolites (*69*). Although phoslactomycins were active against phosphatase 2A in mammalian systems (*70*), the fungicidal mode of action (MoA) is unknown and would likely be a novel MoA for crop protection.

The newly identified bioactive compounds had novel structural characteristics relative to the known phoslactomycins and were named phoslactomycin H and phoslactomycin I (Figure 9). Phoslactomycin H and I exhibited antifungal activity against multiple plant pathogenic fungi and were able to control fungal disease on plants. It was also observed that phoslactomycin I was more active than phoslactomycin H indicating that the lactone ring was important for the activity. In order to further study this chemistry two compounds, phoslactomycin E and phoslactomycin 4B were obtained from Kevin Reynolds at Portland State University. Phoslactomycin E is a natural compound while phoslactomycin 4B was generated by precursor-directed fermentation of a phoslactomycin biosynthetic mutant (*70*, *71*). Of the four compounds tested, phoslactomycin 4B demonstrated the most potent activity against plant pathogenic fungi. Phoslactomycin H was the least potent.

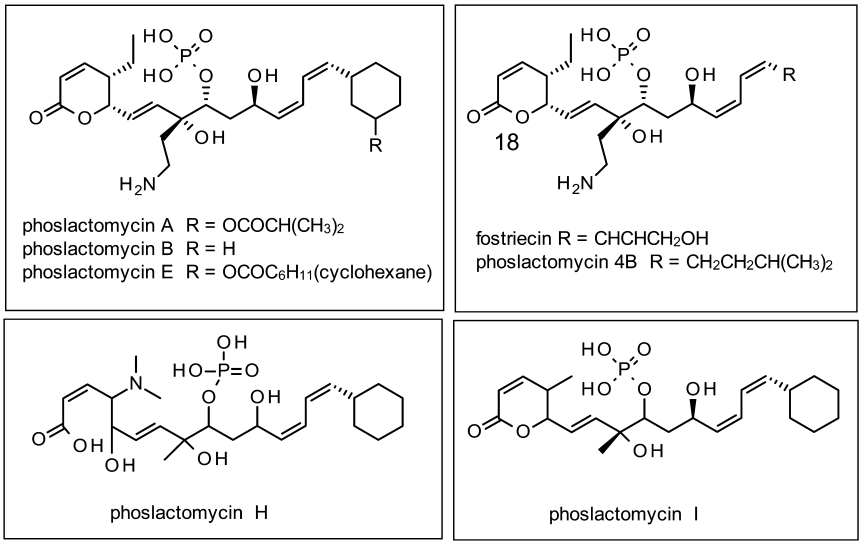

Figure 9. Structure of fostriecin and phoslactomycins.

Both phoslactomycin H and I showed *in vitro* anti-fungal activity against 3 plant pathogens: *Phytophthora infestans*, *Septoria tritici* and *Ustilago maydis*. Pathogen specificity was observed with phoslactomycin I which was active *in vivo* against the oomycete *P. infestans* providing 87% control at 111 ppm and against the basidiomycete *Puccinia recondita* providing 88% control at 200 ppm. Lack of disease control was observed against the imperfect fungus *Alternaria solani*. The lack of *in vivo* activity of phoslactomycin I against *S. tritici* and *Cochliobolus sativus* may be due to the observed phytotoxicity.

Conclusion

Natural products from bacteria are an important source of novel leads for development of crop protection fungicides. It has been estimated that the number of undiscovered natural products from nature far exceeds the number discovered to date (*72*). One important source of new natural products is cryptic biosynthetic pathways that can be induced in *Streptomyces* by antibiotics and stress factors or by *in vivo* growth of entomopathogenic bacteria. Novel bioactive natural compounds with appropriate physical characteristics can be utilized in crop protection as fermentation products. However, chemical synthesis has been demonstrated to be an effective way to build useful physical characteristics into lead molecules. Natural products are a productive source of novel bioactive scaffolds for use as synthetic starting points for development of crop protection fungicides.

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