

Semiochemicals in Pest and Weed Control

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Semiochemicals in Pest and Weed Control

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Preface

Control of insect pests and invasive weeds has become very sophisticated. Knowledge of the interaction between insects and host plants is critical to development of effective control strategies that are friendly to the environment. We must also understand how insects communicate with each other. What are the semiochemicals (signal substances such as sex pheromones) and how do they function? Research efforts in this fast-growing field involve organic chemists, analytical chemists, entomologists, biochemists, microbiologists, formulation experts, and statisticians.

This book has chapters taken from the symposium *Semiochemicals in Pest Management and Alternative Agriculture* presented at the New Orleans, Louisiana, American Chemical Society National Meeting held in the spring of 2003. This symposium was sponsored by the ACS Division of Agricultural and Food Chemistry with the aim of providing scientists with a forum for presenting research on semiochemicals.

Our goal is to inform the reader of current efforts in research regarding the discovery and use of semiochemicals as part of an integrated pest or weed management program. After a brief overview chapter, a section describes the discovery of a variety of new semiochemicals. The next section describes research aimed at efficient control of the Formosan termite. Deployment of semiochemicals, along with pesticides, is discussed in this section. The final section describes efforts to develop formulations of microbial pesticides that resist sunlight degradation.

Acknowledgments

We express our thanks to the authors and their employers who have shared with us the details of their interesting results at the

symposium and in the published chapters. We hope that you our readers will find the chapters interesting, useful, and stimulating. We also thank Bedukian Research Inc. and the ACS Division of Agricultural and Food Chemistry for their generous support of the symposium resulting in this publication.

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Chapter 1

Semiochemicals in Pest and Weed Control: An Introduction

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Section 1: Discovery of New and Useful Semiochemicals

Semiochemicals are natural substances produced and used by animals and plants to communicate. The term “Semio” comes from Greek and means “sign”. Examples of semiochemicals are insect pheromones (substance produced by an insect to attract members of its own species or notify members of its own species that danger is present – an alarm) and allelochemicals (used for communication between individuals belonging to different species, such as plants and insects or plants and other plants) (*1*). The weed suppressive activity of sorghum (Chapter 5) is an example of an allelochemical. The remaining chapters in this section deal with mosquito-detering activity (Chapter 1) or the insect pheromone type of semiochemical (Chapters 2-4). Insect pheromones can be used as part of an integrated pest management program or to monitor dispersal of a beneficial insect used to control invasive weeds, such as the Saltcedar leaf beetle to control saltcedar (Chapter 3).

Steps in accomplishing the goal of utilizing pheromone semiochemicals from insects to control pests and invasive weeds include: 1) establishing laboratory cultures of the insect species having economic impact on agriculture

or potential use as a biocontrol agent against an invasive weed; 2) demonstrating the existence of a semiochemical by use of a suitable bioassay; 3) obtaining and purifying the compounds using bioassay-directed fractionation procedures; 4) structure elucidation using microscale chemical derivitization (e.g. derivatives to locate double bonds) and state-of-the-art instrumental methods such as 2D-NMR, FTIR, and GC/MS; 5) total synthesis of the compounds for positive identification and for comparison of activity with natural compounds; 6) evaluation of synthetic semiochemicals or blends thereof under field conditions as potential agents to monitor or control the target insect pest or to monitor dispersal of biocontrol agent populations; 7) formulation and deployment of semiochemicals, along with chemical or biological pesticides, to control agriculturally important insect pests or formulation and deployment of semiochemicals, along with beneficial insects, to control invasive weeds. Important issues arise in each of the above steps that need to be resolved. The deployment of biological pesticides has its own special set of problems that will be discussed in the final section of the introduction and in section three of this book.

Semiochemicals are naturally present in only very minute quantities and are sometimes unstable at ambient temperature or in the presence of light (e.g. polyunsaturated aldehydes). Discovery of important and useful semiochemicals is often very difficult because of the scarcity of the compounds and requires considerable technical skill. General semiochemical research techniques can be found in reference texts on chemical ecology (2, 3).

Commercialization of semiochemicals for use in crop protection or in the fight against invasive weeds has an important cost issue. A company must make a profit on any business activity in order to survive in a highly competitive marketplace. For this reason, synthetic pathways to the target compounds must be short and contain robust reactions. Elegant but difficult chemistry must be replaced by operationally convenient reactions. Tedious and expensive fractional distillation or chromatographic purification must be eliminated or at least minimized. These "facts of life" are usually neglected in the synthetic chemical literature so they are presented here.

Section 2: Control of Formosan Termite

Since this symposium was held in New Orleans, it was appropriate to describe research aimed at efficient control of the Formosan subterranean termite (*Coptotermes formosanus*). The Formosan subterranean termite is among the most devastating urban pests in the world wherever it has spread. Its management costs the U.S. alone hundreds of millions of dollars a year.

The removal of chlordane and other pesticides for pest control in the late 80's has led to a search for effective, environmentally friendly compounds with antitermite activity. A survey of the literature focusing on the search for antitermite semiochemicals shows that much of the work involves bioassay guided isolation using one or several termite species. Unfortunately, it is also very clear from the literature that any given compound or material active against a termite species may very well have a different type of activity against a different termite (4-6). The implications are that all previous work done with termite species other than *C. formosanus* has to be reviewed if one is to apply it to the Formosan termite. An added problem in using results from past studies is the lack of standardization in performing antitermite bioassays and in reporting results. Nor could there really be any expectation of uniformity since so many variables are involved: location of test (e.g., laboratory, field), size, duration, substrate choice, and choice of method of application of test compound, to name just a few. Different types of activity have been measured but frequently only one type of activity is reported. Toxicity might be measured in a variety of ways and is subject to a large number of variables including the volatility of the test compounds, their stability, their interaction with the substrate in which they are applied, or any other number of variables. Alternatively, repellency or resistance of a given substrate or compound can be measured, each measurement also with its own sets of variables. To complicate things, the perceived significance of the observed results may vary over time. A multitude of likely antitermitic compounds or natural substrates may have been discounted in past studies because they did not compare well to contemporaneous pesticides now banned, discontinued, or with restricted use because of their excess toxicity (7). Finally, assays are performed sometimes only after the compounds are isolated by a chemist or other scientist and might reflect such varied factors as ease of isolation from the natural source, predominance in the natural source, or even field of expertise of the isolating scientist.

Semiochemicals are a promising source of compounds with antitermite activities. Many currently used pesticides are in fact derived from a handful of naturally existing semiochemicals. Some of the most striking examples are, of course, the pyrethroids and the nicotinoids. Many highly effective insecticides including termiticides have, indeed, been obtained using either of the natural pyrethrins or nicotine as parent compounds. For the most part, *C. formosanus* antitermite studies are focused either on investigating trees and their active components for resistance or toxicity, testing known natural compounds with activities against other insect species, or investigating other organisms such as termite enemies, parasites, or even the termites themselves in the search for active principles. Many studies exist detailing the resistance and toxicity of woods from hundreds of different tree species towards termites (8, and references therein). The literature contains several instances of antitermite compounds being isolated from some of these woods, but also from other

natural sources (9-12). In addition, natural compounds with structures similar to those of other active antitermite semiochemicals are also being studied and providing a number of new, highly active, compounds for termite management as well as providing additional information on structure-activity relationships that might lead to even more potent or versatile agents (13,14).

Semiochemicals remain an attractive research subject with lots of promise. The chemical diversity found in these semiochemicals are likely to yield active compounds with previously unknown modes of action. Indeed, the range of natural compounds investigated for *C. formosanus* antitermite activity spans a wide range of chemical classes: sapogenins, bisnorditerpenoids, furanoterpenoids, monoterpenoids, sesquirosefurans, diterpenes, phenolics, bicyclic ketones, quinones, diazo compounds, bicyclic sulfides, avermectin B1, podophyllotoxin, rotenone, hinokinin, and essential oils (8, and references therein). Modes of action of interest for activity against termites include pesticidal, repellent, attractant, as well as any activity that leads to the ability to modify or manage any aspect of termite physiology or behavior. It is known, for instance, that the behavior of the Formosan subterranean termite is directed by a number of semiochemicals produced by other termites of the colony (15). Identification of trail-following pheromones, for instance, would be in line with the goal of obtaining compounds to manage termite behavior, and several lines of research are looking into these possibilities (16). Other semiochemicals of termite origin being investigated stimulate feeding, and/or arrest the foraging behavior. Finally, an interesting source of semiochemicals currently being investigated, deriving from other organisms with which *C. formosanus* interacts, involves fungi and fungi infested woods and lignin. (17,18). Semiochemicals derived from these research efforts might lead to compounds that can be used to affect, for instance, termite tunneling behavior.

Section 3: Developing Formulations of Microbial Pesticides to Resist Sunlight Degradation

Many options are available where chemistry contributes to agriculture, and more specifically, to production of field crops. Most notably is the development and use of chemical pesticides for control of harmful weeds, insects and diseases. Unfortunately, a few pesticides contribute to adverse environmental and human health effects and promote an adverse public view of pesticides in general. DDT is the quintessential example of a beneficial insecticide, which saved many human lives by killing insects such as mosquitoes, lice and fleas that transmit serious human diseases like malaria, typhus (19), but was also contributed to environmental contamination because of the phenomenon known as biological magnification in the food chain. Without delving into a specific

discussion about the pros and cons of chemical pesticides, the crux of the matter is that agriculture continues to promote the development and use of environmentally friendly pesticides in order to combat the prevalent public condemnation of chemical pesticides. Microbial-based pesticides are one biological answer to the counter the chemical pesticide perception. Entomopathogenic microbes have many characteristics that make them a suitable replacement for some insecticides. Microbial pesticides are the result of developing natural disease causing organisms such as bacteria, fungi, and viruses into target specific pesticides. Beneficial characteristics of microbial pesticides include specific activity against the target insect, disease development resulting in death of the host, potential for commercial production using in vitro methods, and natural transmission resulting in epizotics (natural disease progression) of the target pest (references). Unfortunately, microbial agents are sensitive to environmental exposure, most specifically exposure to solar radiation and more specifically UV radiation. Here again, application of the appropriate chemistry may improve the pest control by extending the residual activity of the microbes when applied to the field environment.

Microbial pesticides include bacteria, viruses, fungi, and nematodes, and are part of broader group known as biological control agents (which also includes fermentation products and chemically derived pheromones). Microbial agents used as pesticides have the benefit of specificity, user and environmental safety, diversity of target pests among a variety of agents, registration ease, and common delivery equipment with current chemical pesticides. In their book entitled *Biopesticides, use and delivery*, Hall and Menn (20) state that biopesticides represented 1.3% of the total pesticide sales in 1995. Although this represents a small fraction of the market, Menn (21) forecast a growth rate for biopesticides to be 10-15% per annum in contrast to 2% for chemical pesticides. Therefore, the future is bright for the development of biological pesticides, but only if technical difficulties can be overcome. Technical difficulties are often specific to each pest control agent. Requirements for successful development of commercial products include inexpensive production, shelf storage (propagule efficacy), efficacy, ease of application, and residual activity. Limited commercial success has attributed to difficulty of production, sensitivity to UV light and desiccation, requirement of high humidity for infection, insufficient performance over a wide range of environmental conditions and lack of appropriate formulation (22). Sensitivity to UV light has been demonstrated for many microbial agents.

Formulation development for microbial pesticides is often the results of efforts from two opposing directions. One direction is from the production side that begins with production of the active agent that provides a technical product with specific characteristics. For example the fungus *Beauveria bassiana* is produced by solid media culture and provides a powdered product containing a high concentration of hydrophobic spores. In contrast, the bacteria *Bacillus*

thuringiensis is produced by liquid fermentation that provides a technical product in liquid slurry form. Yet a baculovirus agent is produced in vivo and provides a technical product of virus particles in homogenized insect parts. From the other side, formulations are developed backwards beginning with the application environments specific to the pest control situation. For example, many microbes, including baculoviruses and bacteria, must be eaten by target pests to be infective. European corn borers are often controlled by the application of granular formulations applied to corn, which grows in such a way that the pesticide is funneled into the whirl and leaf axils of the plant where the young larvae often feed. Other pests such as cabbage loopers are better controlled by spray or dust applications that uniformly coat the plant leaves where these insects feed. In contrast to these pathogens that need to be ingested, many fungal pathogens, like *B. bassiana*, infect their hosts through the integument, and thus applications must target the insect. Therefore, the final formulation must link the technical material with the final application directed at the target pest. The “trick” for developing a useful formulation is to make this link between the pathogen and target in the most economical and effective way possible, which considers the cost of production for the manufacturer and the final efficacy for the end user.

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Chapter 2

Chemistry and Applications of Mealybug Sex Pheromones

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Abstract— The sex pheromone of the vine mealybug *Planococcus ficus* has been identified as a single component, (*S*)-lavandulyl senecioate **2a**. Males were equally attracted to either (*S*)-**2a** or racemic **2a**, indicating that the unnatural enantiomer does not inhibit male behavioral responses. Female mealybugs also produced (*S*)-lavandulol **1**, but mixtures of racemic **1** with racemic **2a** were less attractive to male mealybugs than racemic **2a** alone. In field trials, lures loaded with 100 µg doses of racemic **2a** attracted males for at least 12 wk. From a broader perspective, aspects of the pheromone chemistry and biology of four mealybug and five related scale insect species are discussed. The pheromones are all terpenoid esters, and all but one are characterized by an unusual linkage between isoprene units. In addition, each species produces unique structures, to the extent that no two species even produce stereoisomeric forms of the same basic skeleton. Aspects of the biology of these insects also are discussed in relation to practical applications of their pheromones for monitoring insect populations, and for pheromone-based control strategies.

Introduction

The mealybugs (Homoptera: Pseudococcidae) are a family of scale insects that are characterized by a covering of waxy material that gives them their characteristic “mealy” appearance. The newly hatched nymphs, known as crawlers, are quite mobile and can rapidly infest plants and fruits. Later stage nymphs and adult females are sessile, attaching themselves to plants with their mouthparts to feed on plant juices. The adult females produce sex attractant pheromones to attract the winged and very short-lived adult males to mate. Both sexes are typically ~2-4 millimeters long in the adult stages.

Economic losses from mealybug infestations in North American vineyards have increased dramatically over the past decade, with the vine mealybug, *Planococcus ficus* Signoret (Homoptera: Pseudococcidae), and the grape mealybug, *Pseudococcus maritimus* (Ehrhorn) (Homoptera: Pseudococcidae), being the two most important pest species (1,2). The vine mealybug has a broad distribution, being found in southern Europe, the Middle East, southern Africa, and parts of North and South America. Vine mealybug was first reported in California about 10 years ago (3), and since then it has spread through most of the grape-growing regions of California (2), causing severe economic damage to infested vineyards. The growth of sooty molds on the sugary secretions of the mealybugs (known as honeydew) and the buildup of insects in grape clusters render the fruit unmarketable (4). Vine mealybug has 4-6 generations per year in California, and it has also been reported to infest a variety of other crops, including figs, apples, citrus, dates, bananas, avocados, and mangos (5). Furthermore, vine mealybugs transmit viral diseases of grapevines (6-9).

Mealybug infestations in vineyards are difficult to manage for several reasons. First, mealybug control with insecticides has been inconsistent because the mealybugs are located primarily in protected sites such as leaf axils and bark crevices where pesticide penetration may be difficult (10,11). Pesticides also must be used with care to minimize deleterious effects on beneficial insects. Second, methods of monitoring most mealybug species consist of laborious examination of plants for live mealybugs, detection of honeydew and sooty mold, or even monitoring the ant species that “farm” the mealybugs for their honeydew (11,12). The development of faster, simpler, and more effective sampling methods based on pheromone-baited traps would offer distinct advantages for monitoring these pest insects.

Our overall project goal was to identify and develop pheromones that could be exploited for the management of the four major mealybug pests in California vineyards. We report here the identification, synthesis, and results of field tests of the sex pheromone of the vine mealybug. Within the space of two years, the pheromone-baited traps have become the cornerstone of methods of monitoring this pest in California, and they are also being developed for sale and use in South Africa, the Middle East, and South America.

Materials and Methods

Identification of the Pheromone

A colony of vine mealybug was obtained from Foothills Agricultural Research, Corona, CA, and reared on organically grown butternut squash (*Cucurbita moschata*). Squash were infested with newly-hatched mealybug crawlers. One wk later, the squash were sprayed with a 10 ppm solution of the insect growth regulator pyriproxyfen in 0.1% aqueous Triton-X, which selectively killed most of the males before they matured. The infested squash, with primarily mature virgin females, were then placed in 5L glass aeration chambers, and purified air was passed through the chamber at ~3L/min, with the volatiles being collected from the effluent air on a 2.5 cm X 4 mm ID column of Porapak-Q. Collectors were changed every 3-5 d, and compounds were eluted with pentane. Extracts were analyzed by GC-MS in electron impact mode (70 eV), using a Hewlett-Packard 6890 GC equipped with a DB-5 column (20 m x 0.25 mm ID, J&W Scientific), and interfaced to an H-P 5973 mass selective detector. Extracts were also analyzed on an H-P 5890 GC equipped with DB-WAX and DB-17 columns (each 20 m x 0.32 mm ID). Control collections were made under identical conditions with uninfested squash.

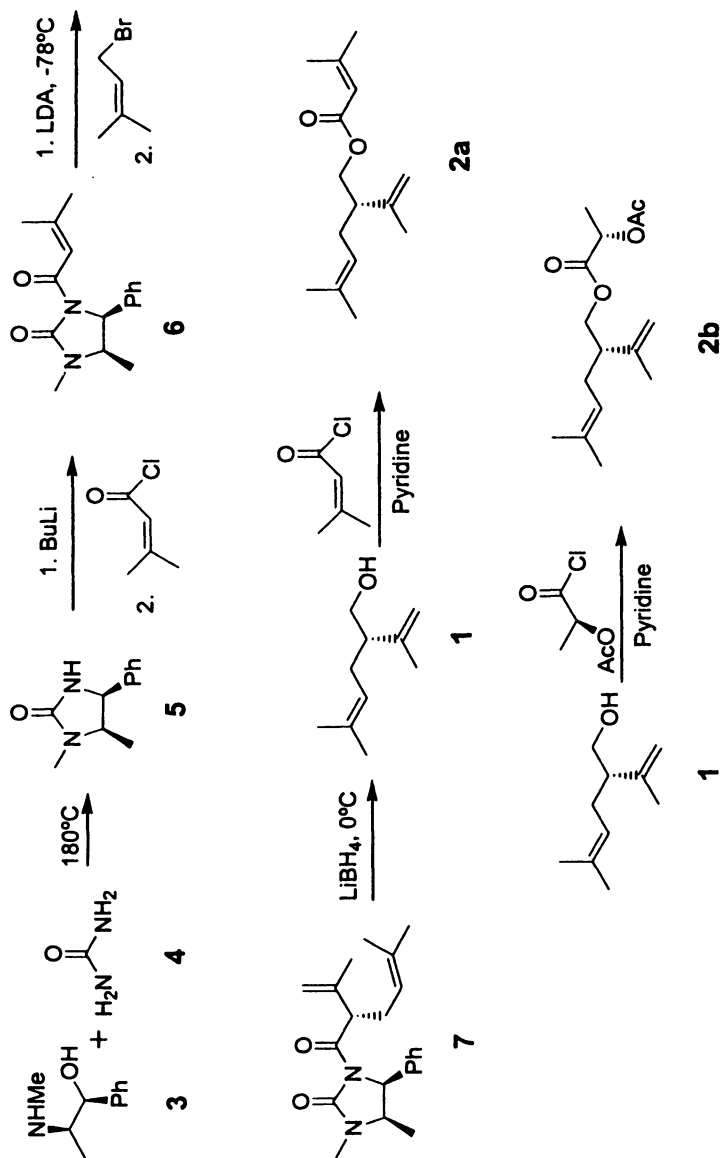
An aliquot of the extract was concentrated almost to dryness under a gentle stream of nitrogen, then hydrolyzed by stirring 4 hr with 100 μ l of 0.2 M NaOH in aqueous 95% ethanol. The mixture was then extracted with pentane and analyzed by GCMS as described above.

Pheromone chemicals.

NMR spectra were obtained on a Varian INOVA 400 instrument, at 400 MHz, in CDCl₃. Flash chromatography was carried out with 230-400 mesh silica gel.

(*S*)-(+)-Lavandulyl senecioate **2a** was synthesized in 37.2% yield in four steps (Scheme 1) by modification of a literature procedure for the synthesis of (*S*)-(+)-lavandulol **1** (13). Thus, the chiral auxiliary (4*S*,5*R*)-1,5-dimethyl-4-phenylimidazolidin-2-one **5** was prepared by heating a mixture of (1*S*,2*R*)-(+)-ephedrine hydrochloride **3** and urea **4** under argon at 180-185°C for 1.25 h (14), during which time the mixture melted with the evolution of gas and then began to thicken and precipitate solids. After cooling to room temp, the crude product was mixed with 100 ml of water while heating gently to soften the viscous mass of solids, resulting in a white slurry. After filtration, the solids were stirred with

Scheme 1. Synthesis of vine mealybug pheromone.



100 ml of 1 M HCl for 15 min, filtered, resuspended in 100 ml water, and filtered again. The resulting solids were dissolved in 80 ml of hot ethanol, and recrystallized by chilling overnight at -20°C . Filtration yielded 9 g of pure **5**. Concentration and recrystallization of the liquor from 28 ml of hot ethanol yielded a further 2.4 g of pure **5**, with 4.5 g of impure product remaining in the filtrate.

(4S,5R)-1,5-Dimethyl-4-phenyl-3-(3'-methyl-2'-butenoyl)-imidazolidin-2-one (**6**). An ice-bath cooled solution of *(4S,5R)*-1,5-dimethyl-4-phenylimidazolidin-2-one **5** (2.00 g, 10.5 mmol) in 21 ml dry THF was treated dropwise with *n*-butyllithium (10.5 mmol in hexanes). The resulting solution was stirred at 0°C for 0.5 h, then 3,3-dimethylacryloyl chloride (1.25 g, 10.5 mmol in 5 ml THF) was added dropwise, and the mixture was stirred 1 h at 0°C . The reaction was quenched with saturated aqueous NH_4Cl and extracted 3 times with CH_2Cl_2 . The organic layer was washed with brine, dried over Na_2SO_4 , concentrated, and the crude product was purified by flash chromatography (hexane/ethyl acetate, 1:1), yielding 2.62 g of **6** as white crystals (90% yield). ^1H NMR (400 MHz, CDCl_3) δ 0.79 (d, $J = 6.6$ Hz, 3H), 1.93 (d, $J = 1.2$ Hz, 3H), 2.05 (d, $J = 1.2$ Hz, 3H), 2.81 (s, 3H), 3.87 (dq, $J = 6.6$ Hz, 8.4 Hz, 1H), 5.34 (d, $J = 8.4$ Hz, 1H), 7.14-7.18 (m, 2H), 7.24-7.34 (m, 3H). ^{13}C NMR (100 MHz, CDCl_3) δ 14.99, 21.11, 27.88, 28.19, 53.88, 59.21, 117.19, 126.92, 127.88 (2C), 128.46 (3C), 136.95, 156.00, 165.01.

(4S,5R,2'S)-1,5-Dimethyl-4-phenyl-3-[2'-(1-propen-2-yl)-5'-methyl-4'-hexenoyl]imidazolidin-2-one (**7**). A THF solution of LDA (1.5 M; 6.3 ml, 9.4 mmol) was added dropwise to a solution of **6** (2.56 g, 9.4 mmol) in 13 ml THF at -78°C . After stirring 30 min at -78°C , 2-methyl-4-bromo-2-butene (1.40 g, 9.4 mmol, in 6.5 ml THF) was added dropwise and the mixture was warmed to 0°C over 12 h. The reaction was quenched with 20 ml 1 M aq. HCl, extracted 3 times with CH_2Cl_2 , the combined organic layers were washed with water, then dried, concentrated, and flash chromatographed (hexane/ethyl acetate, 3:1), yielding 2.40 g of **7** (75%) as a 6:1 ratio of diastereomers. The desired diastereomer was purified by recrystallization of 0.5 g of the crude product from hexane:ether (5.5 ml of 10:1 mixture) at -20°C , yielding 0.404 g of white crystalline **7** (diastereomeric purity $> 99\%$ ^1H NMR). ^1H NMR (400 MHz, CDCl_3) δ 0.77 (d, $J = 6.4$ Hz, 3H), 1.44 (s, 3H), 1.58 (s, 3H), 1.81 (s, 3H), 2.12-2.22 (m, 1H), 2.39-2.49 (m, 1H), 4.72 (dd, $J = 6.0$ Hz, 8.8 Hz, 1H), 4.83-4.95 (m, 3H), 5.27 (d, $J = 8.4$ Hz, 1H), 7.10-7.40 (m, 5H). ^{13}C NMR (100 MHz, CDCl_3) δ 14.95, 17.57, 21.21, 25.68, 28.19, 30.05, 50.24, 53.42, 59.63, 112.88, 121.70, 126.92, 127.79 (2C), 128.32 (3C), 133.02, 136.85, 143.92, 155.59, 172.89.

(S)-2-(1-Propen-2-yl)-5-methyl-4-hexen-1-ol (*Lavandulol* **1**). **7** (0.20 g, 0.6 mmol) in 0.6 ml THF was added dropwise to a solution of lithium borohydride (0.014 g, 0.6 mmol) 0.3 ml THF at 0°C , the mixture was stirred at 0°C for 12 h,

then quenched with 0.5 ml saturated aqueous NH_4Cl and extracted 3 times with CH_2Cl_2 . The combined organic extracts were dried, concentrated, and distilled under vacuum (bp ~ 56 – 59°C , 0.5 mm Hg), yielding (*S*)-lavandulol **1** (0.064 g, 71%) as a colorless oil. ^1H NMR (400 MHz, CDCl_3) δ 1.60 (s, 3H), 1.68 (d, $J = 0.8$ Hz, 3H), 1.69 (dd, 1.2 Hz, 3H), 1.97–2.15 (m, 2H), 2.22–2.32 (m, 1H), 3.49 (dd, $J = 10.8$ Hz, $J = 8.0$ Hz, 1H), 3.56 (dd, $J = 10.8$ Hz, $J = 5.3$ Hz, 1H), 4.79–4.82 (m, 1H), 4.90–4.94 (m, 1H), 5.04–5.10 (br t, $J = 7.2$ Hz, 1H). ^{13}C NMR (100 MHz, CDCl_3) δ 17.82, 19.47, 25.74, 28.35, 49.94, 63.60, 113.18, 121.98, 132.81, 145.39. Spectra matched those of the racemic compound obtained from commercial sources.

Derivatization of Racemic and (S)-Lavandulol.

(*S*)-(–)-2-acetoxypropionyl chloride (0.010 g, 0.06 mmol) (**15**) was added to a solution of (*S*)-lavandulol **1** (0.005 g, 0.03 mmol) and pyridine (0.008 g, 0.10 mmol) in 0.10 ml ethyl ether. The mixture was stirred 1 h at room temp, then quenched with water and extracted with ether. The ether extract was washed with brine, dried, and concentrated. The procedure was repeated with racemic lavandulol (see below). The products **2b** were analyzed by GCMS (DB-5, $50^\circ\text{C}/1$ min, $5^\circ\text{C}/\text{min}$ to 140°C , hold at 140°C). MS (70 eV) m/z 136 (26), 121 (32), 115 (6), 107 (10), 93 (89), 87 (18), 80 (15), 69 (100), 65 (2), 53 (8), 43 (59), 41 (32).

(*S*)-(+)-Lavandulyl Senecioate (**2a**). 3,3-Dimethylacryloyl chloride (0.035 g, 0.30 mmol) in 0.5 ml dry ethyl ether was added to (*S*)-lavandulol **1** (0.030 g, 0.20 mmol) in 1 ml ether at 0°C , the mixture was warmed to room temp and stirred overnight, then quenched with water and extracted 3 times with ether. After washing the ether extract with brine, drying, and concentration, the residue was Kugelrohr distilled (bp ~ 75 – 79°C , 0.08 mm Hg), yielding (*S*)-(+)-**2a** (0.038 g, 78%) as a colorless oil. $[\alpha]_{\text{D}}^{22} = +8.55^\circ$ (c 0.703, hexane). ^1H NMR (400 MHz, CDCl_3) δ 1.59 (s, 3H), 1.68 (s, 3H), 1.70 (d, $J = 0.8$ Hz, 3H), 1.88 (d, $J = 1.6$ Hz, 3H), 2.02–2.22 (m, 2H), 2.14 (d, $J = 1.2$ Hz, 3H), 2.41 (q, $J = 6.8$ Hz, 1H), 4.00–4.12 (m, 2H), 4.74 (d, $J = 0.8$ Hz, 1H), 4.80–4.84 (m, 1H), 5.03–5.09 (m, 1H), 5.64–5.68 (m, 1H). ^{13}C NMR (100 MHz, CDCl_3) δ 17.79, 19.94, 20.19, 25.73, 27.36, 28.67, 46.14, 65.10, 112.24, 116.08, 121.72, 132.82, 145.09, 156.41, 166.73. The NMR and mass spectra matched those of the racemic compound (**16**).

Racemic lavandulol was prepared by hydrolysis of lavandulyl acetate (TCI America, Portland, OR) in 1 M NaOH in 95% ethanol. Racemic lavandulyl senecioate was synthesized by esterification of lavandulol as described above.

Field Trials.

Field trials were conducted in California vineyards infested with vine mealybugs. Lures were prepared from grey rubber septa (11 mm; The West Co., Lititz, PA) loaded with hexane solutions of the pheromone components, with butylated hydroxytoluene stabilizer added at 1% of the pheromone dose. Control lures were treated with solvent and stabilizer only. Triangular Pherocon Delta IID sticky traps (Trécé Inc., Salinas, CA) with a grid pattern printed on the trap bottom to aid counting were hung from vine-cane support wires at a height of ~1.5 m, in randomized block design with a minimum spacing of 10 vines between traps within each row and 5 rows between each block, with blocks replicated 6 times. Traps were replaced weekly or biweekly as required. The following field experiments were conducted:

Blend Ratios. Blends of 0, 1, 3.3, 10, 33, or 100 μg of racemic lavandulol with 100 μg of racemic lavandulyl senecioate were tested from 2 to 23 May 2001, with traps and lures replaced on 16 May. In this and subsequent trials, data was transformed $[(x + 0.5)^{1/2}]$ and analyzed by one-way ANOVA, followed by a means separation test (Student-Newman-Keuls test, $\alpha = 0.05$).

Dose. Having determined that lavandulyl senecioate was the sole component of the attractant, doses of 0, 10, 33, 100, 333, and 1000 μg of racemic lavandulyl senecioate (6 replicates) were tested from 13 June to 7 July 2001, with traps and lures replaced on 21 June.

Field Longevity of Lures. Grey rubber septa were loaded with 100- μg doses of racemic lavandulyl senecioate. One quarter of the lures were stored at -20°C immediately, and the remainder were placed outdoors in indirect light. One-quarter of the total number were retrieved and frozen 1, 2, and 3 wk later. The attractiveness of the fresh and aged lures was compared in a heavily infested vineyard beginning 14 August 2001, with traps counted approximately weekly until 18 September, then biweekly until the experiment was terminated on 12 October 2001 due to natural decline in the mealybug population density as the overwintering period approached.

Comparison of Racemic and Chiral Pheromone. Lures, replicated 6 times, were loaded with either 25 μg of (*S*)-(+)-lavandulyl senecioate, or 50 μg of the racemic material. Traps were set out on 21 September and collected on 28 September 2001. Trap counts were compared with a two-sample *t*-test.

Comparison of Pheromone Trap Catches with Mealybug Densities. Pheromone trap catches were compared with mealybug densities determined using manual sampling protocols (11), in 3 vineyards near Del Rey, CA. At each site, 3-5 plots of 5 adjacent vines (in a single row) with moderate-to-heavy vine mealybug infestations were selected by looking for ant activity, which is strongly associated with mealybugs (12). A trap baited with a 100- μg dose of racemic pheromone was placed on the middle vine of each 5-vine set. Traps

were deployed in May and June and were counted weekly. On each vine in the 5-vine sets, mealybug densities were estimated on 12 July 2001 by counting all mealybug stages anywhere on the vine (trunk, cordon, canes, leaves, and fruit) for 5 min, with vine sections searched that showed indications of mealybug presence (e.g., ant activity, honeydew). Damage to fruit was rated on 15 September by using a 0-3 scale: 0, no mealybug damage; 1, honeydew present but the bunch salvageable; 2, honeydew and mealybugs present and part of the bunch salvageable; and 3, total loss of the bunch. On each vine, nine grape bunches were sampled. For each five-vine set, mealybug mean density and damage were compared with mean pheromone-baited trap catches by using regression analysis. For damage, data from all 5-vine plots in each vineyard were combined (as were pheromone trap catches) because damage levels among vines varied dramatically.

Results

Identification and Synthesis of the Pheromone.

Chromatograms of the extracts from squash infested with virgin female vine mealybugs showed two significant insect-produced compounds (Figure 1). The smaller and earlier eluting of the two components **1** had a weak molecular ion at m/z 154 (<1%), with significant fragments at m/z 136 (2%, M^+ -18), 123 (9%, M^+ -CH₂OH), 111 (24%, M^+ -C₃H₇), 93 (17%, M^+ -H₂O-C₃H₇), 69 (90%, C₅H₉⁺), and 41 (100%, C₃H₅⁺), indicative of a monoterpene alcohol. The compound was tentatively identified as the monoterpene alcohol lavandulol (**1**) by comparison with database spectra. The identification was verified with retention time matches on 3 GC capillary columns (DB-5, DB-17, and DB-WAX), and the mass spectrum matched that of an authentic standard prepared by hydrolysis of commercial lavandulyl acetate (TCI America).

The second, later eluting component **2a** had a weak molecular ion at m/z 236 and a base peak of m/z 83, characteristic of a 5-carbon unsaturated ester fragment. Upon base hydrolysis, the compound yielded lavandulol, confirming that it was an ester, with the acid moiety of the ester being a 5-carbon monounsaturated carboxylic acid. In addition, the difference in retention times between the model compounds geraniol and geranyl tiglate, and between lavandulol and the unknown ester were almost identical, suggesting that the ester was probably conjugated. Because most of the pheromones known from related scale and mealybug species are terpenoids, there were three likely possibilities, (lavandulyl tiglate, senecioate, or angelate), corresponding to the acid portion being a 2,3- or a 3,3-dimethylacrylate structure. Lavandulyl angelate was synthesized by base-catalyzed transesterification of methyl

angelate with lavandulol, and the other two esters were prepared by esterification of lavandulol with the corresponding acid chlorides and an amine base in ether. However, the choice of amine base for the esterification was critically important: use of 4-dimethylaminopyridine caused extensive deconjugation of the acid moiety, presumably via initial formation of a conjugated ketene from the acyl chloride, followed by nucleophilic attack on the carbonyl carbon by lavandulol and reprotonation of the resulting intermediate on the α carbon rather than the γ carbon (17). In contrast, reaction of senecieryl chloride and lavandulol in ether at 0° with pyridine as the base (18) and warming to room temp overnight gave lavandulyl senecioate **2a** as a single component. The mass spectra (Figure 2) and retention times of the synthetic and insect-produced lavandulyl senecioate matched closely, whereas those of lavandulyl angelate and tiglate were markedly different. In particular, the latter two compounds gave a base peak at m/z 93 instead of m/z 83 amu. To our knowledge, this is the first report of **2a** as a natural product, although **1** has been previously reported from both plant oils and an insect (the carrion beetle, *Necrodes surinamensis*) (19).

Racemic **1** and **2a** were not resolved into their two enantiomers on a Cyclodex B chiral stationary phase GC column (J&W Scientific), and so the absolute configurations of insect-produced **1** and **2a** were determined by derivatization with a chiral derivatizing reagent followed by GC analysis of the resulting diastereomers on an achiral column. Thus, racemic lavandulol, a sample of (*R*)-(-)-lavandulol, and a hydrolyzed sample of the insect-produced compound were derivatized with acetyl (*S*)-lactic acid chloride and pyridine in ether to form the acetyl lactate ester(s) (15). The diastereomeric derivatives from racemic lavandulol were resolved almost to baseline, with the derivative from (*R*)-lavandulol (isolated from lavender oil) being the later eluting peak. The derivative of insect-produced **1** produced only one peak, that matched the retention time of the derivative of (*S*)-(+)-lavandulol. This was further confirmed by sequential injections of mixtures of the derivatized insect-produced **1** with the derivatized (*R*)-(-)- and racemic lavandulol, conclusively demonstrating that insect-produced **1** and **2a** both have the (*S*)-configuration.

(*S*)-(+)-Lavandulyl senecioate **2a** was synthesized as shown in Scheme 1. Thus, the anion of **5** was acylated with 3,3-dimethylacryloyl chloride, giving **6** in 90% yield. The stereogenic center at C-2 of (*S*)-lavandulol was established by stereoselective alkylation of **6**, producing a 6:1 ratio of diastereomers **7**. The desired diastereomer was purified by recrystallization, yielding chemically and stereoisomerically pure **7** (75%). Reduction of **7** with LiBH₄ gave (*S*)-lavandulol **1** (71.1%), with recovery of most (63%) of the chiral auxiliary **5**. To verify the enantiomeric purity of alcohol **1**, racemic and chiral **1** were derivatized with (*S*)-2-acetoxypionyl chloride and pyridine in ether. The diastereomeric derivatives **2b** from racemic lavandulol were resolved almost to

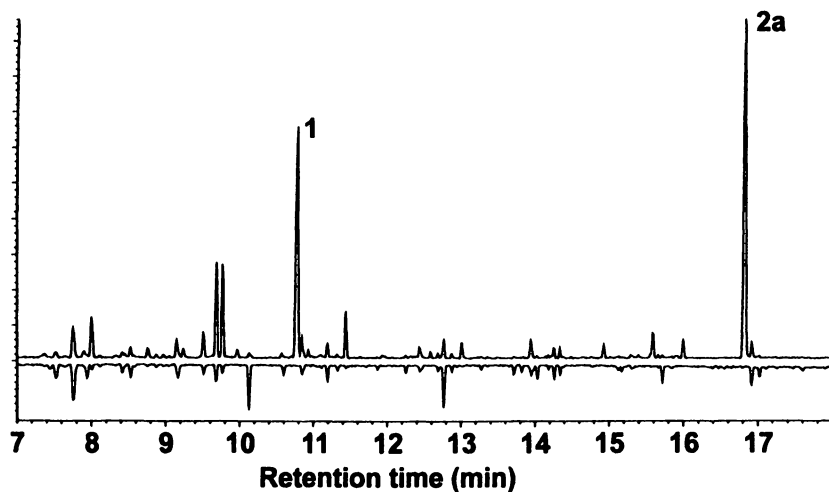


Figure 1. GC chromatograms of volatiles collected from mature female vine mealybug on butternut squash (top) and from uninfested squash (bottom, inverted). Lavandulol 1 and lavandulyl senecioate 2a are indicated.

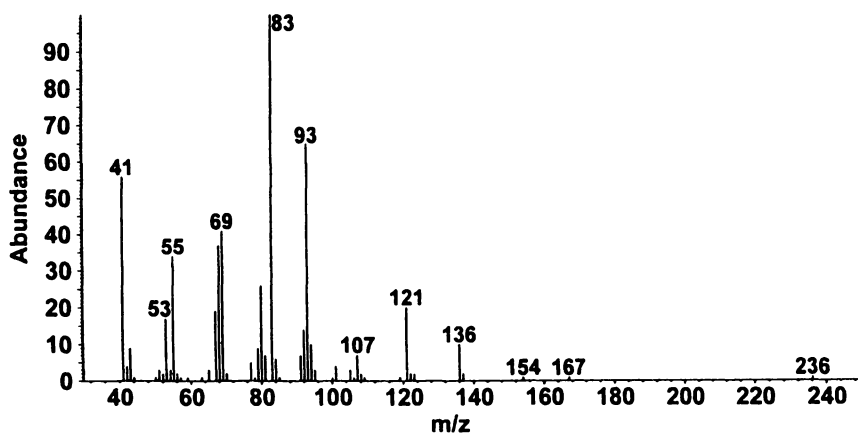


Figure 2. EI mass spectrum (70 eV) of lavandulyl senecioate 2a.

baseline on capillary GC, whereas the derivative from (*S*)-lavandulol gave a single symmetrical peak, indicating a chiral purity of >99%.

Having verified the stereoisomeric purity of alcohol 1, a portion was converted to the chiral pheromone, (*S*)-(+)-lavandulyl senecioate 2a (78% yield), by treatment with senecioid chloride and pyridine in ether (16).

Field Trials.

In the first field trial, increasing proportions of lavandulol in the lure blends significantly decreased trap catches (Figure 3). Thus, lavandulol appears to deter or inhibit attraction of males, even though it is present in extracts from female mealybugs. Blank control traps caught only six males and were not included in the statistical analysis for this experiment.

The second field test evaluated the attractiveness of doses of the racemic pheromone ranging from 10 to 1,000 μg . There were no differences in the attractiveness of any of the lures (one-way ANOVA: $F = 0.64$; $df = 4, 25$; $P = 0.64$), with mean trap catches over all treatments varying from 155 to 200 insects per trap.

Because lavandulyl senecioate is chiral, and vine mealybug females produce exclusively the (*S*)-enantiomer, the relative attractiveness of pure (*S*)- and racemic lavandulyl senecioates was tested. Traps baited with either the chiral or racemic pheromone were equally attractive to male mealybugs (racemic, 373 ± 175 [mean \pm SD]; (*S*)-enantiomer, 286 ± 122 ; two-sample *t*-test, $P = 0.34$), clearly demonstrating that the “unnatural” enantiomer is neither inhibitory nor synergistic.

Lures that had been loaded with 100- μg doses of the racemic pheromone and then field-aged for periods of 0-3 wk before deployment were equally attractive to male mealybugs (ANOVA, followed by Tukey's HSD tests; Figure 4). Furthermore, the lack of differences persisted for 8 wk, from mid-August to mid-October when the trial was halted due to autumn decline in mealybug populations. Over the course of this trial, >250,000 male mealybugs were caught in the pheromone-baited traps. Thus, rubber septa loaded with 100- μg doses of racemic pheromone may be effective lures for 12 wk or more.

Finally, we compared estimates of mealybug density and economic damage determined with pheromone-baited traps versus the more labor-intensive visual sampling method. The results showed a significant and positive correlation between pheromone-baited trap catches and visual sampling methods for mealybug density ($y = 1.65 + 1.10x$; $r^2 = 0.48$; $df = 1,9$; $P = 0.019$) and ratings of economic damage ($y = -35.17 + 73.74x$; $r^2 = 0.73$; $df = 1,3$; $P = 0.093$) (20).

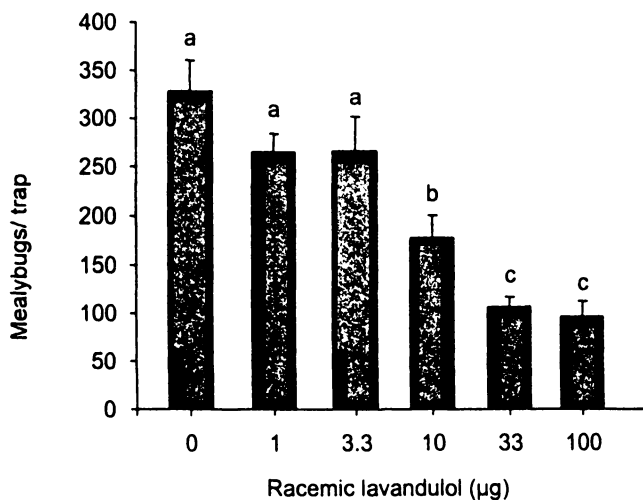


Figure 3. Numbers of male vine mealybugs caught in traps baited with blends of racemic lavandulol and lavandulyl senecioate ranging from 0:100 to 100:100 µg. Note: Y-axis is a log scale. Bars surmounted by different letters are significantly different.

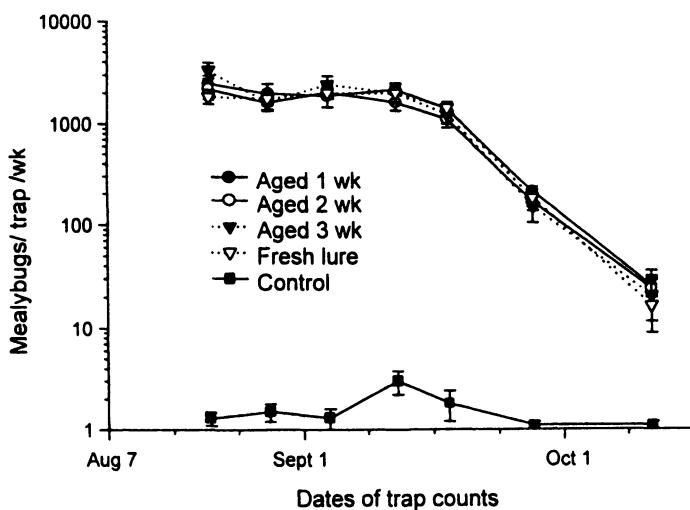


Figure 4. Male vine mealybugs caught in traps baited with racemic lavandulyl senecioate, with lures aged for periods of 0 to 3 wk before use. The y-axis is a log scale. Controls were lures treated with solvent only. For a given sampling date, there were no differences between treatments, but all treatments were always different from the solvent controls.

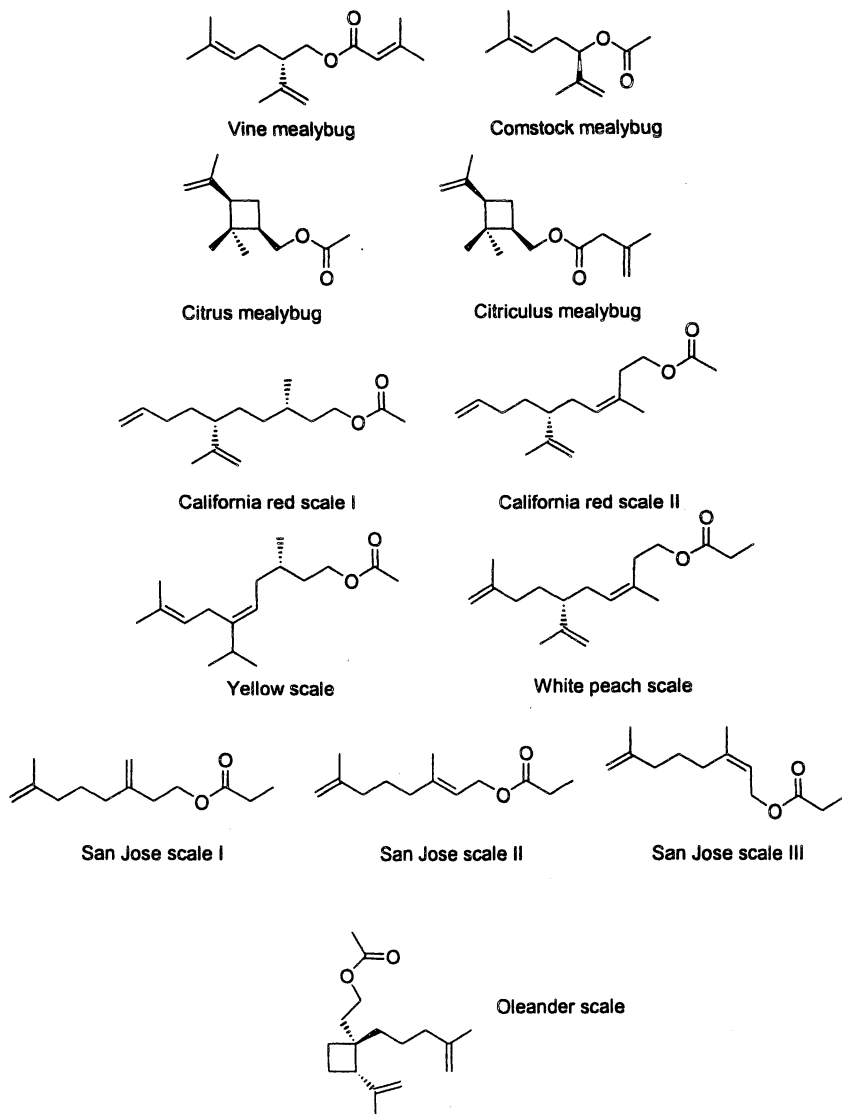
Discussion

In field trials, racemic lavandulol mixed with lavandulyl senecioate significantly inhibited attraction of male vine mealybugs, despite the fact that lavandulol is produced by females. Thus, the role of lavandulol remains unclear. Although it clearly is not required as a component of the sex attractant pheromone, as evidenced by the large trap catches with lavandulyl senecioate as a single component, it is possible that male mealybugs may be sensitive to the enantiomeric composition of lavandulol, despite not being sensitive to the wrong enantiomer of lavandulol senecioate. Alternatively, lavandulol may mediate other aspects of the mating behavior. For example, at appropriate doses, it might have a role in short-range interactions between males and females, as was found with a compound produced by females of the white peach scale, *Pseudaulacapsis pentagona* (Targioni-Tozzetti) (Homoptera: Diaspididae) (21). The sex attractant pheromone of white peach scale consists of (3Z,6R)-3,9-dimethyl-6-isopropenyl-3,9-decadienyl propanoate, and the corresponding alcohol inhibited male attraction in field trials, but enhanced the copulatory responses of males in laboratory bioassays (21,22).

To date, pheromones have been reported from only four mealybug species, the vine mealybug *Planococcus ficus* (16), the Comstock mealybug *Pseudococcus comstocki* (23), the citrus mealybug *Planococcus citri* (24), and the citriculus mealybug, *Pseudococcus cryptus* (25) (Scheme 2). The pheromone of the obscure mealybug *Pseudococcus viburni* has recently been identified and synthesized (J.G.M., manuscript in prep.), and work is in progress on the identification and synthesis of the pheromones of several other species, including the longtailed mealybug *P. longispinus*, the grape mealybug *P. maritimus* (J.G.M., unpublished data), and the pink hibiscus mealybug *Maconellicoccus hirsutus* (A. Zhang, pers. comm.). The pheromones of five related diaspidid scale species have also been identified (Scheme 2). The structures of all the identified mealybug and diaspidid scale species are esters of mono- or sesquiterpene alcohols. Furthermore, most of the structures are characterized by an unusual linkage between the isoprene units, with the “tail” of one isoprene unit being connected to the second carbon of the next unit, rather than to the “head”. This motif is found in the pheromones of all of the species except San Jose scale.

It is also interesting to note that for all nine of these mealybug and diaspidid scale species, the male insects are readily attracted to mixtures of the pheromone component(s) with other stereoisomers. This is in distinct contrast to the pheromone-mediated behavior of insects in other orders, such as moths and beetles, in which the males are frequently acutely sensitive to trace amounts of stereoisomers of the pheromone components, including both geometric isomers and enantiomers. The reason for this is that insects such as moths and beetles usually make use of a limited number of pheromone components, with species-

Scheme 2. Structures of known mealybug and diaspidid scale insect pheromone components.



specific signals being generated by combining subsets of shared pheromone components in unique blends. In moths and beetles, the specificity of the signal is further enforced by males of one species being strongly inhibited by minor components in the blend of closely related, sympatric species.

The fact that closely related scales and mealybugs, some of which may occur on the same host plants, appear to be indifferent to the presence of “unnatural” stereoisomers in synthetic pheromones has important implications. First, it suggests that these insects do not share pheromone components across species, and that each species creates a species-specific pheromonal signal by producing one or more unique structures, eliminating any possibility of cross-attraction between species due to one or more shared components. This conjecture is supported by the fact that all of the pheromone components from the nine species listed above, several of which are closely related congeners, are produced by only a single species. Furthermore, to date no species has been shown to use a pheromone component that is a stereoisomer of a component produced by another species. Thus, there would seem to be no reason for males of the various species to detect and become sensitive to the pheromone components of even closely related species.

From a practical viewpoint, the demonstrated insensitivity of male mealybugs and diaspidid scales to stereoisomeric mixtures is enormously useful, because it means that “technical grade” pheromone consisting of a mixture of isomers is as effective as a trap bait as the pure compound. Thus, it is not necessary to develop stereospecific syntheses for production of the pheromones on a commercial scale. Considering the complexity of some of the structures, this is a tremendous economic advantage which has rendered it both possible and economically feasible to use the pheromones of these insects for monitoring their populations. In the best cases, such as that of the vine mealybug, for which racemic pheromone can be produced on a multikilogram scale for a couple of dollars a gram or less, it may also provide opportunities for safe and economical control of these types of insects using pheromone-based strategies, such as mating disruption, mass trapping, or attract-and-kill. Several peculiarities of the biology of these types of insects may make them particularly susceptible to pheromone-based control strategies. First, the male insects are fragile and live for only a few hours to a few days (22). Furthermore, males are nonfeeding, with no functional mouthparts, and so their energy reserves are limited and cannot be replaced. In the presence of synthetic pheromone, males activate and begin flying, and rapidly consume this energy reserve and die. Thus, the overall effect of the pheromone is to remove males from the system relatively rapidly (by terminal exhaustion), analogous to what might be accomplished with an insecticide, but with none of the deleterious side-effects of an insecticide.

Second, male mealybugs and scales are acutely sensitive to their pheromones. For example, in the vine mealybug results presented here, pheromone lures loaded with 100 μg of racemic pheromone (i.e., 50 μg of the

active ingredient) still attracted males after 12 wk in the field. Analogous results have been obtained with male yellow scale, for which lures consisting of rubber septa impregnated with 5 μ g of racemic pheromone are effective for monitoring populations (26). This suggests that it should be possible to use small quantities of pheromone (a few grams per acre season-long) to achieve good control of pest populations, helping a pheromone-based control strategy be economically competitive or even superior to control of these insects with insecticides.

Third, female mealybugs and scales are sessile and wingless.

Consequently, in areas treated with pheromone for mating disruption, immigration of mated females from outside the treated area is not an issue. This is in distinct contrast to mating disruption of highly mobile lepidopteran pests, in which immigration of mated females (as evidenced by relatively high levels of damage along borders of treated plots) can be a significant problem.

Fourth, field trials with pheromone-baited traps for vine mealybug have shown that the traps catch negligible numbers of the parasitic wasps that can be helpful in controlling the mealybugs (20). On a broader scale, use of pheromone-based strategies for control of mealybugs also would eliminate problems caused by insecticide-related outbreaks of secondary pests, as well as eliminating reentry restrictions for vineyard workers, environmental contamination, and other problems inherent with pesticide use. Large-scale field trials are in progress to assess the feasibility of using pheromones for both sampling and control of vine mealybugs over large areas.

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Chapter 3

Practical Syntheses of Selected Insect Pheromones

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Practical examples of several insect pheromone syntheses are presented. Examples of key reactions in synthetic pathways include: kinetic versus thermodynamic control of cycloheptanone dimethylation (flea beetles), *in situ* oxidation/Wittig olefination (saltcedar leaf beetle), preparation of a 1,4-diol using a four-carbon homologating agent (currant stem girdler), preparation of trisubstituted alkenoic acids (cowpea weevil), and regiospecific epoxidation followed by cleavage of the epoxide to form a key aldehyde intermediate (cereal leaf beetle).

Sesquiterpene Pheromones from Flea Beetles—A Practical Synthesis Based on Standard Methods

A series of sesquiterpene pheromone components based on the himachalene ring system were recently identified from *Phyllotreta* and *Aphthona* flea beetles (1). The compounds are produced only by male beetles but both sexes are attracted to them. Biologically, the compounds together serve as an “aggregation” pheromone. A synthesis of the racemic compounds was devised (Figure 1) using standard methods (2). For the discussion here, the synthesis is considered in four general sections, each with its own special issues. These sections are: 1) conversion of the starting material (cycloheptanone 7) into dimethylcycloheptenone 10; 2) addition of the methyl and 3-ketobutyl side chains to 10; 3) completion of the Robinson annulation to form bicyclic ketone 1 (the first of the pheromone structures); and 4) elaboration of pheromone structures 2-6 from 1.

For the first section, reaction of methyl iodide with the enolate of cycloheptanone under thermodynamic conditions (*t*-BuOK in *t*-BuOH, 3) eventually gave predominantly 2,2-addition, as planned, but the reaction did not easily go to completion. Three “repetitions” of the procedure were required before the starting ketone and monomethyl intermediate were satisfactorily consumed (2). To prepare for a subsequent repetition, the current reaction product was simply filtered to remove KI, concentrated by rotary evaporation, and then added to fresh reagent. Bromination of the dimethyl ketone to 8, with liquid bromine in ether (4), and subsequent dehydrobromination to 9, with LiBr and Li₂CO₃ in DMF (5), went smoothly (2).

The second section included the addition of the final methyl group at carbon 6 of ketone 10 and the 3-ketobutyl group at carbon 7. Two acceptable schemes were found for accomplishing these alkylations. In the first scheme (unpublished), the initial step was to react bromoketal 11 (6) with the enolate of 10 formed with NaH in DMSO (7). The next step was to methylate the cyclic enone by conjugate addition using a cuprate reagent (8). Finally, the ketal protective group was removed by heating with dilute HCl in MeOH. A disadvantage of this scheme was that the product from each step had to be isolated before proceeding to the next step. In the second scheme, however, the conversion from ketone 10 to a diketone suitable for cyclization (16) could be done in one pot. Here, methylation by conjugate addition was the first step, and the resulting copper enolate was used directly to drive the second addition (9), rather than being quenched with water. The bromoketal 11 was not reactive enough for this reaction, but the Michael acceptor, 2-(trimethylsilyl)-1-buten-3-one, 15 (10) reacted smoothly. Furthermore, this reagent did not lead to the multiple Michael additions and complex product mixture that likely would have resulted from using methyl vinyl ketone instead of 15 (11). Regents 11 and 15 required similar amounts of effort to prepare; thus the second scheme was deemed preferable.

The third section of the synthesis was cyclization of the intermediate diketone (14 or 16) to pheromone 1. This reaction required fairly harsh conditions to proceed, such as 3.5 N KOH in refluxing EtOH (2). Silyl diketone 16 converted rapidly to 14 upon treatment with base; thus both schemes for the second stage of the synthesis ultimately produced 1 from exactly the same intermediate. The key issue for the third stage was the relative stereochemistry at the two asymmetric centers. Of these, the ring-junction was enolizable, both before and after cyclization. Fortunately, the equilibrium mixture under the cyclizing conditions contained about 97% of the desired diastereomer, regardless of the stereochemical composition before the reaction.

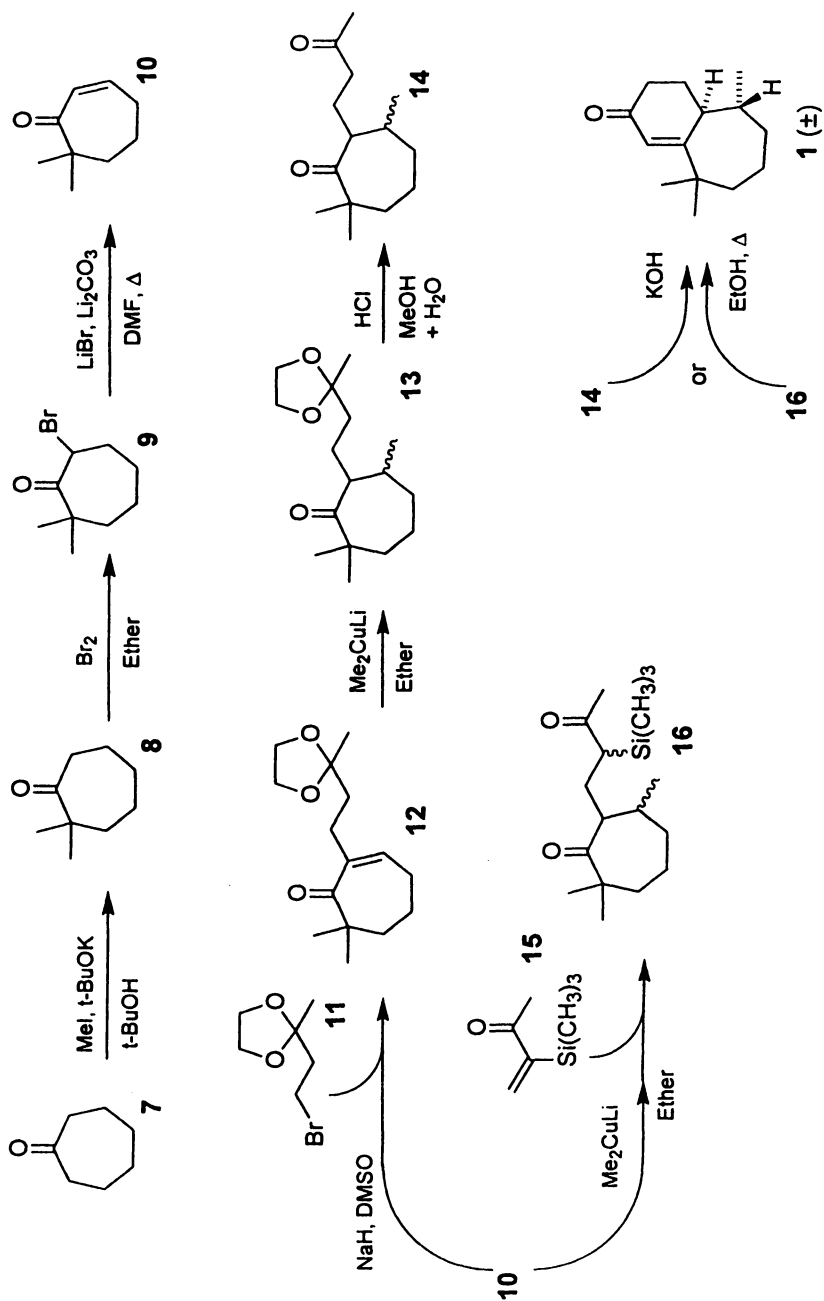
Finally, bicyclic ketone 1 could be readily converted to pheromone components 2-6. Alcohols 2 and 3 were both prepared when 1 was treated with methyllithium in ether (12). Then either 2 or 3, or a mixture of both, could be readily dehydrated to a mixture (about 50:50) of hydrocarbons 4 and 5, simply by stirring with a strong cation exchange resin in ether (2). When a mixture of 4 and 5 was allowed to equilibrate in warm formic acid and methanol, the ratio shifted to greater than 80% 4 (13). If desired, these isomers could be separated by column chromatography on silica gel containing silver nitrate (2). Alternatively, ketone 1 could be converted directly to 5 with a Wittig reaction, using methyl(triphenyl)phosphonium bromide and butyllithium in THF (14). The aromatic himachalene 6 could be prepared by heating 4 or 5, or a mixture of these, with chloranil (2,3,5,6-tetrachloroquinone) in benzene (15).

This overall synthetic approach has provided the racemic pheromone components for biological testing. The compounds have been formulated so that the proportions emitted from dispensers accurately mirrored those from live male beetles, and these blends have been demonstrated to attract both male and female beetles under field conditions (in preparation).

Saltcedar leaf beetle pheromone blend synthesis

Diorhabda elongata Brulle (Coleoptera: Chrysomelidae) is being used for the biological control of saltcedar (*Tamarix* spp), which is an invasive weedy tree found along rivers in the western United States (16). The compounds, (2*E*,4*Z*)-2,4-heptadien-1-ol (17) and (2*E*,4*Z*)-2,4-heptadienal (18), are a major part of the pheromone of *D. elongata* and have potential use in monitoring dispersal of this species from sites of release (17).

A concise synthetic pathway (Figure 2), leading to compounds 17 and 18, was developed (17). Commercially available (*Z*)-2-penten-1-ol (19) was partially oxidized to intermediate (*Z*)-2-penten-1-al, which was immediately trapped by a stabilized ylide, (carboethoxymethylene)triphenylphosphorane,



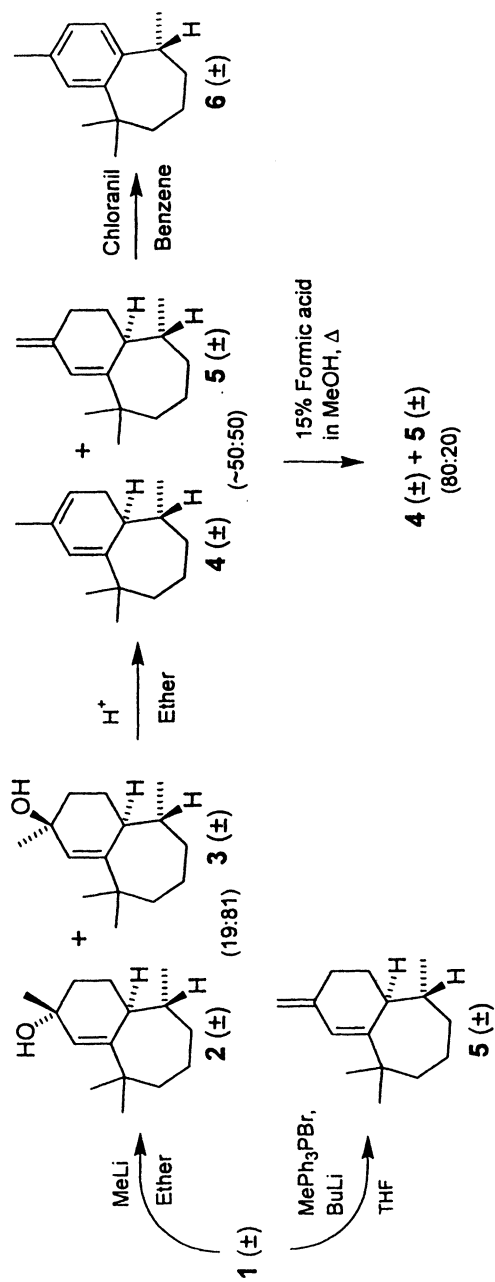


Figure 1. Synthesis of sesquiterpene pheromones of flea beetles.

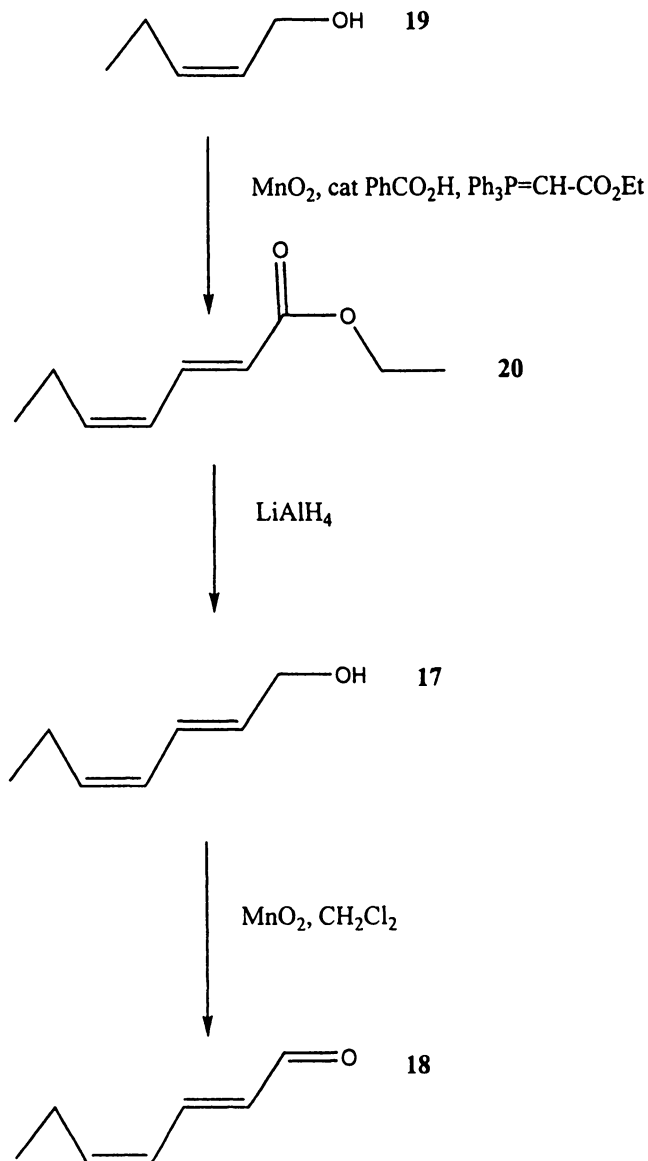


Figure 2. Synthesis of the saltcedar leaf beetle pheromone blend, composed of various heptenoic acids (1-6).

before the *Z*-conjugated aldehyde intermediate could isomerize to the more thermodynamically stable *E*-isomer (18). The resulting ethyl-(2*E*,4*Z*)-2,4-heptadienoate (20) was reduced with LiAlH₄ to give the (2*E*,4*Z*)-dienic alcohol 17. Mild partial oxidation to aldehyde 18 was accomplished with MnO₂ (17). Compound 18 should be prepared shortly before use because the 4*Z*-double bond slowly isomerizes to 4*E*, even when stored in a freezer at -20°; the amount of 4*E*-isomer increased from 6% to 10% when stored for four months.

Currant stem girdler pheromone synthesis

The currant stem girdler, *Janus integer* Norton (Hymenoptera: Cephidae), is a sawfly pest of currants and gooseberries in North America. Damage from *J. integer* is caused by tunneling of larvae in canes of red currants and also by the girdling habit of the adult female which results in the death of young shoots (19). Availability of a synthetic sex pheromone for *J. integer* would substantially improve chemical management of this cryptic sawfly pest by providing an 'early warning' to growers, allowing targeted application of an insecticide prior to crop damage (20). Cossé et al. (21) reported the occurrence of a female-specific compound from *J. integer*, which they identified as a lactone, (*Z*)-9-octadecen-4-olide (21). The absolute configuration of natural enantiomer was subsequently determined to be *R* (20).

The racemic lactone was prepared in five steps from 1,4-butanediol 22 (Figure 3). The symmetrical diol 22 was mono-protected as a tetrahydropyranyl (THP) ether 23, using a biphasic mixture containing 1,4-butanediol, 3,4-dihydro-2*H*-pyran (DHP), CH₂Cl₂, and a catalytic amount of 0.1 N aqueous HCl (22). Compound 23 migrates from the aqueous phase as it is formed, thus reducing the chance of further derivatization to the diether (22).

The free hydroxyl function of 23 was oxidized with a dilute solution of pyridinium dichromate (PDC) in CH₂Cl₂ containing anhydrous MgSO₄ as a water scavenger, to give the aldehyde 24 (23). The four-carbon homologating agent 24 allows direct preparation of 1,4-diols after deprotection. Grignard reaction of 24 with *Z*-5-tetradecenyl magnesium bromide (20), followed by deprotection afforded the 1,4-diol 25. We subsequently discovered that the yield of the Grignard reaction can be improved by as much as 20% if the reaction is quenched at 0° instead of allowing it to warm to rt.

The THP protective group was removed with NH₄Cl in refluxing methanol (24). The 1,4-diol 25 was oxidized directly to the lactone 21 with tetrapropylammonium perruthinate (TPAP) and 4-methylmorpholine-*N*-oxide as co-oxidant in CH₂Cl₂ (25).

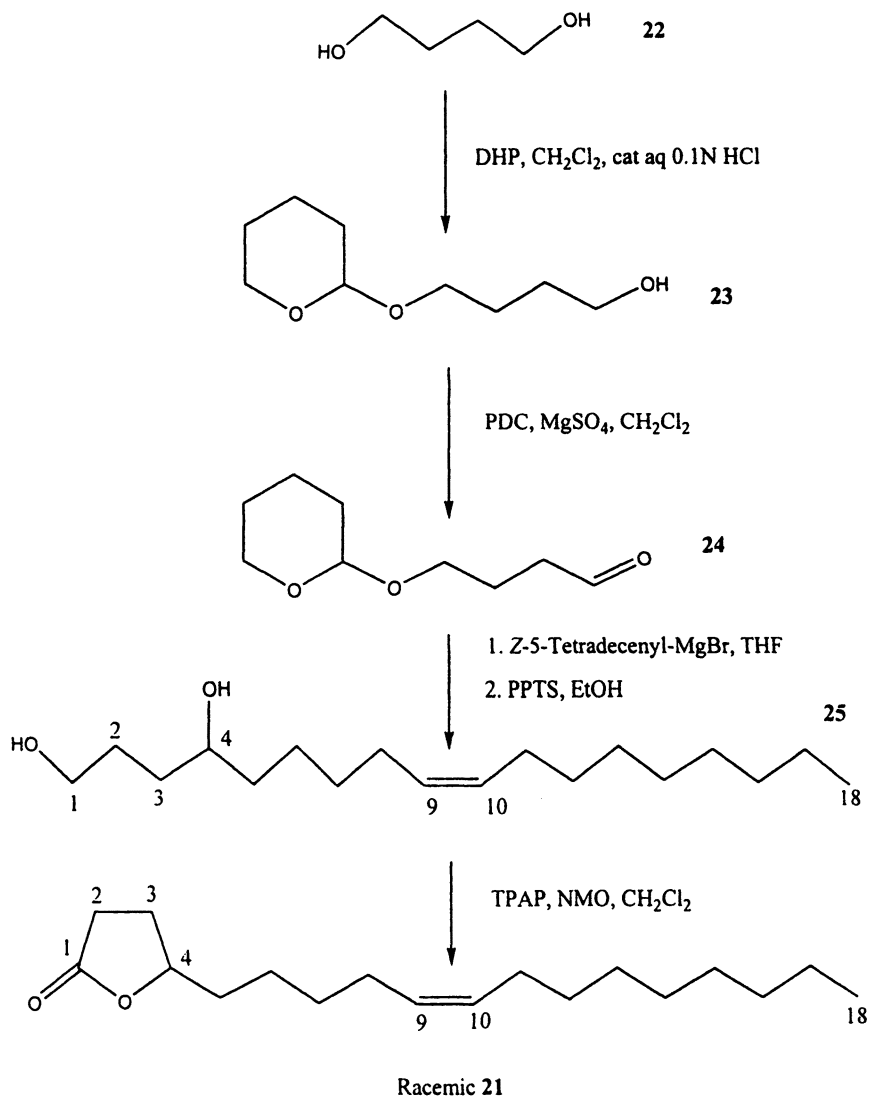


Figure 3. Synthesis of racemic (Z)-9-octadecen-4-olide (21), the sex pheromone of the currant stem girdler.

The enantioselective synthesis of lactone, (*R*)-**21**, was accomplished in five steps from (*S*)-(+)-glutamic acid (**26**). As shown in Figure 4, **26** was oxidized to the lactone carboxylic acid **27** with NaNO_2 and HCl (**26**). Reduction of **27** to alcohol **28** was done with borane-methyl sulfide complex (**27**). Tosylation of **28** with TsCl in the presence of $\text{Me}_2\text{N}(\text{CH}_2)_3\text{NMe}_2$ at $0-5^\circ$ (**28**) gave ester **29**. Tosylate **29** was converted to chiral epoxide **30** with NaOMe in anhydrous MeOH at room temperature (**29**). The final reaction was a Grignard cross-coupling between chiral epoxide **30** and *Z*-4-tridecenyl magnesium bromide (**20**). Cyclization to the lactone occurred during workup. The desired lactone (*R*-**21**) was obtained, but the yield was low (*ca.*1%); purity was >99% by GC after HPLC. Sufficient material was prepared to allow determination of the chirality of the natural pheromone by chiral GC and GC/EAG comparison of the synthetic *R*-enantiomer versus the natural pheromone.

Cowpea weevil pheromone blend synthesis

The cowpea weevil, *Callosobruchus maculatus* (F.) is a serious pest of stored dried seeds of legumes. Female cowpea weevils produce a sex pheromone blend that elicits orientation and sexual behavior in males (**30**). The blend is composed of the following 8-carbon acids: (*Z* and *E*)-3-methyl-2-heptenoic acid (**31**), (*Z* and *E*)-3-methyl-3-heptenoic acid (**32**), and 3-methyleneheptanoic acid (**33**). The primary difference between compounds **31**, **32** and **33** is the position of the double bond. Preparation of these compounds for planned cowpea weevil behavioral studies required three separate synthetic routes (Figure 5). Both *Z*- and *E*-isomers are needed for ongoing studies. Eventually, the isomers of **31** and **32** will be separated by preparative gas chromatography on a sub-milligram scale. Syntheses of **31** and **32** were devised to acquire nearly a 50/50 mixture of *Z*- and *E*-isomers.

Compound **31** was prepared in two steps from 2-hexanone (**34**). Reaction of **34** with the enolate of triethylphosphonoacetate (TEPA), in hexane (**31**), gave an almost quantitative yield of (*Z* and *E*)-Ethyl-3-methyl-2-heptenoate (**35**). The isomeric esters were hydrolyzed to **31** with methanolic KOH (**32**).

Compound **32** was prepared in four steps from 4-hydroxy-2-butanone (**36**). Protection of the alcohol function of **36**, as a tetrahydropyranyl (THP) ether **37**, was accomplished with dihydropyran (DHP) and NH_4Cl catalyst in refluxing THF (**24**). Wittig reaction of **37** with the ylide from butyl(triphenyl)phosphonium bromide (*n*-BuLi, THF) resulted in alkylidenation to form **38**, but the yield was less than 5%, even with dropwise addition of the ylide to the aldehyde at -78° . Removal of the THP group, followed by oxidation of the alcohol directly to the acid under neutral conditions (PDC in DMF, **33**) afforded **32**. Mild oxidation conditions are required to prevent migration of the double bond from the 3-position to the 2-position.

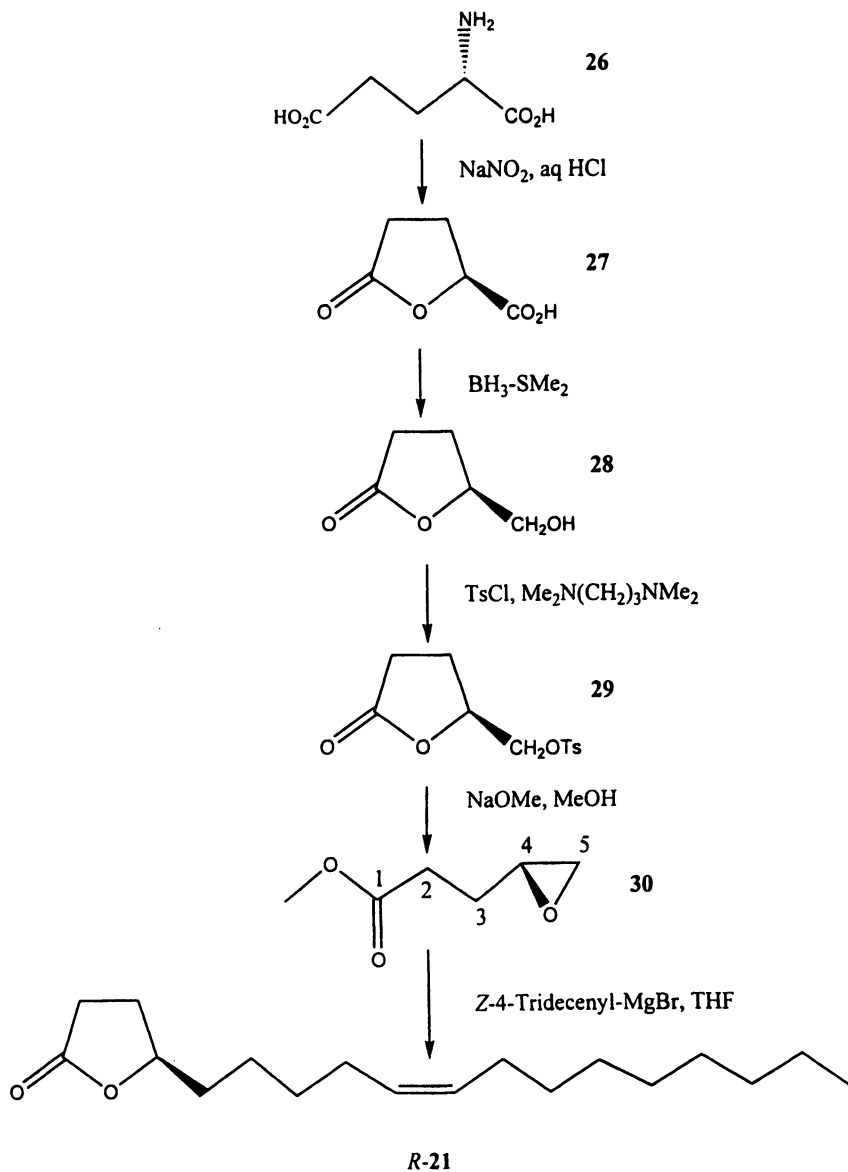


Figure 4. Enantioselective synthesis of *(R, Z)*-9-octadecen-4-olide (**R-21**).

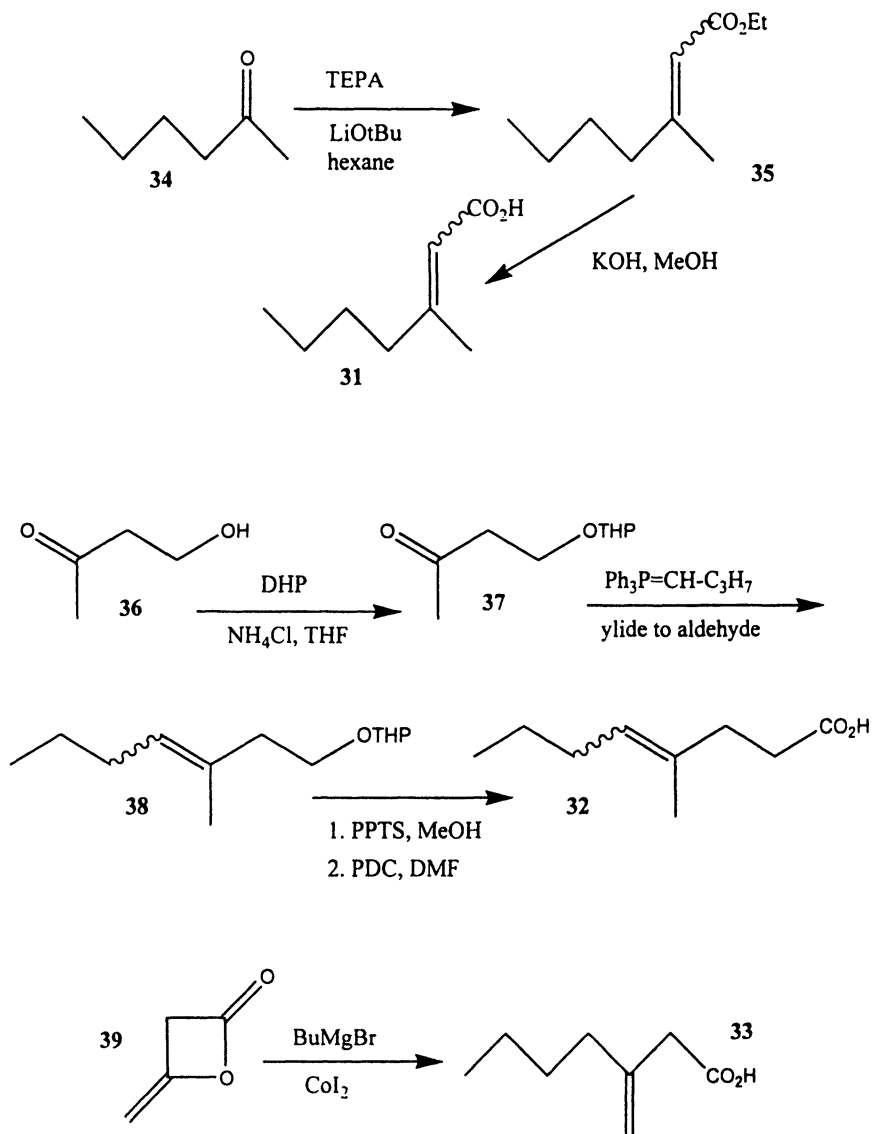


Figure 5. Synthesis of the cowpea weevil pheromone blend (31, 32, 33).

The final compound, **33**, was prepared by a Grignard cross coupling reaction between diketene (**39**) and *n*-butylmagnesium bromide in the presence of anhydrous cobalt iodide (**30**, **34**). It is noteworthy that the Grignard reagent attacks the methylene carbon instead of the carbonyl carbon in the presence of cobalt iodide.

Optimized synthesis of the male-produced aggregation pheromone of the cereal leaf beetle, *Oulema melanopus* (Coleoptera: Chrysomelidae).

In earlier studies, the male-derived aggregation pheromone (*E*)-8-hydroxy-6-methyl-6-octen-3-one (**40**), was identified from cereal leaf beetles (**35**), followed by the successful demonstration of the behavioral activity of **40** in the field (**36**). Compound **40** was made in a six step synthesis (**35**) using geraniol (**41**) as a convenient starting material (Figure 6). The hydroxyl group of geraniol was protected as THP ether **42** (**37**), followed by regioselective epoxidation to give compound **43** (**38**). The epoxide was cleaved to the aldehyde **44** (**39**), followed by a Grignard addition (**40**) to afford compound **45**, oxidation (**33**) to compound **46**, and removal of the THP group (**37**) to give the final product **40**. This initial synthesis was sufficient for the pheromone identification and the initial field test, but the relatively low overall yield (7%) and purification steps of the intermediates made the synthesis of this pheromone not practical for monitoring beetle populations on a larger scale.

The two main issues with the developed synthesis were the cleavage of the epoxide **43** and the lability of the aldehyde (**44**). The cleavage step needed relatively large amounts of solvent due to the very low solubility of the periodic acid in ether, while, the breakdown of the remaining 5 to 10% of **44** after the Grignard addition became a major source of impurities in later reaction steps, forcing several, yield lowering, open column chromatography steps for cleanup of intermediates and the final product.

The epoxide **43** was cleaved with periodic acid. Periodic acid is soluble in water, but water in any of the reaction steps was thought to cause the removal of the THP protecting group due to possibility of an acidic environment. However, **43** could be cleaved with the addition of a small volume of periodic acid dissolved in water without any evidence of loss of the THP protecting group. In the improved preparation of **44**, a solution H₅IO₆ (12.9 g, 56.7 mmol) in water (8 mL) was added dropwise to a solution of **43** (14.4 g, 56.7 mmol) in ether (250 mL) and the mixture was stirred at room temperature. The reaction was monitored by GC and was 100% complete after 3 hours. At this point the

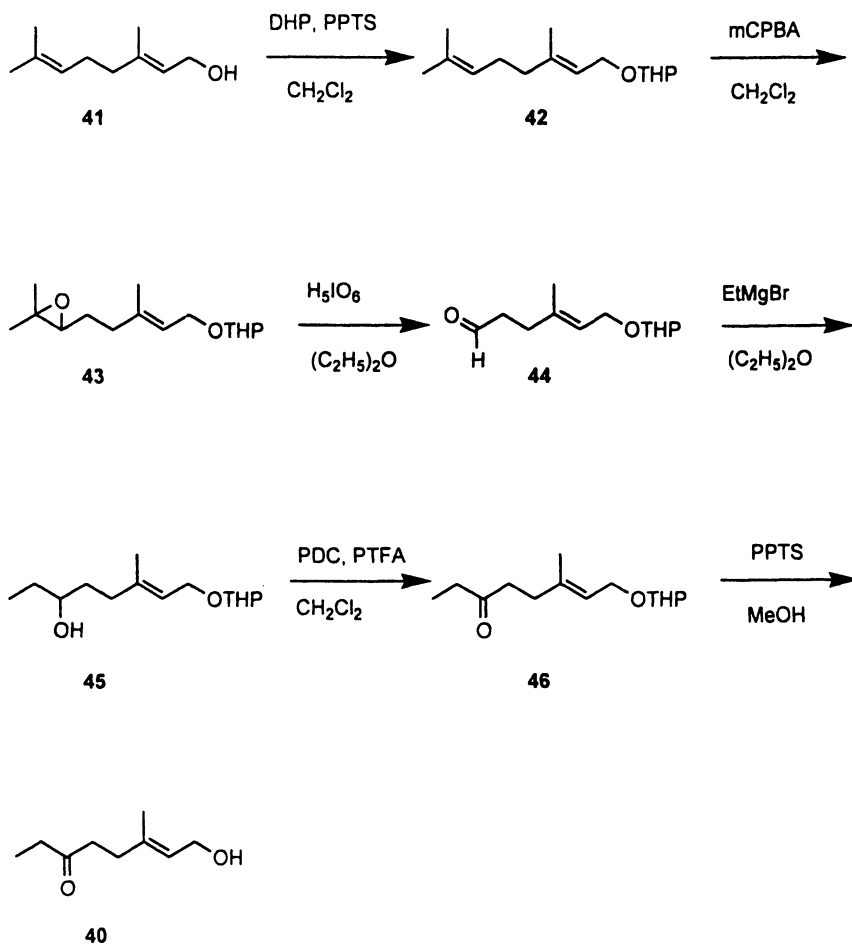


Figure 6. Synthesis of (*E*)-8-hydroxy-6-methyl-6-octen-3-one (40), the male-produced aggregation pheromone of the cereal leaf

reaction mixture had turned from a milky white color to an all clear solution. After workup (35), the yield of 44 was 73%.

The preparation of 45 from 44 was also improved, to avoid subsequent purification problems. The remaining aldehyde 44, after the Grignard addition, could be completely removed by adding a solution of crude 45 (6 g after workup but prior to distillation, 35) in hexane (20 mL) to 20 mL saturated aqueous NaHSO₃ solution. After stirring the mixture for 2 hours at room temperature, the layers were separated and the organic layer was washed successively with water and ether, and dried over anhydrous Na₂SO₄. The solvent was removed by rotary evaporation and 45 (4.9 g) was obtained as a colorless oil (purity 75% by GC). Kugelrohr distillation of 45 was not required prior to oxidation to 46. The formation of troublesome aldehyde breakdown products was eliminated by using the new procedure.

Substituting the above mentioned procedures in the six step synthesis (35) afforded 40 in a 21% overall yield with only a single open column chromatography step at the end.

In summary, practical examples of several insect pheromone syntheses are presented. Most of the chemistry is based on standard reactions.

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Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

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Chapter 4

Molecular Basis of Pheromone Reception and Signal Inactivation in Moths and Beetles

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Insects perceive semiochemicals with remarkable selectivity and sensitivity. The process starts with detection of odorants by specialized sensilla such as sensilla trichodea in moths and sensilla placodea in scarab beetles. These sensilla house odorant receptor neurons (ORNs) whose odorant receptors (ORs) are compartmentalized by an aqueous solution (sensillar lymph). Odorant- and pheromone-binding proteins (OBPs and PBPs) serve as liaison between the external environment and the ORs. Recent studies are unveiling the functions of PBPs, their structural features, in particular, binding specificity determinants, and molecular details of the protection and release of pheromones. By contrast, the molecular basis of pheromone signal inactivation is *terra incognita*; two hypotheses are yet to be tested: enzymatic degradation and “molecular trapping.” Esterases and other insect antennae-specific pheromone-degrading enzymes have been reported, but to date they have never been fully characterized. Antennae-specific esterase(s) from the Japanese beetle have a significant preference for the pheromone, (*R*)-japonilure, with slower degradation of the behavioral antagonist, (*S*)-japonilure. These findings led to the hypothesis that kinetics of pheromone inactivation plays a significant part in the chiral discrimination by the Japanese beetle.

Chemical communication is essential for insects in general. Female moths, for example, advertise their readiness to mate and reproduce by releasing sex pheromones, which are utilized by male moths in odorant-oriented navigation towards females. Although chemical communication is overt, females tend to be “shy” and produce only minute amounts of these pheromones, probably avoiding “chemical conspicuousness” and, consequently, predation. Once released from the pheromone glands, the semiochemicals are diluted in the environment and mixed with a myriad of physiologically irrelevant compounds. Males are able to find “a needle in a haystack,” i.e., they can detect small amounts of pheromones buried in complex mixtures because of the remarkable sensitivity and selectivity of their olfactory system. The ultimate refinement in selectivity is the ability to detect enantiomers of a pheromone and process them with diametrically opposed functions, such as attraction and “inhibition.” For example, males of the Japanese beetle are strongly attracted to the female-produced (*R*)-japonilure [(*R,Z*)-5-(dec-1-enyl)oxacyclopentan-2-one] (1), whereas its antipode, produced by females of another species sharing the same habitat in Japan (2), is a behavioral antagonist. Largely, insects detect the natural pheromones with extremely high sensitivity, but the thresholds for even slightly modified pheromones are some orders of magnitude higher, if they are at all active. The sensitivity of the insect olfactory system is highlighted by the ability of the male silkworm moth, *Bombyx mori*, to distinguish within one second 170 nerve impulses generated by the female-produced sex pheromone (bombykol) from 1,700 spontaneous nervous impulses (3). This is such a low signal-to-noise ratio that it is close to the theoretical limit for a detector. Another unique feature of the insect olfactory system is its ability to inactivate chemical signals on a millisecond timescale. Odorant-oriented navigation requires that the olfactory system be reset between pockets of pheromones, which are separated by clean air. Whether this is achieved by enzymatic degradation or “molecular trapping” of stray pheromone molecules is still a matter of considerable debate. This review being based on a lecture I presented at the ACS National Meeting held in New Orleans may be biased toward the work in my own lab, but provides a somewhat concise overview of the current understanding of the molecular basis of pheromone binding, release, and inactivation.

Pheromone Reception

Perception of pheromones involves the reception of the semiochemicals by specialized apparatus in the periphery, such as antennae (and other chemosensory organs, in some cases, such as tarsi, palpi and proboscis), processing of semiochemicals-derived signals (action potentials) in the antennal

lobes, integration with other stimulus modalities in the protocerebrum, with ultimate translation into behavior (Figure 1).

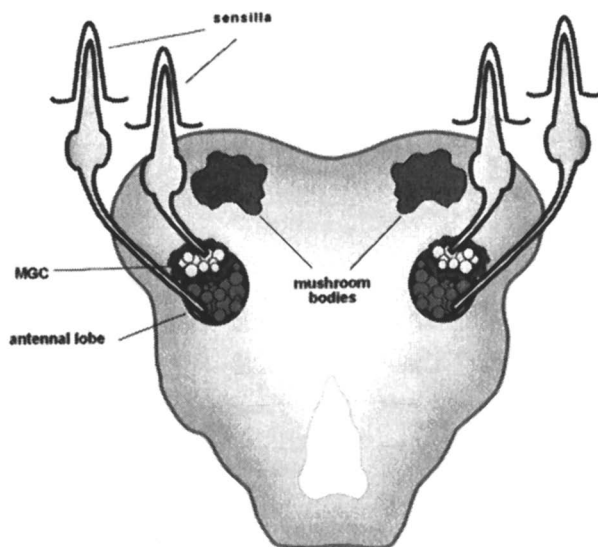


Figure 1. Schematic view of the overall olfactory processing in insects. Pheromones and other semiochemicals are detected by specialized sensilla on the antennae, where the chemical signal is transduced into nervous activity (action potentials). The olfactory receptor neurons in the semiochemical-detecting sensilla are connected directly to the antennal lobe (at the same side as the antenna), which houses the general glomeruli and in some cases the pheromone-processing macroglomerular complex (MGC). In the antennal lobes the semiochemicals-derived electrical signals are processed and information is sent out (through projection neurons) to the mushroom bodies in the protocerebrum. Olfactory information is then integrated with other stimulus modalities, a decision is made and conveyed to the motor system.

In moths (Lepidoptera), the pheromone-detecting sensilla are hair-like structures (sensilla trichodea), which are extensions from the antennae cuticle, but in scarab beetles they are olfactory plates (sensilla placodea), flat plates distributed over the internal part of the lamellae (4-6). Their external appearances are quite different, but internal structures resemble those of sensilla trichodea to some extent (Figure 2). In the Japanese beetle, each pheromone-detecting sensillum placodeum houses two olfactory receptor neurons (ORNs) accompanied by three auxiliary cells (thecogen, trichogen, and tormogen) (6). On the basal end of the sensillar (receptor) lymph cavity, the somata of the ORNs send axons that join the axon bundles near the basement membrane and

are connected to the antennal lobe. On the apical end, the somata project elongated inner and outer dendritic segments that run in parallel and are enveloped by a trichogen and a tormogen cells. The outer dendritic segments branch out and form cluster of terminal ends in outer cuticular pockets bearing groups of minute pores, the port of entry for pheromones and other semiochemicals. In other words, each sensillum placodeum has dendritic branches of ORNs (and presumably olfactory receptors) just below the flat cuticle surrounded by the sensillar (receptor) lymph. In contrast to sensilla trichodea, sensilla placodea have numerous microvilli of tormogen cells projecting towards the cuticle of the sensilla and approaching the terminal ends of the dendrites. This structure suggests that microvilli supply secretory materials right up to the terminal ends of the outer dendritic segments with minimal circulatory requirements (6).

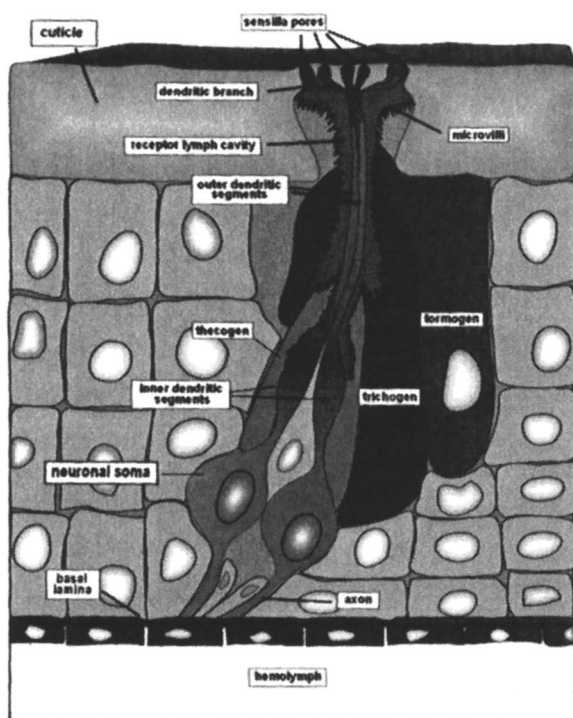


Figure 2. Schematic drawing of a pheromone-detecting sensillum placodeum. In the Japanese beetle, these pheromone detectors house two olfactory receptor neurons (ORNs); one specialized for the detection of pheromone (5,7,8) and the other tuned to the behavioral antagonist (5,7,8).

Despite the differences in the internal morphology of these types of pheromone-detecting sensilla, a common feature is the occurrence of dendrites (bearing ORs) in close proximity to cuticular pores. In general, the receptors are protected from hydrophobic molecules by an aqueous layer (sensillar lymph) surrounding the dendrites in which the ORs are attached. To reach these ORs, hydrophobic pheromone molecules are carried by odorant-binding proteins (OBPs), which are named pheromone-binding proteins (PBPs) when there is evidence that they bind (specifically) pheromone(s). The OBP-odorant complex is water soluble and, thus, can diffuse through the sensillar lymph and reach the membrane-bound ORs.

Pheromone Binding and Release

Recent structural biology and biochemical studies with the pheromone binding protein from the silkworm moth, *B. mori* (BmorPBP), have revealed the mechanism of binding and release of pheromones by PBP (9-13). Pheromone-binding proteins engulf pheromones in a hydrophobic cavity inside their structures (10) (Figure 3).

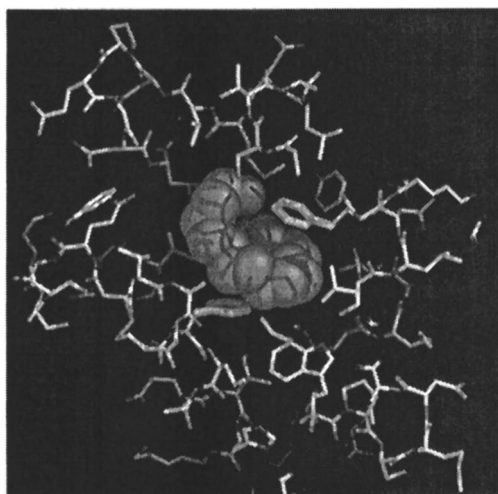


Figure 3. Structure of BmorPBP-bombykol complex highlighting bombykol in the binding cavity sandwiched by two phenyl alanine residues. (See page 1 of color insert.)

At low pH values, such as those present on the negatively-charged surfaces (9,11,12), the BmorPBP-bombykol undergoes a remarkable conformational

change leading to the release of the ligand (13). A C-terminal sequence, which has an unstructured conformation in the BmorPBP-bombykol complex rearranges to form a regular α -helix (Figure 4) that occupies the binding pocket (13).

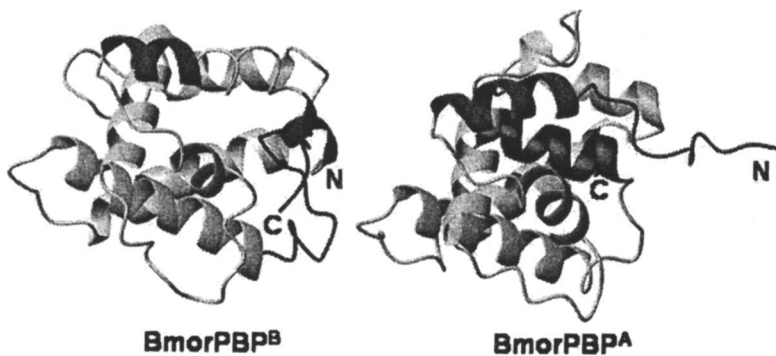


Figure 4. Structures of the BmorPBP, at the pH of the sensillar lymph (BmorPBP^B) and the acid form (BmorPBP^A). An unstructured conformation in the C-terminal sequence of the protein (C in BmorPBP^B), rearranges at lower pH to form a regular α -helix (C in BmorPBP^A) that occupies the pheromone-binding pocket thus “ejecting” the pheromone to the receptor. Note that the N-terminus (N), which formed as α -helix in BmorPBP^B, is unfolded in BmorPBP^A. (See page 1 of color insert.)

Some pieces of evidence suggest that PBPs are essential for the dynamics of the olfactory system. Expression of a *Drosophila* OR in *Xenopus* oocytes showed that activation of the receptor was orders of magnitude slower than normal *in vivo* function (14). In this heterologous system (devoid of OBPs), activation of the *Drosophila* receptors occurred on the scale of seconds rather than the milliseconds typical of the intact system (8). This difference is probably due to the absence of OBPs. On the other hand, kinetic studies (15) have demonstrated that the pH-dependent conformational changes in PBPs associated with “unloading” the pheromone molecule occurs in less than 5 msec.

Mode of Action of OBPs

There is growing evidence in the literature that insect PBPs (e.g, BmorPBP) bind, solubilize, carry, and deliver pheromones to the pheromone receptors. BmorPBP is predominantly expressed in the male antennae (16) and binds to bombykol, a cognate ligand with some degree of specificity (9). BmorPBP is

specifically localized in the long sensilla trichodea of males (17). Females possess the same type of sensilla but rather than PBP they express a general OBP (GOBP). The long sensilla trichodea in male *B. mori* have been demonstrated to be the pheromone detectors (18), whereas in females they respond to benzoic acid and linalool (19). BmorPBP undergoes a pH-dependent conformational change (9) and the surfaces of dendrites are negatively-charged (20,21), thus, generating localized low pH. Evidence from structural biology (see above) demonstrates that the low pH (as expected near the surface of dendrites) triggers the formation of an additional C-terminal α -helix that fills the binding pocket leaving no room for pheromone in the binding cavity. Binding assays showed that BmorPBP binds bombykol at the sensillar lymph pH but not at low pH as on the surface of dendrites. Based on this body of information, I proposed the model depicted in Figure 5 for the mode of action of OBPs.

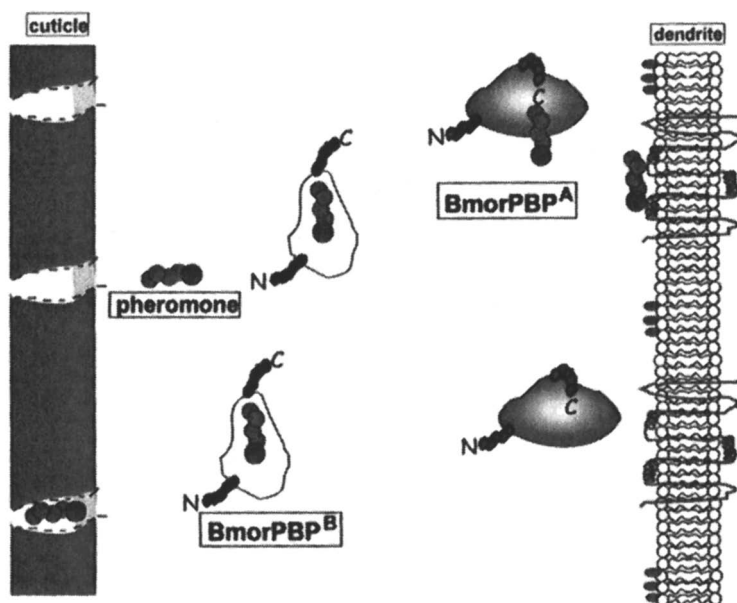


Figure 5. Proposed mechanism for pheromone binding and release. The odorant receptors are located on the surface of the ORN dendrites (right), surrounding by a sensillar lymph. Pheromones are transported from the openings (left) of the sensilla (pore tubules) to the ORs by PBPs. At the sensillar lymph pH, PBP^B binds to a pheromone. At negatively-charge surfaces (lower pH) a newly formed α -helix in PBP^A "takes over" the binding pocket thus ejecting the pheromone to the ORs.

First OBPs uptake compounds entering the sensillar lymph through pore tubules, bind physiologically relevant ligands, encapsulate them, and ferry these semiochemicals to the ORs. Then, OBPs deliver the chemical signal by a conformational change upon interaction with negatively charged sites in the dendrites. In this model, OBPs participate in the selective transport of pheromones and other semiochemicals to their ORs. The selectivity of the system is likely to be achieved by “layers of filters” (15), i. e., by the participation of compartmentalized OBPs and olfactory receptors. It seems that OBPs transport only a subset of compounds that reach the pore tubules. Some of these compounds may not bind to the receptors compartmentalized in the particular sensilla. The ORs, on the other hand, are activated by a subset of compounds, as indicated by studies in *Drosophila*, showing that a single OR is activated by multiple compounds (14). If some receptor agonists reach the pore tubules but are not transported by OBPs, receptor firing is prevented. In other words, even if neither OBPs nor ORs are extremely specific, the detectors (olfactory system) can show remarkable selectivity if they function in a two-step filter. Another group of olfactory proteins is the chemosensory proteins (CSPs), whose function(s) is (are) yet to be clarified. CSPs differ from OBPs in their disulfide structures (15).

Pheromone Degradation

The dynamics of the odor-oriented navigation in insects requires – in addition to sensitivity and selectivity - a fast process for inactivation of chemical signals. Immediately after stimulation of the receptors the detectors can be switched on and off on a millisecond timescale. It has been suggested that peripheral interactions rather than intracellular signaling processes determine the temporal precision of insect olfactory system (22). Several quantitative models of peripheral interactions have been proposed, particularly after the discovery of PBPs and pheromone-degrading enzymes (PDEs) (23). Recent structural biology studies (see above) suggest that PBPs not only ferry the ligands to their receptors, but also protect the chemical signals from PDEs.

The literature on the inactivation of chemical signals is dichotomous. One school favors the hypothesis that the rapid inactivation of chemical signals is an enzymatic process regulated by PDEs. The other school favors another molecular process in which “stray” pheromone molecules would be trapped by olfactory proteins (OBPs or the like) before being degraded (catabolized) by PDEs. These controversies can be addressed only when recombinant PDEs become available and kinetics studies can be accurately carried out in cell-free system mimicking the *in vivo* conditions. Hitherto, this has not been possible because PDEs are expressed in such low amounts making the collection of

enough purified protein for characterization (by Edman degradation or MS-MS) a daunting task. If these PDEs are indeed fast enough, as previously suggested (24), and they show substrate-specificity, their hypothesized role in signal inactivation will be supported.

Studies on the degradation of enantiomers of japonilure by extracts from tissues of scarab beetles indicate that antennae-specific esterase(s) from the Japanese beetle showed a significant preference for the pheromone, (*R*)-japonilure, over the behavioral antagonist.

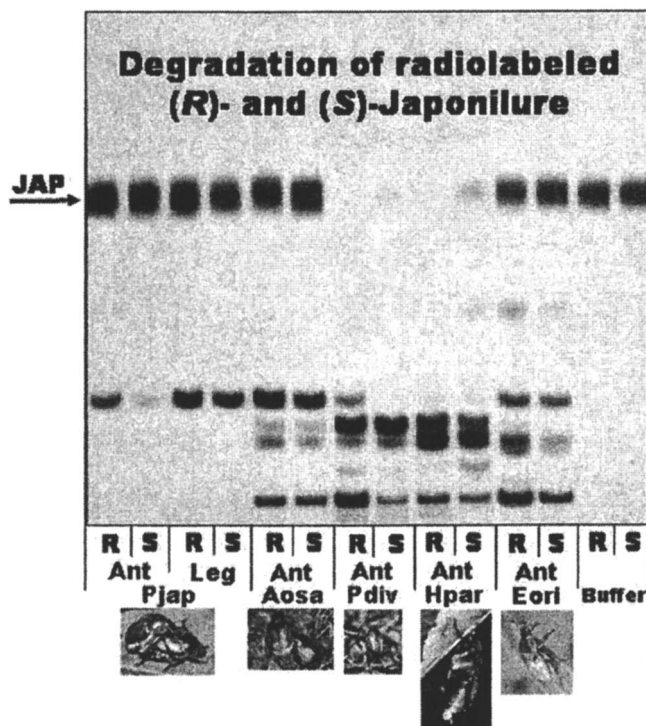
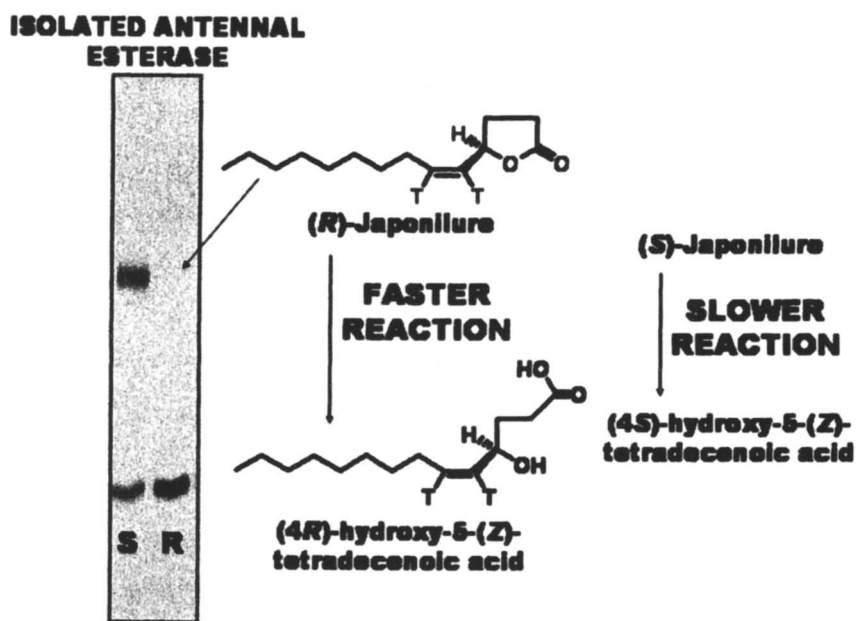


Figure 6. TLC plate showing degradation of (*R*)- and (*S*)-japonilure by esterases from legs (Leg) and antennae (ANT) of the Japanese beetle (*Pjap*), *A. osakana* (*Aosa*), *P. diversa* (*Pdiv*), *H. parallela* (*Hpar*), and *E. orientalis* (*Eori*). Note the slower degradation of the behavioral antagonist, the (*S*)- enantiomer, by the sensilar esterase(s), but not with enzyme(s) from legs. Neither (*R*)- nor (*S*)-japonilure is degraded in control experiments (buffer).
(See page 2 of color insert.)

Protein extracts from antennae and legs of the Japanese beetle, the Osaka beetle, *Anomala osakana*, and the other scarab beetle species (*Phyllopertha diversa*, *Holotrichia parallela*, and *Exomala orientalis*) were incubated either

with (*R*)- or (*S*)-japonilure prepared by tritiation of the alkyne intermediates. After extraction with organic solvent, the reaction mixtures were analyzed by TLC (Figure 6).

Comparison of the profiles of the protein extracts from legs and antennae, analyzed by native PAGE, with the proteins visualized by the naphthyl acetate method, showed a clear antenna-specific band. No corresponding bands were detected in the extracts from legs, even when larger amounts of sample were loaded in the gels, (data not shown). The antenna-specific band was isolated directly from the gel, electroeluted, and concentrated. Incubation of the enantiomers of japonilure with the partially isolated esterase(s) showed faster degradation of the pheromone (Figure 7).



*Figure 7. Faster degradation of the Japanese beetle pheromone, (*R*)-japonilure, than the behavioral antagonist, (*S*)-japonilure, by esterase(s) isolated from the Japanese beetle antennae.*

Mass spectral analyses indicate that japonilure underwent opening of the lactone ring to yield the corresponding hydroxyacid. Although the behavioral antagonist, (*S*)-japonilure, underwent degradation into (*4S*)-hydroxy-5-(*Z*)-tetradecenoic acid, this reaction was clearly slower than the corresponding reaction with the pheromone.

These findings suggest that esterases found in legs (and possibly other non-olfactory tissues) are not specific, but sensillar esterases may have evolved for the specific degradation of pheromones. Work in progress in my lab is aimed at testing the hypothesis that PDEs are not only involved in inactivation of chemical signals, but also in chiral discrimination.

Reverse Chemical Ecology

Largely, bioassay-oriented approaches are applied in chemical ecology studies for the identification of pheromones and other semiochemicals, but the complex behavior of some insect species has retarded progress. The knowledge generated by fundamental research programs aimed at understanding the molecular basis of insect olfaction has provided the foundation for practical applications. Like molecular-based drug discovery, OBPs can be utilized for the screening of attractants since these protein bind to physiologically relevant compounds. The pharmaceutical industry approach is based on receptor-drug interactions because delivery of water-soluble drugs may not be a problem. With semiochemicals, functional expression of ORs for *in vivo* binding studies is technically difficult. In addition, the essential role of OBPs to bind, solubilize, and transport hydrophobic pheromones to the ORs makes them a feasible molecular target. It is necessary, however, to identify, clone and obtain functional expression of OBPs that are indeed expressed in the antennae or other olfactory tissue. As discussed elsewhere (25), the number of functionally expressed OBPs may be much lower than the number of genes encoding OBP-like proteins. In the *Drosophila* genome, the OBP-gene family comprises as many as 51 putative OBPs, whereas only seven of them have been demonstrated to be expressed specifically in olfactory organs of *Drosophila* adults (25). Though not trivial, expression of all functional OBPs is doable and essential in “bullet-proof” screening of semiochemicals. This process is justified for cases in which semiochemicals are sorely needed, but conventional chemical ecology approaches have failed.

This reverse chemical ecology approach is the molecular equivalent of electrophysiology-based approaches, such as electroantennogram (EAG) and gas chromatography linked to a an electroantennographic detector (GC-EAD). Although these “short-cut bioassays” can not replace behavioral bioassays, they may narrow down the list of candidate compounds to be tested in wind tunnels, olfactometers, field trappings, etc. Likewise, binding of a candidate compound to an OBP does not necessarily imply a physiological function. However, test compounds with low or no binding affinity can be eliminated from futher tests as they can not reach the ORs.

Work is now in progress in my lab towards the identification of mosquito repellents and attractants by OBP-based screenings. We have isolated OBPs from various *Culex* species, vectors of West Nile Virus, clone the cDNAs encoding these proteins, and generated recombinant proteins for binding studies. The development of binding assays for throughput screening of candidate semiochemicals is underway.

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Chapter 5

Chemical Basis for Weed Suppressive Activity of Sorghum

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The weed suppressive activity of *Sorghum* species has been associated with phytotoxic compounds that are exuded from the roots, and contain primarily sorgoleone (**1**). The concentration of **1** ranges from about 40 to 800 µg/mg root extract, based on quantitative analysis of seven genetically different *Sorghum* accessions. Minor constituents were isolated, including novel resorcinolic lipids. Analogues of **1** with the aliphatic side chain varying in number of carbon and double bonds have also been isolated by thin layer-argentation chromatography. The minor constituents that have been tested inhibit photosystem II electron transport, indicating that they contribute to weed suppressive activity of *Sorghum*. In studying the biosynthesis of **1**, 2-¹³C-acetate was found to also get incorporated into 4,6-dimethoxy-2-[(8'Z,11'Z)-8',11',14'-pentadecatrienyl]-1,3-benzenediol, providing significant information on the possibility of altering the biosynthesis of **1** to a more phytotoxic and stable methylated resorcinolic lipid.

Introduction

Several *Sorghum* spp. are used as cover crops due to their weed suppressive activity (e.g. 1, 2). Because of its potential use in agriculture as a source of natural pesticides, we have undertaken studies to understand the chemical and biochemical aspects underlying the plant growth inhibiting properties of sorghum. We have focused on root exudates, based on reports that they contain phytotoxic compounds (3, 4, 5), which consist of approximately 85 – 90% (w/w) sorgoleone (1) (6). A capillary mat production system was developed that enabled growing sorghum with massive roots and collecting large quantities of exudates. This system was utilized to grow seven genetically diverse sorghum accessions under uniform conditions, and provided considerable amounts of exudates that were analyzed for their constituents. The amount of 1 in the exudates was quantitated, and minor constituents were isolated and their structures characterized. In further studies, the biosynthesis of 1 was investigated by feeding ¹³C-labelled precursors, and determining the position of incorporation by ¹³C-nuclear magnetic resonance (NMR) spectroscopy. Interestingly, feeding with 2-¹³C-acetate resulted in incorporation of the labelled precursor in one of the minor resorcinolic lipid constituents of the exudate. Results of our studies are presented.

Materials and Methods

General. UV spectra were obtained on a Shimadzu UV-3101 PC spectrophotometer. IR spectra were recorded with an ATI Mattson Genesis FT-IR instrument. GC-MS was performed on a JEOL (JEOL USA, Inc., Peabody, MA) GCMate II system. HR-ESIMS was obtained from a Bruker FT-MS, BioApex 3.0T spectrometer. NMR experiments were carried out on either a Bruker Avance DRX (500 MHz) or DPX 300 (300 MHz) instrument (Bruker, Billerica, MA) using standard Bruker software (XWINNMR version 1.3). For thin layer chromatography (TLC), aluminum-backed, 10 X 20 cm, 0.2 mm thick silica F₂₅₄ plates (EM Science, Gibbstown, NJ) were used. For preparative layer chromatography (PLC), Merck Si gel F₂₅₄ 20 x 20 cm, 1.0 mm thick plates (VWR Scientific, Atlanta, GA) were used. All solvents used were HPLC grade (Fisher Scientific, Suwanee, GA).

Plant material. The seven sorghum accessions studied and quantitated for sorgoleone content included two weed species, shattercane (*Sorghum bicolor* (L.) Moench) and johnsongrass (*Sorghum halepense* (L.) Pres.) (both from Azlin Seed Service, Leland, MS); two cultivated forage type sorghum/sudangrass hybrids, SX15 and SX17 (*S. bicolor* x *S. sudanense* (L.) Moench) (Poaceae)

(both from Monsanto Company, St. Louis, MO); two cultivated grain sorghum species, 8446 and 855F (*S. bicolor* (L.) Moench) (both from Pioneer Hi-Bred International, Inc., Des Moines, IA); and a cultivated sweet sorghum, Della (*S. bicolor* cv. Della) (Southern States Cooperative, Inc., Richmond, VA).

Sorghum cultivar SX17, *S. bicolor* x *S. sudanense* (L.) Moench (Poaceae), DeKalb Hybrid SX17 (DeKalb, IL) was used for the isolation of active constituents, as well as for biosynthetic studies.

Growth and harvesting. Seven sorghum accessions were used for quantitation of sorgoleone. Seeds were processed and grown using a capillary mat production system as reported (7). Two separate but identical root exudate collection systems were constructed. Each system contained six individual screens (28 cm x 43 cm). Each screen was considered a replicate, thus totalling twelve replicates for each accession. Roots, which grew through the holes in the screens, were excised from below with a blade and exudates were collected by extraction as described herein.

For the isolation of constituents by argentation chromatography, a modified capillary mat system was used (8). For biosynthetic studies, seeds were grown in Petri dishes (9).

Extraction. Roots were immersed in CHCl_3 or CH_2Cl_2 for 1-5 minutes, and the extraction was repeated. The extracts were decanted through a fluted glass funnel lined with Whatman #1 filter paper to remove root debris. The crude extract was concentrated under reduced pressure (Büchi Rotovapor, Brinkmann Instrument, Westbury, NY) at 30 °C, dried under a stream of nitrogen, and stored at -20 °C.

Preparative layer chromatography (PLC). Minor constituents were isolated by PLC on a glass backed plate developed twice in hexane-isopropanol (9:1, v/v). Bands were detected under UV at λ_{254} nm and the compounds were eluted from scraped sections of the silica gel with CHCl_3 -MeOH (19:1).

Argentation TLC. Aluminum-backed 10x20 cm, 0.2 mm thick silica F254 plates (EM Science, Gibbstown, NJ) were washed in EtOAc-MeOH (9:1 v/v) and activated at 80 °C (20-30 min) or 62 °C (30-35 min), to improve adsorption of AgNO_3 onto the silica. Plates were developed (twice) in a TLC tank containing a saturated (5%) solution of AgNO_3 in MeOH-water (95:5, v/v). Plates were air-dried 1-16 h and activated several times at 70-80 °C (total activation time of 2.5-3 h). The mixture of 1, 4, 5, 6, 7 eluted from PLC was dissolved in 100-500 μL of CH_2Cl_2 and spotted across the origin of a AgNO_3 -

impregnated plate. Plates were developed twice in hexane-acetone- CH_2Cl_2 (7:2:1, v/v/v). Bands were scraped from the plate and eluted with CHCl_3 , and concentrated and dried.

Quantitation of 1. Quantitation was performed by high performance liquid chromatography using a Hewlett Packard 1050 system. One mg of root exudates were dissolved in 1 mL of acetonitrile. Components of the root exudates were separated on an Alltech EPS C18 column 100 Å 3 μ (150 mm length x 4.5 mm internal diameter). The mobile phase was eluted at a flow rate of 2 mL/min as follows (solvent A is 2.5% acetic acid in water, solvent B is acetonitrile): 0-15 min 45% A / 55% B isocratic; 15-22 min linear gradient from 55% to 100% B; 22-25 min 100%B; 25-26 min 100% to 55% B; 26-30 min 45% A / 55% B isocratic. The peaks were monitored at λ 280 nm, and the volume of injection was 20 μ L. Quantitation was based on a calibration curve of an external standard of purified 1.

Phytotoxicity assay. Compounds were tested for phytotoxic activity in a 24-well microtiter plate, using atrazine as a positive control. Lettuce seeds were incubated in the wells on filter paper impregnated with test compounds. Seeds were grown in growth chamber at 25°C with a 16-hr photoperiod at 400 $\mu\text{mol}/\text{m}^2/\text{sec}$ photosynthetically active radiation for 4 days. Treatments were in duplicates. Compounds were dissolved in acetone and tested at final concentrations of 0.1 – 3 mM in a final volume of 200 μ L (10% acetone in H_2O) in the wells. Seedlings were rated on a scale of 0 (no symptoms) to 5 (seedling death).

Preparation of spinach thylakoid membranes. The oxygen evolution assay was performed on spinach thylakoid membranes, prepared as previously described (10), except that in testing for activity of 4 - 7, after the initial homogenization, the filtrate was centrifuged for 10 instead of 5 min at 4 °C and 1100 x g. The membrane preparation was diluted to a chlorophyll concentration of 4 mg/mL and stored at -80 °C. The preparation was diluted in resuspension buffer for the assays, to a final chlorophyll concentration of 1.5-1.6 mg/mL.

Oxygen evolution assays. Assays were performed at 30 °C with a computer-controlled oxygen electrode (Hansatech Instruments, Norfolk, England). A fiber-optic light source (Fiberoptic specialties, Inc., Palmetto FL) provided saturating light (2400 $\mu\text{mol}/\text{m}^2/\text{s}$), and a temperature controlled circulator (Neslab RTE-101, Newington, NH) connected to the oxygen electrode cell kept the temperature at 30 °C throughout the assay. The assay protocol was as previously described (10), except that in assaying 4 - 7, decyl plastoquinone was omitted from the assay buffer, and the rate of oxygen evolution was measured for

25 s over the linear portion of the curve. Compounds were stored as 33 mM stocks in 100% EtOH at 4 °C. Dilutions, prepared in EtOH, ranged from 0.1 to 10 mM and gave final assay concentrations of 0.01 to 3 μM. Dose-response curves were fitted to a four-parameter logistic function (11).

Labeling studies. Seeds of sorghum cultivar SX17 were surface sterilized in 20% Clorox bleach (final concentration of 1% NaOCl) for 10 min and rinsed with deionized water. Seeds were poured into a funnel lined with cheesecloth and allowed to dry overnight in a laminar flow hood. Twenty seeds (ca. 500 mg) were placed in 20 x 100 mm sterile polystyrene Petri dishes (Falcon) over the surface of sterile Whatman #1 filter paper (90 mm diameter). Five mL of sterile water was added to the dish, and the seeds were covered with a second sterile filter paper. The dishes were sealed and incubated in the dark at 25°C.

A solution of 2-¹³C-acetate (99% ¹³C; Sigma-Aldrich, Milwaukee, WI) was prepared as a 1:1 or 1:3 ratio of ¹³C: ¹²C isotopes in 1 mM stock solution. This labeled precursor was added to the Petri dishes on the fourth day. The Petri dishes were opened and the upper filters discarded. Excess water in the dishes was removed and replaced with 2 mL of 1 mM 2-¹³C-acetate. The same procedure was repeated on the fifth and sixth day. At the end of the labeling period, the roots were excised, and **1** was extracted as described above. Labeling procedures were done under low-intensity green light to prevent the formation of anthocyanins by the sorghum roots.

NMR analysis - ¹³C-NMR experiments were carried out in CDCl₃ on a Bruker Avance DPX 300 (Bruker, Billerica, MA) instrument using standard Bruker software (XWINNMR version 1.3), with the following variables: 30° pulse (2.17 μs), repetition time, 3.0 s; spectral width, 15.1 kHz, temperature, 27°C. The decoupling pulse was 90 μs at 18 db. ¹³C-NMR spectra of labeled and unlabeled compounds were obtained under the same experimental conditions.

Results and Discussion

HPLC analysis of the root extracts of seven sorghum accessions showed very similar profiles when monitored at λ280 nm, with the largest peak corresponding to that of **1** eluting at around 8.2 min (see Fig. 1 for representative profiles; see also 12). The HPLC method developed showed baseline separation of the minor constituents from **1**, thus allowing more accurate quantitation. With the exception of Johnsongrass, the amount of **1** in the extracts was about 55 to 80% (Table 1). These values are lower than those reported previously (6). This

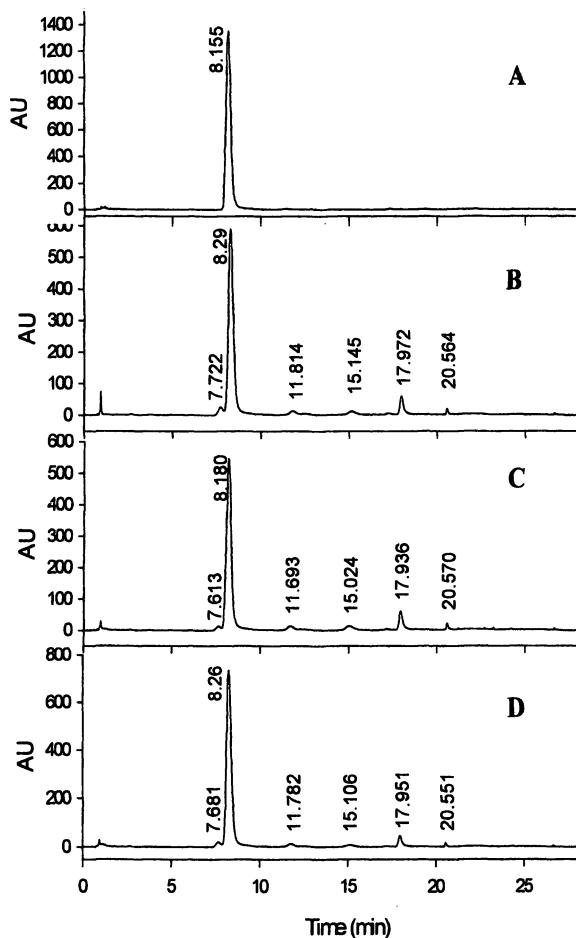


Figure 1. HPLC Chromatogram of (A) *Sorgholeone*, (B) *S. bicolor* cv. 8446, (C) *S. bicolor* cv. Della, and (D) *S. bicolor* cv. Shattercane monitored at $\lambda 280$ nm. Peak at 7.8, 8.2, 11.7, 15.1, and 20.5 are compounds **8**, **1**, **2**, **3**, and **7**, respectively. The peak at 17.9 min has yet to be characterized.

result triggered efforts to isolate and identify othe constituents in the exudates, and determine their phytotoxic activity.

A typical thin layer chromatogram of a sorghum root extract on a normal silica gel plate appears as in Fig. 2A. By PLC, constituents were isolated and structurally characterized by spectroscopic means (see 10 and 11 for details on structure elucidation). The largest spot (Rf 0.3) which appears as a single spot

Table 1. Sorgoleone content of *Sorghum* accessions.

Accession	Sorgoleone $\mu\text{g}/\text{mg}$ root extract
SX-15	797.51
SX-17	745.19
8446	595.40
855F	683.60
Della	548.38
Shattercane	731.23
Johnsongrass (sample 1)	37.70
Johnsongrass (sample 2)	76.96

was determined not to be pure **1** from NMR, although **1** comprised the majority. The components of the mixture were separated on silica gel plate treated with AgNO_3 (Fig. 2B), and isolation of the individual compounds became possible. These compounds were determined to be analogues of **1** with varying number of carbon and double bonds in the aliphatic side chain by GC-MS and NMR (9).

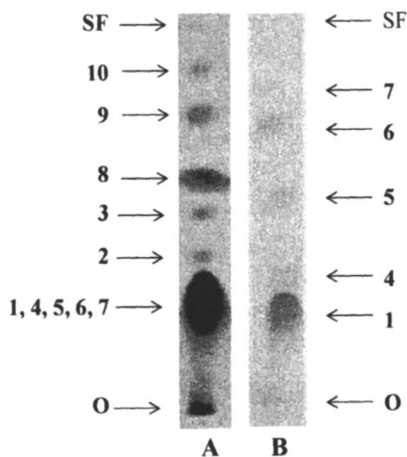
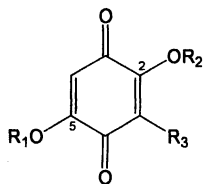


Figure 2. (A) Composite TLC of phytotoxins from root extracts of sorghum on normal silica gel plate showing elution of **1** – **10**, developing solvent hexane:isopropyl alcohol (90:10); (B) representative TLC of congeners of **1** on silica gel impregnated with 5% AgNO_3 showing separation of **1**, and **4** – **7**, developing solvent hexane:acetone:methylene chloride (7:2:1). O, origin; SF, solvent front.

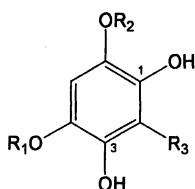
The compounds that we have identified from sorghum root extracts other than **1** are 5-ethoxysorgoleone (**2**), 2,5-dimethoxysorgoleone (**3**), 2-hydroxy-5-methoxy-3-[(8'Z,11'Z,14'Z)-8',11',14'-heptadecatrienyl]-2,5-cyclohexadiene-1,4-dione (**4**), 2-hydroxy-5-methoxy-3-[(8'Z,11'Z)-8',11'-pentadecadienyl]-2,5-cyclohexadiene-1,4-dione (**5**), 2-hydroxy-5-methoxy-3-[(8'Z)-8'-pentadecenyl]-2,5-cyclohexadiene-1,4-dione (**6**), 2-hydroxy-5-methoxy-3-pentadecyl-2,5-cyclohexadiene-1,4-dione, also known as dihydromaesanin (**7**), 4,6-dimethoxy-2-[(8'Z,11'Z)-8',11',14'-pentadecatrienyl]-1,3-benzenediol (**8**), 4-methoxy-6-ethoxy-2-[(8'Z,11'Z)-8',11',14'-pentadecatrienyl]-1,3-benzenediol (**9**) and 6-ethoxy-3-[(10'Z,13'Z)-10',13',16'-heptadecatrienyl]-1,2,4-benzenetriol (**10**) (Fig. 3).

The activity of these compounds in inhibiting photosystem II (PS II) electron transport in spinach thylakoid membranes was tested (except for **4** and **10**, due to limited amount of material available). With the exception of **2**, **3** and **9**, the activities of these compounds are almost equal (Tables 2 and 3), indicating that neither the number of double bonds nor whether a 15- or 17-carbon aliphatic side chain was present affected PS II electron transport inhibition. A generalization can also be made that an ethoxy substituent reduces activity. Compound **1** inhibits photosynthetic electron transport by competing for the Q_B binding site of the natural substrate plastoquinone on PSII (*13*, *14*, *15*). It is noteworthy that **8** and **9**, while not bearing a quinone moiety are also able to inhibit electron transport, with **8** showing activity equal to **1**. Enough material was available for **1**, **2**, **3**, and **8** to allow testing for inhibition of lettuce germination. Interestingly, **8** showed greater phytotoxic activity than **1** in this assay (Table 4).

The phytotoxins identified from the root extracts noticeably fall into two chemical classes, benzoquinone and resorcinolic lipids. The occurrence of the resorcinolic compounds can be traced back to the biosynthesis of **1**. By retrobiosynthetic NMR experiments, we determined that **1** was synthesized by cycloaddition of three acetate units to an existing hexadecatrienyl-fatty acyl-CoASH (**9**) forming a key resorcinolic lipid intermediate 5-[(8'Z,11'Z)-8',11',14'-pentadecatrienyl]-1,3-benzenediol (**11**) (Fig 4). Methylation of **11** yields 4,6-dimethoxy-5-[(8'Z,11'Z)-8',11',14'-pentadecatrienyl]-1,3-benzenediol (**12**), which upon exposure to air is oxidized to **1**. Whereas we have not been able to isolate labeled **11**, labeled **8** was isolated from feeding with 2-¹³C-acetate. ¹³C-NMR provided evidence of the incorporation, showing the enhancement of the carbon signals of C-1/C-3 and C-5 of **8** (Fig. 5). Because **8** is most likely the result of further methylation of **12**, the incorporation provides further support for the biosynthetic route leading to **12** (Fig. 4). Compound **8** has been previously reported to enhance the activity of **12** as a *Striga* germination chemical signal, by stabilizing the autooxidation of **12** (*16*).



	R ₁	R ₂	R ₃
1	CH ₃	H	(CH ₂) ₇ -(CH=CH-CH ₂) ₂ -CH=CH ₂
2	CH ₂ CH ₃	H	(CH ₂) ₇ -(CH=CH-CH ₂) ₂ -CH=CH ₂
3	CH ₃	CH ₃	(CH ₂) ₇ -(CH=CH-CH ₂) ₂ -CH=CH ₂
4	CH ₃	H	(CH ₂) ₇ -(CH=CH-CH ₂) ₃ -CH ₃
5	CH ₃	H	(CH ₂) ₇ -(CH=CH-CH ₂) ₂ -CH ₂ CH ₃
6	CH ₃	H	(CH ₂) ₇ -CH=CH-(CH ₂) ₅ CH ₃
7	CH ₃	H	(CH ₂) ₁₄ CH ₃



	R ₁	R ₂	R ₃
8	CH ₃	CH ₃	(CH ₂) ₇ -(CH=CH-CH ₂) ₂ -CH=CH ₂
9	CH ₃	CH ₂ CH ₃	(CH ₂) ₇ -(CH=CH-CH ₂) ₂ -CH=CH ₂
10	H	CH ₂ CH ₃	(CH ₂) ₉ -(CH=CH-CH ₂) ₂ -CH=CH ₂

Figure 3. Structures of phytotoxic constituents from root extracts of sorghum.

Table 2. PSII inhibitory activity of compounds 1, 2, 3, 8 and 9^{a,b}.

Compound	I ₅₀ (μM)
1	0.09 ± 0.01
2	1.07 ± 0.09
3	0.20 ± 0.02
8	0.09 ± 0.01
9	0.23 ± 0.03

^aMeans ± standard deviation of 3 replicates.

^bAdapted from 10 and 11.

Table 3. PSII inhibitory activity of of compounds 1, 5, 6 and 7^{a,b}.

Compound	Expt. 1, I ₅₀ (μM)	Expt. 2, I ₅₀ (μM)
1	0.083 ± 0.0049	0.065 ± 0.011
5	0.103 ± 0.017	0.105 ± 0.013
6	0.109 ± 0.022	0.117 ± 0.010
7	0.104 ± 0.032	0.110 ± 0.007

^aMeans ± standard deviation of 3 replicates.

^bAdapted from 8.

Table 4. Inhibition of lettuce germination^a.

Compound	Concentration (mM)				
	3.33	1.0	0.3	0.1	0.03
1	5	4	3	1	0
2	ND	4	2	1	0
3	ND	4	3	1	0
8	5	5	4	3	ND
Atrazine	5	4	4	4	0

^aVisual rating based on a scale of 0 (no effect) to 5 (seedling death).

ND = not determined.

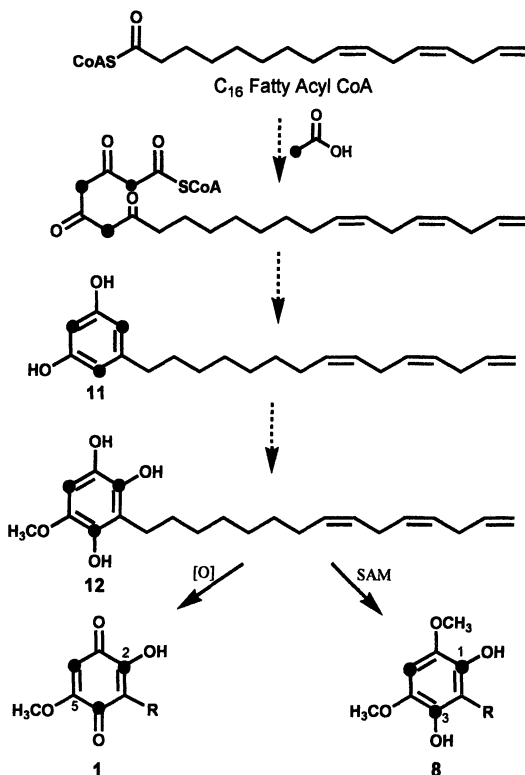


Figure 4. Biosynthesis of phytotoxins from sorghum root extract. R = (CH₂)₇-(CH=CH-CH₂)₂-CH=CH₂; SAM, S-adenosylmethionine.

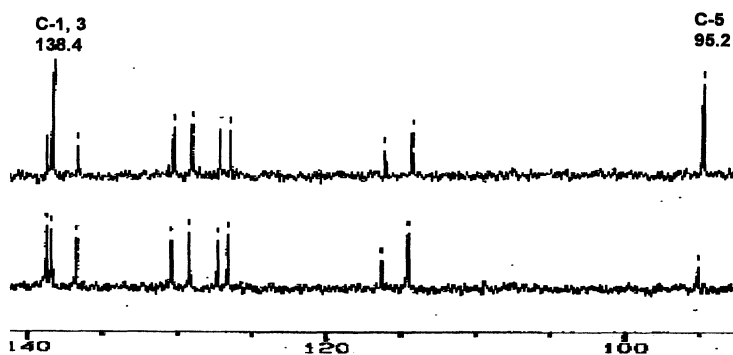


Figure 5. ¹³C-NMR spectra of **8**, unlabeled (lower trace) and labeled (upper trace), 95–140 ppm, showing incorporation of 2-¹³C-acetate in C-1/3 and C-5.

In conclusion, we have shown that sorghum root exudates contain **1** and a number of minor benzoquinone and resorcinolic lipid constituents that are also phytotoxic. Biosynthetic studies provided evidence that **8** shares the same biosynthetic route as **1** through **12**. Only a single methylation step by a specific *O*-methyl transferase is required to prevent the oxidation of **12**. An important avenue that can be explored is the manipulation of the pathway which will favor the synthesis of **8**, a more stable constituent that is also more phytotoxic than **1**.

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Chapter 6

Assessment of Natural Products for Control of Formosan Subterranean Termites

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Numerous plant species have been reported to be resistant to attack from subterranean termites. Many of these plants and extracts derived from them were tested for leads as potential natural product based pesticides. Forty plant and tree extracts reported to be active against termites displayed poor activity against the Formosan subterranean termite at rates < 0.5% wt/wt. Most naphthoquinones were active with a non-polar substitution in the 2-position. Anthroquinones generally had little termiticidal activity. None of the plant extracts or natural products tested were sufficiently active to be considered useful for control of the Formosan subterranean termite without structural modification.

Introduction

Man has used natural products to fight insect pests competing for food and fiber and affecting his health for thousands of years. Some of the natural insecticides are minerals, which are mined, such as arsenic, diatomaceous earths, and borates. Other natural insecticides are derived from plant parts or plant extracts such as chrysanthemum flowers, pyrethrums, and nicotine. There has always been some risk associated with these pest management strategies, but when confronted with plague and pestilence, careful use of pesticides was deemed a better option than death from starvation or disease.

In the middle of the 20th century, the synthetic development of DDT and other chlorinated hydrocarbons (C.H.), increased insecticidal activity well beyond that of most natural products. Problems arose with bioaccumulation of C.H. residues in the food chain, human fat tissue, mother's milk, as well as the development of insecticide resistance. It became obvious there were limitations to synthetic technology as well. The modification of a natural product, for example, from chrysanthemum flowers and their pyrethrum extracts (1) to pyrethroids such as allethrin, resmethrin, permethrin (2), and deltamethrin created a model in which insecticides are created from the skeleton of insecticidally active natural molecules. Thus, the avermectin, abamectin, ivermectin family of pesticides originated from compounds produced by the soil bacterium, *Streptomyces avermitilis* (3), and the commercially successful chloronicotinyl insecticides, though not derived from nicotine, are chemically related (4). Both pyrethroids and chloronicotinyls are currently used commercially as termiticides. We have previously provided a detailed review of natural products as pesticidal agents for control of the Formosan subterranean termites, *Coptotermes formosanus* Shiraki (5).

The purpose of this research was to determine whether various plants or woods and their extracts had an effect on Formosan subterranean termites to warrant further evaluation for termite control applications. Based on activity of wood extracts, an extensive series of substituted quinones was evaluated to gain insight into the relationship of substitutions to termiticidal activity for synthesis of more active compounds.

Materials and Methods

Termites from three colonies of *C. formosanus* were obtained from field sites in New Orleans, Louisiana, from bucket traps (6), and maintained on spruce (*Picea* sp.) slats (10 x 4 x 0.5 cm) under conditions of ca. 100 % relative humidity (R.H.) and 26.6° C. Termites were identified using keys for soldier identification from Scheffrahn and Su (7) and Su, et al. (8).

Extracts were collected from plants or trees as follows. Dried plant materials (>100 g) were ground into powder and extracted for 1 minute in a blender consecutively with pentane, acetone, pentane: acetone: water (54: 44: 2), methanol, and water followed by soxhlet extraction with pentane, acetone, and methanol for 1 week with each solvent. Solvents were removed under vacuum except for water that was removed by freeze-drying. For whole plant treatments, tissues were pulverized using a coffee bean grinder and/or mortar and pestle and applied as a thin layer on filter paper. Quinones were purchased from commercial sources and dissolved in acetone.

Compounds were dissolved in an appropriate solvent. One hundred μ L of the solution was pipetted onto 2.5 cm. diam. Whatman #1 filter paper. The solvent was allowed to evaporate from the filter paper for several hours. Treated filter paper disks were placed in plastic Petri dishes (35 x 10 mm) and moistened with 100- μ L water. Twenty *C. formosanus* workers (3rd instar or greater as determined by size) and a single soldier were placed on each treatment. Treatments were replicated 3 times with termites for each replicate originated from a different *C. formosanus* colony. Petri dishes were maintained at ca. 100 % R.H. and 26° C. Filter paper disks receiving water alone served as controls. It was previously determined that the filter paper treated with various solvents alone had no discernible effect on termite mortality or consumption.

Daily termite mortality was evaluated for 3 weeks. Cumulative daily mortality (mean and standard deviation) was calculated for each treatment. Treatments are compared using ANOVA and means separated with the SNK means separations test, following transformation to arcsine square root percent mortality (9). Actual percent mortality is reported in the tables.

Tested extracts and whole plants are listed in Tables I and II and tested quinones listed in Tables III and IV.

TABLE I. LIST OF HERBACEOUS PLANTS TESTED AND THOSE PREVIOUSLY REPORTED AS HAVING ACTIVITY AGAINST TERMITES

HERBACEOUS PLANTS TESTED AGAINST <i>C. formosanus</i>	TREES SPECIES REPORTED (5, 11, 12, 13, 14, 15)	TOXIC TO <i>C. formosanus</i>	RESISTANT TO TERMITES
<i>Flourensia cernua</i>	<i>Thuja standishii</i>		<i>Amyris elemifera</i>
<i>Sorghum bicolor</i>	<i>Mezilaurus itauba</i> ^a		<i>Aniba ovalifolia</i>
<i>Cornus florida</i>	<i>Ocotea cymbarum</i>		<i>Brosimum paraense</i>
<i>Hydrangea macrophylla</i>	<i>Calophyllum brasiliensis</i> (Beautyleaf) ^a		<i>Callitris glauca</i>
<i>Artemisia annua</i>	<i>Pouteria chiricana</i>		<i>Chlorophor tinctoria</i>
<i>Parthenium argentatum</i>	<i>Copaifera multijuga</i>		<i>Gaiacium officinale</i>
<i>Lepidium meyenii</i>	<i>Enterolobium cyclocarpum</i> (Ear tree) ^a		<i>Licaria canella</i>
<i>Cimicifuga racemosa</i>	<i>Pithecellobium mangense</i>		<i>Maclura pomifera</i>
<i>Podophyllum peltatum</i>	<i>Diploporis purpurea</i> ^a		<i>Ocotea rodiaei</i>
<i>Oryza sativa</i>	<i>Sweetia nitens</i> (Itaubarana) ^a		<i>Peltogyne pubescense</i>
<i>Cassia bauhinioides</i>	<i>Sweetia panamensis</i> (Chichipate) ^a		<i>Piratinera guianensis</i>
<i>Drymaria pachyphylla</i>	<i>Paramachaerium gruberi</i>		<i>Platymiscium ulei</i>
<i>Callicarpa americana</i>	<i>Platymiscium pinnatum</i> (Vencola) ^a		<i>Tabebuia capitata</i>
<i>Euonymus americana</i>	<i>Platymiscium ulei</i>		<i>Taxodium distichum</i>
<i>Nicotiana trigonophylla</i>	<i>Conocarpus erectus</i>		<i>Zollernia paraensis</i>
<i>Hibiscus cannabinus</i>	<i>Laguncularia racemosa</i>		
<i>Passiflora</i> sp.	<i>Hippomane mancinella</i>		
<i>Piper</i> sp.	<i>Cedrela odorata</i> (Spanish cedar) ^a		

Usnea sp.
Parmotrema sp.
Peltigera sp.
Cladonia sp.
Lentinula sp.
Ganoderma sp.
Pleurotus sp.
Flavoparmelia sp.
Punctelia sp.
Cladonia sp.
Vulpicida canadensis
Parmelia sulcata
Letharia vulpina

Guarea longipetiola
Cordia alliodora (Bojon)^a
Calycophyllum candidissimum^a

^a Commercially available

**TABLE II. WOOD AND WOOD EXTRACTS:
 MORTALITY OF *C. formosanus* ON FILTER PAPER**

TREATMENT (5.0% WT/WT)	% MORTALITY (MEAN ± S.D.) ^a		
	3	11	21
1A. <i>Platymiscium pinnatum</i> (pentane)	0A	0A	6.3±12.5A
1B. <i>Platymiscium pinnatum</i> (acetone)	0A	0A	11.3±22.5A
1C. <i>Platymiscium pinnatum</i> (MeOH)	0A	0A	0A
2A. <i>Cedrela odorata</i> (pentane)	11.3±22.5A	16.3±32.5A	27.5±48.6AB
2B. <i>Cedrela odorata</i> (acetone)	0A	0A	1.3±2.5A
2C. <i>Cedrela odorata</i> (MeOH)	0A	0A	0A
3. <i>Chlorophora tinctoria</i> (wood flour)	0A	1.3±2.5A	7.5±8.7AB
4A. <i>Brosimum paraense</i> (pentane)	1.3±2.5A	1.3±2.5A	13.8±24.3A
4B. <i>Brosimum paraense</i> (acetone)	0A	1.3±2.5A	23.8±47.5A
4C. <i>Brosimum paraense</i> (PAW54:44:2)	0A	2.5±5.0A	5.0±7.1A
4D. <i>Brosimum paraense</i> (MeOH)	0A	1.3±2.5A	2.5±2.9A
4E. <i>Brosimum paraense</i> (H ₂ O)	1.3±2.5A	5.0±7.1A	5.0±7.1A
5A. <i>Diplotropis purpurea</i> (pentane)	0A	0A	2.5±5.0A
5B. <i>Diplotropis purpurea</i> (acetone)	0A	0A	8.8±10.3A
5C. <i>Diplotropis purpurea</i> (MeOH)	0A	1.3±2.5A	2.5±2.9A
6. <i>Ocotea rodiaei</i> (wood flour)	0A	0A	0A

7. <i>Simaruba amara</i> (wood flour)	0A	0A	0A
8A. <i>Mora excelsa</i> (soxhlet, pentane)	0A	3.8±4.8AB	43.8±26.9AB
8B. <i>Mora excelsa</i> (soxhlet, MeOH/Acetone 1:1)	1.3±2.5A	5.0±7.1AB	11.3±13.2AB
8C. <i>Mora excelsa</i> (soxhlet, H ₂ O)	1.3±2.5A	3.8±4.8AB	28.8±21.8AB
8D. <i>Mora excelsa</i> (soxhlet, Acetone)	0A	3.8±4.8AB	30.0±7.1AB
UNTREATED	0A	1.3±2.5A	1.3±2.5A

^a Means within a column with the same letter are not significantly different, SNK: $P < 0.05$.

TABLE III. MORTALITY OF *C. formosanus* ON NATURAL NAPHTHOQUINONES TREATED FILTER PAPER

TREATMENT (1% WT/WT)	% MORTALITY (MEAN ± S.D.) ^a			
	DAYS			
	3	11	21	
1. naphthoquinone, Figure 1B	11.7±2.9F-J	18.3±10.4E-J	20.0±10.0G-K	
2. 1,2 naphthoquinone	8.3±7.6G-J	11.7±12.6F-J	15.0±10.0H-K	
3. menadione; 2-methyl-1,4-naphthoquinone (vitamine K3)	100.0±0.0A	100.0±0.0A	100.0±0.0A	
4. 2-hydroxy-1,4-naphthoquinone	25.0±13.2E-H	65.0±5.0B-E	81.7±16.1A-E	
5. plumbagin; 2-methyl-5-hydroxy-1,4-naphthoquinone	100.0±0.0A	100.0±0.0A	100.0±0.0A	
6. Juglone: (5-hydroxy-1,4-naphthoquinone)	100.0±0.0A	100.0±0.0A	100.0±0.0A	
7. 2-hydroxy-3-methyl-1,4-naphthoquinone	40.0±7.1CDE	92.5±10.6A	100.0±0.0A	
8. 2-hydroxy-3-(5-methylhexyl)-1,4-naphthoquinone	40.0 (N=1)CDE	90.0 (N=1)AB	90.0 (N=1)A-D	
9. 2-hydroxy-3-(2-methylpropyl)-1,4-naphthoquinone	26.7±12.6D-G	38.3±12.6C-I	76.7±23.6A-G	

10. 2-(2,3-dimethylbutyl)-3-hydroxy-1,4-naphthoquinone	61.7±16.1BC	100.0±0.0A	100.0±0.0A
11. 2-hydroxy-3-(2-methylbutyl)-1,4-naphthoquinone	58.3±28.4BC	100.0±0.0A	100.0±0.0A
UNTREATED	0J	6.3±7.5G-J	6.3±7.5JK

CONTINUED

TABLE III. MORTALITY OF *C. formosanus* ON NATURAL NAPHTHOQUINONES TREATED FILTER PAPER

TREATMENT (1% WT/WT)	% MORTALITY (MEAN \pm S.D.) ^a DAYS		
	3	11	21
12. 2-hydroxy-3-(2-phenylbutyl)-1,4 naphthoquinone	OJ	OJ	3.3 \pm 5.8K
13. 2-dodecylaminomethyl-3-hydroxy-1,4-naphthoquinone	OJ	8.3 \pm 10.4G-J	11.7 \pm 12.6H-K
14. 2-hydroxy-3-(4-methylphenyl)-1,4-naphthoquinone	OJ	21.7 \pm 12.6E-J	25.0 \pm 13.2F-K
15. Iapachol; 2-hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone	OJ	3.3 \pm 2.9G-J	6.7 \pm 5.8JK
16. β -Iapachone; 2H-Naphtho[1,2-b]pyran-5,6-dione, 3,4-dihydro-2,2-dimethyl-	OJ	15.0 \pm 8.7E-J	46.7 \pm 10.4B-K
17. vitamin K; 2-methyl-3-(3,7,11,15-tetramethyl-2-hexadeceny)-1,4-naphthoquinone	OJ	3.3 \pm 2.9G-J	18.3 \pm 23.1H-K
UNTREATED	OJ	6.3 \pm 7.5G-J	6.3 \pm 7.5JK

^a Means within a column with the same letter are not significantly different, SNK: $P < 0.05$.

Results and Discussion

None of the plants or their extracts in Table I had high termiticidal activity against FST below 0.5 % wt/wt. None of the tree woods or their extracts in Table II had high termiticidal activity at even 5.0 % wt/wt even though many had been reported in the literature as having activity against termite species (Table I). Compounds currently registered as termiticides have active ingredients, which are active as low as 0.001 % wt/wt (10).

Preliminary results showed that benzoquinones displayed termiticidal activity when substituted with two groups of different characteristics on opposite sides of the ring, such as methyl on one side and methoxy on the opposite side (Figure 1A).

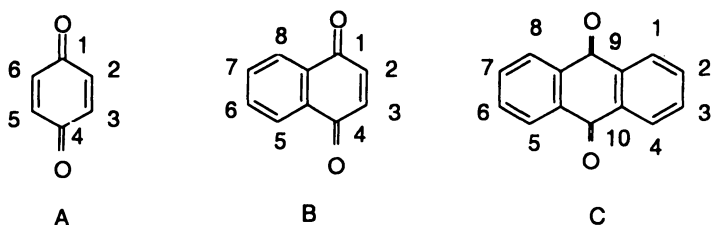


Figure 1. IUPAC numbering scheme Benzoquinone (A), Naphthoquinone (B), and Anthroquinone (C).

With naphthoquinones, termiticidal activity was displayed when the benzene ring fulfilled the steric requirements of the 5-position substitution of the benzoquinone combined with no 2-substitution or a non-polar substitution in the 2-position (Figure 1B, Table III, compounds 3, 5, 6, and 10). Juglone (compound 6) had been previously reported to have activity against *C. formosanus* (18) consistent with the findings of this report. Plumbagin (compound 5) was reported as non-repellent to *Reticulitermes* sp. (20). Also active are naphthoquinones with a 2-hydroxyl substitution and no 3-substitution (modest activity, Table III, compound 4) or a substitution of various numbers of carbons attached to the 4-position (Table III, compounds 7-9, and

TABLE IV. MORTALITY OF *C. formosanus* ON NATURAL ANTHROQUINONES TREATED FILTER PAPER

TREATMENT (1% WT/WT)	% MORTALITY (MEAN ± S.D.) ^a			
	DAYS			
	3	11	21	
1. anthraquinone, Figure 1C	0J	5.0±5.0G-J	8.3±5.8H-K	
2. 2-methylanthraquinone	0J	1.7±2.9IJ	5.0±8.7K	
3. 1-hydroxyanthraquinone	1.7±2.9IJ	35.0±20.0D-I	70.0±8.7A-H	
4. alizarin: 1,2-dihydroxyanthraquinone	0J	6.7±11.6G-J	13.3±14.4H-K	
5. 1,4-dihydroxyanthraquinone	0J	25.0±15.0E-J	31.7±12.6E-K	
6. anthrarufin: 1,5-dihydroxyanthraquinone	0J	5.0±8.7G-J	28.3±25.7G-K	
7. chrysophanic acid: 1,8-dihydroxy-3-methyl-9,10-anthraquinone	35.0±13.2C-F	78.3±7.6A-D	83.3±7.6A-F	
8. purpurin: 1,2,4-trihydroxy-9,10-anthraquinone	1.7±2.9IJ	21.7±12.6E-J	21.7±12.6G-K	
9. emodin: 1,3,8-trihydroxy-6-methylanthraquinone	1.7±2.9IJ	6.7±5.8G-J	8.3±2.9H-K	
10. quinalizarin: 1,2,5,8-tetrahydroxyanthraquinone	6.7±5.8G-J	40.0±13.2C-I	70.0±17.3A-H	

11. 1-hydroxy-2 methyl anthraquinone	OJ	16.7±20.8F-J	30.0±26.5G-K
12. 1,4-dihydroxy-2- methyl anthraquinone	1.7±2.9IJ	10.0±5.0F-J	11.7±7.6H-K
13. 2-(hydroxymethyl) anthraquinone	OJ	11.7±2.9F-J	18.3±5.8G-K
14. 2-ethylanthraquinone	15.0±10.0E-J	38.3±27.5C-I	65.0±25.0A-I
UNTREATED	OJ	6.3±7.5G-J	6.3±7.5JK
15. aloë-emodin: 2-(hydroxymethyl) - 4,5-dihydroxyanthraquinone	5.0±5.0HIJ	18.3±10.4E-J	18.3±10.4G-K
16. rhein: 1,8-dihydroxy-3- carboxyanthraquinone	1.7±2.9IJ	15.0±13.2F-J	38.3±5.8D-K
17. 1,8-dihydroxyanthracenedione	OJ	25.0±8.7E-J	41.7±14.4C-K
18. physcion: 2-methyl-4,5-dihydroxy- 7-methoxyanthraquinone	3.3±5.8IJ	41.7±22.6C-I	46.7±30.6B-K
19. phenanthrenequinone	3.3±2.9IJ	13.3±7.6F-J	16.7±12.6H-K
20. bianthrone	OJ	5.0±0.0G-J	8.3±2.9H-K
21. 2-carboxylic acid anthraquinone	OJ	10.0±10.0G-J	13.3±11.6H-K
22. damnacanthal: 1-methoxy-2- carboxylic acid anthraquinone	OJ	5.0±0.0G-J	5.0±0.0JK
23. 1-amino-4-bromo-2-methyl anthraquinone	10.0±5.0F-J	10.0±5.0F-J	15.0±10.0H-K
24. rufigalic acid: 1,2,3,5,6,7 -hydroxyanthraquinone	OJ	1.7±2.9IJ	1.7±2.9K
UNTREATED	OJ	6.3±7.5G-J	6.3±7.5JK

^a Means within a column with the same letter are not significantly different, SNK: $P < 0.05$.

11). Naphthoquinone (compound 1), 1,2--naphthoquinone (compound 2), and menadione (compound 3) were reported to have termite activity (19) of which we found only menadione active. Lapachol (compound 15) and B-lapachone (compound 16) were also previously reported to have termite activity (16, 17) where we found little. Jugalone (compound 6) previously reported to have activity against *C. formosanus* (18) was also active in this study.

None of the anthroquinones displayed exceptional termiticidal activity, with modest activity seen in four of the compounds (Figure 1C, Table IV, compounds 2, 7, 10 and 14). Chrysophanic acid (Table IV, compound 7) was the most active of the anthroquinones tested.

Substitutions on the quinone rings using different groups at different positions play an important role for their biological activity. Further testing of the most active of the above compound showed little or no activity at < 0.5% wt/wt. These quinones will require additional substitutions or modifications of their structures to have potential for further development as termiticides. Synthetic modifications to the basic skeleton of the natural pyrethroids has led to increases in toxicity by as much as four orders of magnitude. Further research on the most active of these compounds is needed to determine if structural additions or modifications to these naturally occurring quinones can yield sufficiently increased activities to merit their use as termiticides.

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Chapter 7

Semiochemicals of Termite Origin

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The behavior of the Formosan subterranean termite, *Coptotermes formosanus*, is directed by a number of chemicals produced by members of various castes in the colony. We have investigated the semiochemicals produced by termites that are associated with behaviors such as species recognition (cuticular hydrocarbons), trail following (3,6,8-dodecatrien-1-ol), and tandem running or mating. These chemicals were isolated from the termite cuticle, sternal gland, and tergal gland. Compound identities, quantities, and species comparisons were examined.

The Formosan subterranean termite, *Coptotermes formosanus* Shiraki, is a major urban pest that is found in several areas around the world. They were brought to the continental United States from China shortly after WWII and have since spread to several southern states with the highest concentration centered around New Orleans, Louisiana.

A newly formed *C. formosanus* colony begins with a pair of primary reproductives, the king and queen. The queen produces eggs that develop into workers, which further differentiate into soldiers or nymphs. The nymphs then differentiate into alates, which swarm from April to June and begin a new colony again as king and queen. Other termite species are very similar.

During this life cycle, termites use semiochemicals to elicit many of their recognized behaviors (1). Some termite behaviors are listed in Table I, such as those related to recognizing nestmates or finding food or a mate. The associated chemicals are usually produced and stored in glands before use, except the cuticular hydrocarbons that are continuously present on the outer surface or cuticle. Two of the glands that we have studied are the sternal and tergal glands. This paper describes some of our progress in isolating, identifying, and quantitating subterranean termite semiochemicals.

Table I. *C. formosanus* Semiochemicals and Associated Behaviors

<i>Behavior</i>	<i>Source</i>	<i>Compound</i>
Nestmate recognition	Cuticle	Methyl-branched hydrocarbons
Trail following/Mating	Sternal gland	(<i>Z,Z,E</i>)-3,6,8-Dodecatrien-1-ol
Unknown	Tergal gland	Trilinoleoylglycerol

Materials and Methods

Insects

Workers from termite colonies of *C. formosanus* and *Reticulitermes flavipes* were collected from field monitoring stations associated with live oak, cypress, and pine trees on the campuses of the University of New Orleans and USDA, ARS, Southern Regional Research Center, New Orleans, LA and maintained on spruce wood until needed. *Kalotermes approximatus* Snyder was collected from an elm tree in Slidell, LA. *C. formosanus* and *R. flavipes* were collected using underground bucket traps (2). Traps were baited with blocks of spruce, *Picea sp.*, wood. In underground traps infested with *Reticulitermes spp.*, corrugated cardboard rolls were placed in traps along with wooden blocks. Field colonies collected from the same area were delineated based on mark, release, and recapture techniques and on differences in mean worker biomass. Termites were identified to species by using identification keys for soldiers (3). Voucher specimens of soldiers of each colony are stored in 70% alcohol at the Southern Regional Research Center, New Orleans, LA.

C. formosanus alates were collected from light traps placed at three locations in New Orleans, LA from mid April until the first week of June, 2001

– 2003. Samples were collected from the traps the following morning. *R. flavipes* alates were collected during daytime swarms in Slidell, LA. *R. virginicus* alates were collected from wood posts in Poplarville, MS. All samples were sexed and processed within 24 hr.

Instrumentation

HPLC-MS analyses were carried out with a Waters Alliance 2690 HPLC (Milford, MA), with column heater, 996 Photodiode Array Detector, and a Micromass ZMD (Waters, Milford, MA) or Waters Integrity TMD mass spectrometer (Milford, MA). A Luna silica-2, 5 μm , 2.0 x 150 mm (Phenomenex, Torrance, CA) column was used at a temperature of 35°C. The ZMD MS was equipped with an atmospheric pressure chemical ionization (APCI) source and parameters set as: corona voltage, 3.5 kV; cone voltage, 30 V; source block temp, 125°C; APCI heater, 400°C; desolvation gas, 150 L/min; cone gas flow, 100 L/min; scan range, m/z 151-1000. Reversed phase LC-MS was performed using a Beckman HPLC with 126 pump, 168 PDA (Fullerton, CA), and a Thermoquest LCQ mass spectrometer (San Jose, CA). A Luna C18, 3 μm , 2.0 x 50 mm (Phenomenex, Torrance, CA) column was used at ambient temperature. The LCQ MS was equipped with an APCI source and parameters set as: APCI vaporizer temp, 450°C; source current, 5 μA ; sheath gas flow, 80 au; aux gas flow, 20 au; capillary temp, 150°C; capillary voltage, 23 V; scan range, m/z 250-1500.

GC-MS was performed on a Hewlett-Packard 6890 GC with 7683 autosampler and 5973 mass-selective detector (Agilent Technologies, Palo Alto, CA). Electron impact (EI) MS was obtained at 70 eV. A split / splitless injector was used in splitless mode with a purge flow to split at 2.0 min after injection. Chromatograms were run at a constant flow of 0.5 mL /min of He gas. The inlet temperature was set at 250°C. An HP-5MS (5% diphenyl-95% dimethylsiloxane) capillary column (30 m x 0.25 mm, 0.25 μm d_p) was used with temperature programming from 60 °C (1 min hold) to 300°C at 10°C/min with a final 10 min hold. Solvent samples (1 μL) were injected by the autosampler. SPME samples were manually injected by insertion of the fiber into the mass spectrometer inlet until after the purge flow to split occurred. Mass spectra were recorded from m/z 40 to m/z 550.

SPME fibers (100 mm polydimethylsiloxane (PDMS), 70 mm carbowax-divinylbenzene (CW-DVB), and 75 mm Carboxen-PDMS) were obtained from Supelco (Bellefonte, PA, USA).

Cuticular Hydrocarbons

SPME analysis

Headspace: Live termites were placed in a 4 mL vial with septum. A weighed amount of termites was used; 0.22 g was used as an equivalent to 50 termites. A SPME fiber was inserted into the vial (tip of fiber was 1 cm above top of termites) that was then inserted into a sand-filled heating block set at 120°C. After heating for 60 minutes, the fiber was removed.

Direct contact: Fifty termites were placed in a 4 mL vial with septum. The vial was laid on its side at a slight incline so the termites could walk up to the lip of the vial but not reach the septum. The SPME fiber was inserted so as to press against the glass under the termites. After 30 min at 26°C, the fiber was removed. Absorbed chemicals were analyzed by GC-MS.

Hexane extraction analysis

Fifty termites (either living or killed by freezing at -80°C) were placed in a 4 mL vial and 125 μ L hexane was injected onto the termites. After 2 min of slight agitation, the hexane was removed via syringe. The process was repeated with another 70 μ L hexane. A 2 μ L sample of the combined hexane was analyzed by GC-MS.

Dodecatrienol (DTE)

Sternal glands, with minimal amount of cuticle, from 10 female or male alates were excised under a dissecting microscope and placed in 50 μ L hexane. After storing at -20°C for 1 wk, the hexane was removed via syringe and the glands rinsed twice with 30 μ L hexane to give a total volume of 100 μ L. An internal standard, 8,10-dodecadienol (DDD), was added (100 ng / 6.25 μ L ether) followed by addition of 5 μ L of derivatization agent, N,O-bis-(trimethylsilyl)-acetamide (BSA). The solution was concentrated with nitrogen to 20 μ L and then sonicated (Aquasonic model 50D) for 1 hr at 28°C.

For whole body extractions, 100 female or male alates were ultrasonicated (Sonic Dismembrator 60, Fisher Scientific) in 10 mL hexane for 1 min (power level 3). The hexane was filtered (0.45 μ m, PTFE) and stored at -20°C. TMS derivatization was carried out by adding 5 μ L BSA and sonicating (15 min) while concentrating to 95 μ L under a stream of nitrogen. Another 5 μ L BSA was added and the solution was sonicated for 1 hr.

Samples (2 μL) were analyzed by GC-MS with quantitation based on m/z 119 for DTE and m/z 81 for the internal standard. Quantitation of DTE was based on a standard curve of DTE and DDD using m/z 119 and 81 respectively.

Trilinoleoylglycerol

The three terminal segments (8-10) from 50 female or male abdomens were excised and placed on filter paper. All the internal organs and fat bodies were gently removed with fine forceps and the clean tips with tergal glands extracted in 300 μL hexane for 1 hr. The hexane was removed and the tips were rinsed again with 250 μL hexane. The combined hexane extract was concentrated with nitrogen to 100 μL . For individual tergal gland extractions, three terminal abdominal tergites from individual females were cut and placed in a drop of phosphate buffered saline (PBS, 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4). All the internal organs and fat bodies were carefully removed. The area of the 9th and 10th tergites containing the pair of glands was excised and placed in a mixture of hexane, acetonitrile, and 2% NaCl (aq) (200:100:100 μL) and sonicated for 20 sec, three times. The vial was centrifuged for 5 min at 2000 rpm. The hexane phase was removed and concentrated to 100 μL .

Results and Discussion

Cuticular Hydrocarbons

As described earlier (4,5), we have studied nestmate recognition by examining the differences of cuticular hydrocarbons between species, colonies, and castes. Briefly, we developed a methodology for the extraction of termite cuticular hydrocarbons by solid phase microextraction (SPME). Both headspace SPME, where the termites were heated in a vial to 120 $^{\circ}\text{C}$ with the fiber 1 cm above the termites, and direct contact SPME, where live termites walked on the SPME fiber for a certain time period, were studied. As shown in Figure 1, both methods were comparable to the normal hexane extraction method in the types of cuticular hydrocarbons that were extracted. Since SPME requires less manipulation of the sample and may use living termites, it may have advantages.

A clear difference in cuticular hydrocarbon pattern is seen in Figure 2 between three termite species from the New Orleans area by the direct contact SPME method. The direct contact SPME method also demonstrated an unusual

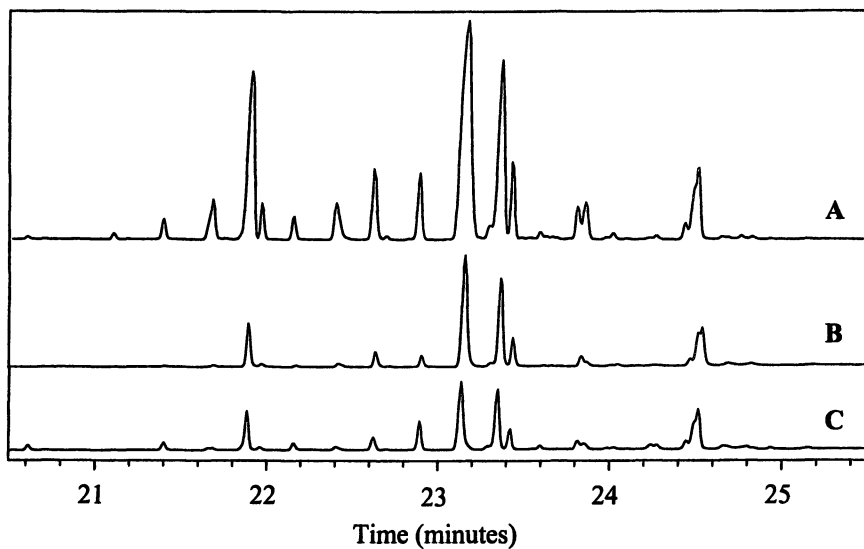


Figure 1. GC-MS detection of termite cuticular hydrocarbons by A) Headspace SPME, B) Direct Contact SPME, and C) Hexane extraction.

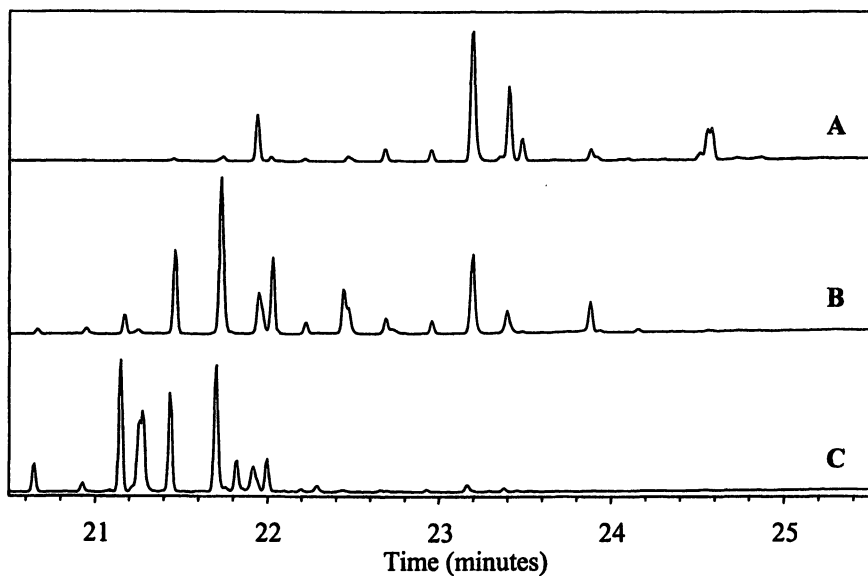


Figure 2. GC-MS comparison of termite cuticular hydrocarbons by species: A) *C. formosanus*, B) *K. approximatus*, and C) *R. flavipes*.

phenomenon, as shown in Figure 3. Since this method used live termites and could be sampled repeatedly, a 2-hr cycle in the production of hydrocarbons was found. The cause or utility of this phenomenon is unknown, though possibly associated to some type of biological clock.

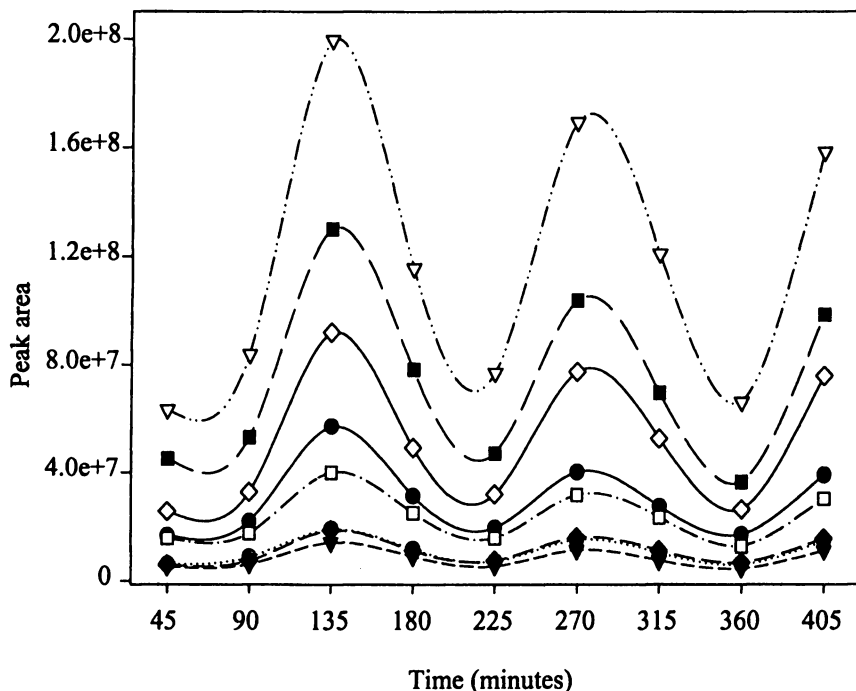


Figure 3. Variation in cuticular hydrocarbons over time for the eight largest GC-MS peaks. In decreasing amounts, 11-,13-MeC27; 2-,4-,6-MeC27 + 9,13-DiMeC27; 13-,15-MeC29 + 13,15-DiMeC29; 2-MeC25; 3-MeC27; 11-,13-,15-MeC28; 2-MeC26; n-C27.

C. formosanus cuticular hydrocarbons were found not to vary significantly between colonies as had been reported previously (6,7). However, some differences were observed in non-cuticular hydrocarbon peaks, between colonies showing a high degree of aggression (unpublished results). Between castes, only the soldier showed a difference in the GC-MS profile, with long chain alkyl amines being observed in addition to the regular hydrocarbons (not shown). Free fatty acids were observed from some samples of workers and may have a seasonality associated with their detection.

Dodecatrienol

(*Z,Z,E*)-3,6,8-dodecatrien-1-ol (*Z,Z,E*-DTE) has been shown to be the trail-following pheromone used by many termite species (8,9,10) including *C. formosanus* (11,12). This pheromone is produced in the sternal gland, which is located on the 5th abdominal sternite. Workers lay a trail of this chemical from a food source back to the rest of the colony to recruit workers and mark the path to the food source.

Besides being used by workers, in some species DTE is also found in alates where it is instead used as a sex pheromone (13,14). *Reticulitermes spp.* exhibit a calling behavior, where the female raises its abdomen to expose the sternal gland and release the sex pheromone (15,16). However, *C. formosanus* alates do not show this calling behavior (17).

Therefore, we examined the levels of DTE from alates of the three species of subterranean termites in the New Orleans area, *C. formosanus*, *R. virginicus*, and *R. flavipes*, as shown in Figure 4. Amounts shown are based on a standard curve of DTE and internal standard, 8,10-dodecadienol. The results

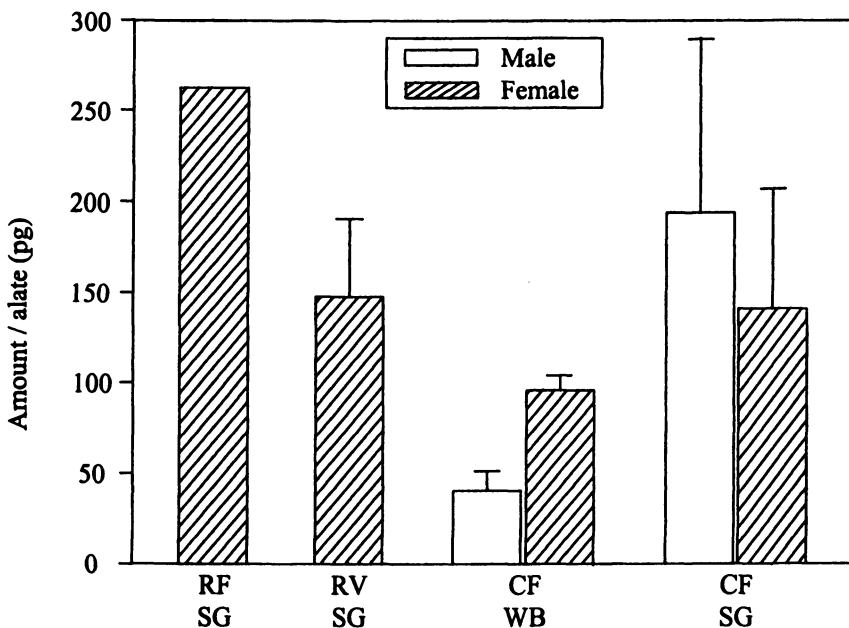


Figure 4. DTE amounts from three subterranean termites species. RF=*R. flavipes*; RV=*R. virginicus*; CF=*C. formosanus*; SG=sternal gland extract; WB=whole body extract.

demonstrate *C. formosanus* alates contain DTE even though they do not show the calling behavior. The extraction of the intact whole body of *C. formosanus* showed a greater amount of DTE in the female than the male as might be expected in a species where the female produces a sex pheromone to attract a male. When only the *C. formosanus* sternal gland was extracted, the male had, statistically, equal amounts of DTE to the female. The difference between the results of the sternal gland and whole body extract may show the contents of the sternal gland are sequestered inside the male alate, not allowing efficient extraction. Or in the natural sense, the contents of the male sternal gland may not be readily released and thus mistakenly attract other males, if it indeed acts as a sex pheromone. The extracts of *R. flavipes* and *R. virginicus* female sternal glands were found to have a similar amount of DTE to that of *C. formosanus*. The detection of DTE from *R. flavipes* has not been reported, but instead tetradecyl propionate was reported as the sex pheromone in this species (16). Tetradecyl propionate was also detected in these samples in very small amounts (not shown).

The possible stereoisomeric differences of DTE were also examined to see if this was the method the three species used to segregate during trail following or swarming. Four diastereoisomeric standards of DTE, containing the Z-configuration at the 3-position, were separated by GC-MS with retention times as shown in Table II. When the DTE isolated from the three alate species, including both male and female of *C. formosanus*, were tested, all corresponded to the same stereoisomer, (ZZE)-DTE. No other stereoisomer was detected.

Table II. 3,6,8 Dodecatrien-1-ol (DTE) stereoisomer determination

<i>R_t</i> (min)	DTE Isomer	Termite Source
23.69	ZZE	RV, RF, CF male and female ^a
23.79	ZEZ	nd ^b
24.03	ZZZ	nd
24.14	ZEE	nd

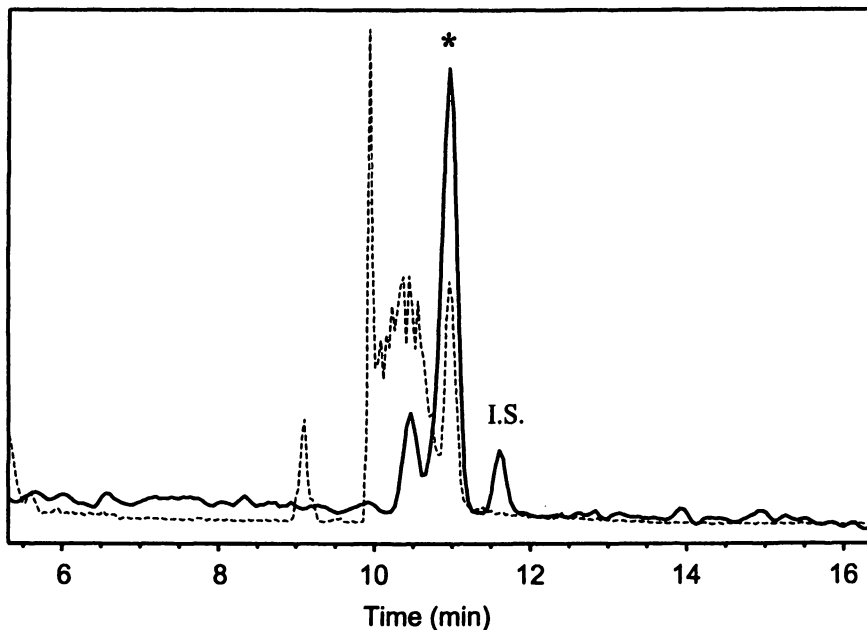
^a Species designations described in Figure 4. ^b Not detected.

Trilinoleoylglycerol

In addition to sternal glands, *C. formosanus* female alates also possess a pair of tergal glands located on the 9th and 10th tergites. This gland is specific to the female and chemically active only during the swarming and nest building period. We therefore examined the contents of the *C. formosanus* tergal gland (18).

Initially, a crude extract of the last several segments of the abdomen from both the female and male *C. formosanus* alate was examined. Using RP-HPLC-MS, a unique peak was detected from the female extract that was not present in the male extract. Silica HPLC-MS showed a similar unique compound from the female extract that could be characterized based on retention time (11.0 min, Figure 5) as a triacylglycerol.

When the excised tergal glands of the female alate were extracted, the predominant compound that was found corresponded to the unique peak found in the abdominal extract (Figure 5). The purity of this compound increased when the tergal gland was more cleanly isolated from extraneous fat bodies.



*Figure 5. Silica HPLC-MS of *C. formosanus* female alate abdominal tip (dashed line) and tergal gland (solid line) extracts. Female-specific peak denoted by *. I.S.=internal standard (trilinolenin).*

The mass spectrum of this peak showed a molecular weight of 879 with fragmentation peaks of 599 and 337. Of the possible triglycerides, only trilinoleoylglycerol (LLL) has the correct molecular weight of 879 with diacylglycerol and monoacylglycerol fragmentation masses of 599 and 337, respectively. Retention time and mass spectrum of authentic LLL (Sigma, St.

Louis, MO) was identical to the isolated compound. The amount of LLL in *C. formosanus* female alates was determined by LC-MS to be 845 ± 205 ng / alate based on LLL standard curves.

Bioassays did not show a positive association between tergal gland extract (or LLL standard) and tandeming behavior, the major behavioral trait observed for alates during the time period of the functional tergal gland. However, an electrophysiological response from the male maxillary palps showed recognition for LLL and tergal gland extract. Female maxillary palps did not detect either LLL or tergal gland extract showing a clear sex-specific response of a gustatory nature. The biological function of trilinolein is under further study.

Acknowledgements

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Chapter 8

Effect of the White Rot Fungus, *Phanaerochaete chrysosporium*, on the Feeding Preferences of the Formosan Subterranean Termite (Isoptera: Rhinotermitidae) for Different Wood Species

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Lignin-degrading fungi alter the chemical composition of wood. The effects of the white rot fungus, *P. chrysosporium* on the feeding preferences of Formosan subterranean termites for Alaska yellow cedar, birch, red oak, and redwood were examined when blocks were decayed for 3, 8, or 12 wk before feeding tests were conducted. There were significant differences in the weight loss of blocks of different wood species due to decay by the white rot fungus, *P. chrysosporium*. Alaska yellow cedar was completely resistant to colonization by *P. chrysosporium*. After 8 wk of fungal decay, there was no difference in the weight loss due to decay in birch and red oak, but weight loss in redwood was significantly less. After 12 wk, weight loss due to fungal decay was significantly greater in birch than in the other three wood species, and weight loss in red oak was significantly greater than in redwood. Wood consumption was greater on birch and red oak than on redwood or Alaska yellow cedar after 3 and 8 wk of decay. In the experiment where blocks were decayed for 12 wk before the feeding test was conducted, the average wood consumption of birch was greater than that of the other three wood species. Termite feeding behavior may have been positively affected by the rate of fungal decay. However, decay by *P. chrysosporium* did not appear to affect the relative feeding preference of Formosan subterranean termites for these four wood species.

Lignin-degrading fungi alter the chemical composition of wood. In some cases, subterranean termites show a feeding preference for decayed wood over sound wood (1). Termite responses to decayed wood depend not only on the species of fungus, but also on the fungal strain, type of wood, decay rate of wood, and the interaction between the type of wood and the decay rate of wood (2). Formosan subterranean termites show a strong preference for spruce *Picea* sp that has been decayed by the white rot fungus, *Phanerochaete chrysosporium* Burdsall, and a litter rot fungus, *Marasmiellus troyanus* (Murrill) Singer (3).

Previous research determined that the relative feeding preference of Formosan subterranean termites for different species of wood was affected by the presence of the fungus, *M. troyanus* (4). In 4-way choice tests, the relative preference of termites for redwood, *Sequoia sempervirens* (D. Don) Endl, compared with red oak, *Quercus rubra* L, birch, *Betula alleghaniensis* Britton, and Alaska yellow cedar, *Chamaecyparis nootkatensis* (D. Don) Spach, increased when the wood was inoculated with the fungus. Redwood and Alaska yellow cedar both have natural resistance to feeding by Formosan subterranean termites (5), whereas red oak and birch are preferred woods (6). These results indicated that the fungus caused chemical modifications of the wood that increased the palatability of the redwood to termites, possibly by breaking down allelochemicals in the redwood that acted as feeding deterrents. In this study, the effects of the white rot fungus, *P. chrysosporium* on the feeding preferences of Formosan subterranean termites for Alaska yellow cedar, birch, red oak, and redwood were examined when blocks were decayed for 3, 8, or 12 wk before feeding tests were conducted.

Materials and Methods

Collection and Maintenance of Termite Colonies and Fungus Cultures.

Formosan subterranean termites were collected from field colonies in New Orleans, LA, using underground bucket traps (7) baited with blocks of spruce wood (*Picea* spp). Termites were kept in the lab in 5.6-L covered plastic boxes containing moist sand and blocks of spruce wood until they were used in experiments. Termites from four colonies (C1, C6, C11, and C12) were used in these experiments.

An isolate of the white rot fungus, *P. chrysosporium* (ATCC #24775) was obtained from the American Type Culture Collection (ATCC, Manassas, VA).

Wood Species. The following types of commercial lumber were obtained from Riverside Lumber Company in New Orleans, LA: spruce, *Picea* sp, yellow birch, *Betula alleghaniensis* Britton, northern red oak, *Quercus rubra* L. redwood, *Sequoia sempervirens* (D. Don) and Alaska yellow cedar, *Chamaecyparis nootkatensis* (D. Don).

Inoculation of Wood Blocks with *P. chrysosporium*. Wood of each species was cut into small rectangular blocks that were 7 cm in length, 1 cm in width and 0.5 cm in height. Wood blocks were oven-dried at 90° C for 24 h and then weighed. Each block was soaked in water for 3 d, wrapped in two layers of aluminum foil with a moist paper towel, and autoclaved for 60 minutes on two consecutive days. After cooling, two blocks of the same wood species were placed side by side in a petri dish (100 mm by 25 mm) containing PDA (Potato Dextrose Agar) inoculated with the fungus. These dishes were placed in an unlit incubator set at a temperature of 25° C for either 3, 8, or 12 wk, depending on the experiment. Wood blocks were removed from the incubator, cleaned, oven-dried at 90° C for 24 h, and weighed to determine the weight loss caused by fungal decay. Weight loss due to fungal decay of wood blocks of Alaska yellow cedar, birch, red oak, and redwood after 3 and 8 wk fungal decay were compared in an experiment using 12 replicates of each wood species. Weight loss due to fungal decay of wood blocks of Alaska yellow cedar, birch, red oak, and redwood after 12 wk of fungal decay were compared in experiments using 24 replicates of each wood species.

Termite Bioassays. Multiple choice tests were conducted using wood blocks of birch, red oak, redwood and Alaska yellow cedar where blocks were decayed by *P. chrysosporium* for either 3, 8, or 12 wk. Bioassays were conducted using rectangular Rubbermaid™ storage containers (14.5 cm X 8.5 cm x 4 cm) (Consolidated Plastics, Twinsburg, Ohio). Each container served as a replicate and was filled with 100 g of sand (Standard Sand and Silica Company, Davenport, FL) moistened with 20 ml of water. Each container had four 2-cm diameter holes, one hole on each side. A 14 ml (17 x 100 mm) polystyrene round-bottom Falcon test tube (Becton Dickinson, Franklin Lakes, NJ) was inserted into each hole and sealed in place using a glue gun. The position of treatment tubes was alternated between replicates to preclude any positional effects. Two hundred termites (190 workers: 10 soldiers) were placed in the center of each container. The termites were able to move freely between the container and the tubes.

A wood block was placed in each tube. Each of the four tubes contained a block from a different wood species. These experiments lasted for 3 wk and at the end of each experiment, the number of termites in each container (replicate) was counted. Wood blocks were cleaned and oven-dried at 90° C for 24 h and then weighed. Weight loss due to termite feeding on decayed blocks was determined by comparing wood weights taken after blocks were decayed for 3, 8, or 12 wk with weights of wood blocks at the end of the feeding bioassays. For experiments where blocks were decayed for 3 or 8 wk, there were 12 replicates, with three replicates each from four termite colonies. For the experiment where blocks were decayed for 12 wk, there were 24 replicates, with six replicates each from four termite colonies.

Statistical Analysis. Differences in weight loss of blocks of different wood species due to fungal decay were compared using a one-way ANOVA where wood species was the factor. In the 4-way multiple choice feeding tests, data were analyzed using a two-way ANOVA where wood species and colony were factors. In cases where there was no significant interaction between wood species and colony and either factor was significant, differences were compared using a Tukey's honestly significant difference (HSD) test. Termite survival after 3 wk was determined. Proportional survival data were transformed by the arcsine of the square root. A one-way ANOVA on survival data was conducted to determine if there were significant colony differences in termite survival.

Results

Weight Loss Due to Fungal Decay. There were significant differences in the weight loss of blocks of different wood species due to decay by the white rot fungus, *P. chrysosporium* (Table I). Alaska yellow cedar was completely resistant to colonization by *P. chrysosporium*. In fact, there were no fungal hyphae of *P. chrysosporium* established on any of the Alaska yellow cedar blocks in any of the tests. After 3 wk of fungal decay, weight loss due to fungal decay was greatest in birch and equivalent in red oak and redwood (Table I). After 8 wk of fungal decay, there was no difference in the weight loss due to decay in birch and red oak, but weight loss in redwood was significantly less. After 12 wk, weight loss due to fungal decay was significantly greater in birch than in the other three wood species, and weight loss in red oak was significantly greater than in redwood (Table I).

Weight Loss Due to Termite Feeding Wood consumption was greater on birch and red oak than on redwood or Alaska yellow cedar after 3 wk (Fig 1) and 8 wk (Fig 2) of decay. In the experiment where blocks were decayed for 12 wk before the feeding test was conducted, there was a significant interaction between the two factors, wood species and colony (Wood Species: $F = 44.9$; $df = 3, 80$; $P = 0.0001$. Colony: $F = 3.9$; $df = 3, 80$; $P = 0.01$. Wood Species*Colony: $F = 4.4$; $df = 9, 80$; $P = 0.001$). Therefore, the two factors could not be analyzed separately. However, the average wood consumption of birch was greater than that of the other three wood species overall (Fig 3a) and for each of the four termite colonies (Fig 3b).

Discussion

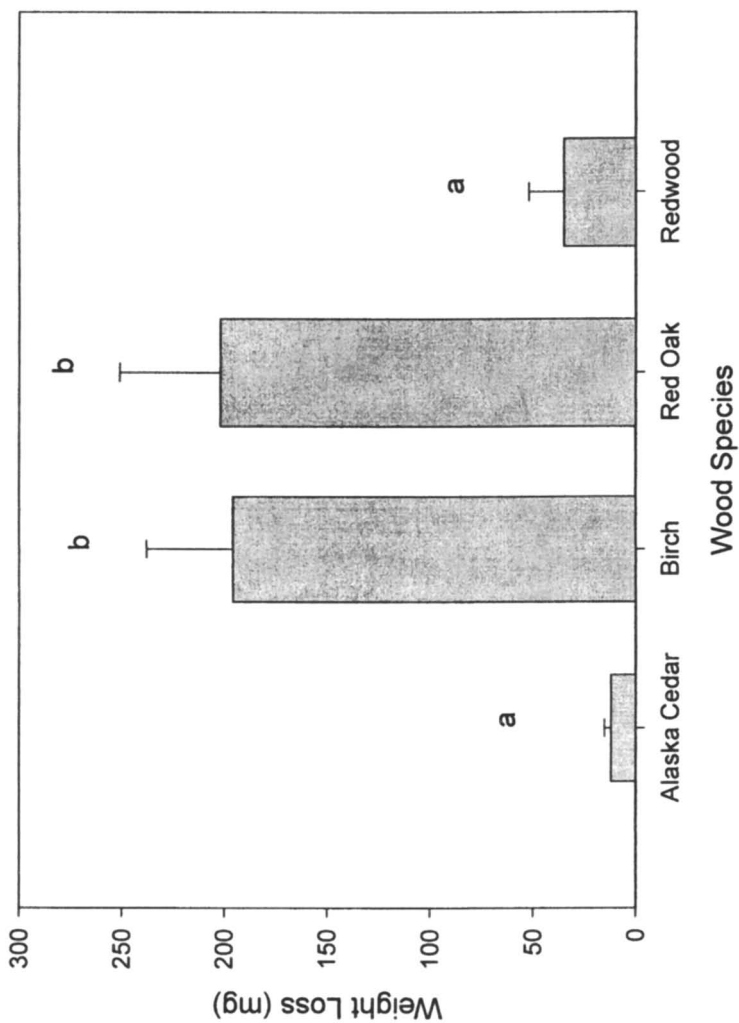
Termite behavior towards decayed wood is affected by the interaction of fungal species and wood species. The results of this study indicate that termite feeding

Table I. Mean (\pm SEM) weight loss (g) of wood blocks from four wood species exposed to *P. chrysosporium* ATCC #24775 .

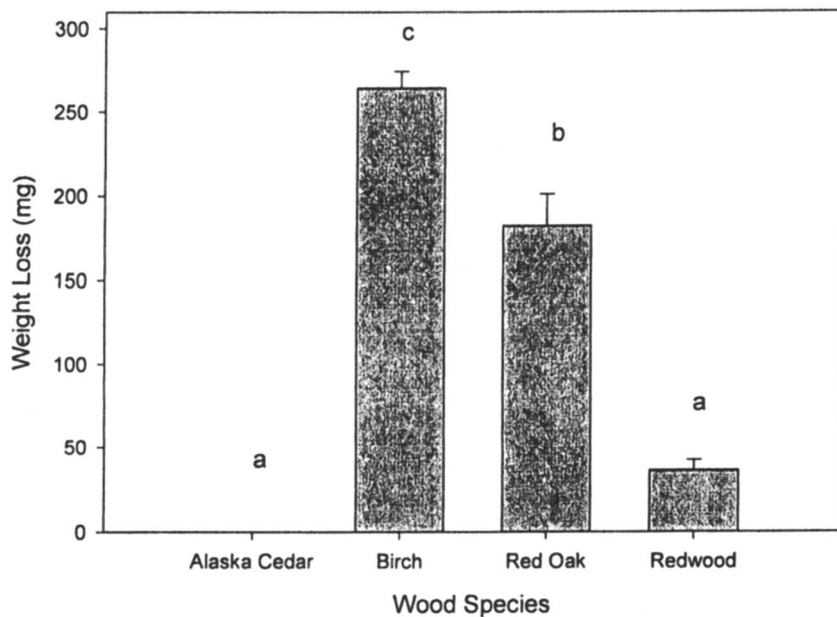
<i>Wood Species</i>	<i>Weight Loss of Wood Blocks (mg)</i>
<i>Experiment 1 (3 wk fungal decay)</i>	
Alaska Yellow Cedar	0.0 \pm 0.0a
Birch	83.0 \pm 12.0c
Red Oak	30.0 \pm 7.0b
Redwood	52.0 \pm 4.0b
<i>Experiment 2 (8 wk fungal decay)</i>	
Alaska Yellow Cedar	0.0 \pm 0.0a
Birch	236.0 \pm 18.0b
Red Oak	193.0 \pm 21.0b
Redwood	36.0 \pm 5.0a
<i>Experiment 3 (12 wk fungal decay)</i>	
Alaska Yellow Cedar	0.0 \pm 0.0a
Birch	330.0 \pm 37.0c
Red Oak	143.0 \pm 9.0b
Redwood	32.0 \pm 12.0a

Means followed by the same letters, within an experiment, are not statistically different, Tukey HSD ($P = 0.05$).

Experiment 1: $F = 24.3$; $df = 3, 44$; $P < 0.0001$. Experiment 2: $F = 68.8$; $df = 3, 44$; $P < 0.001$. Experiment 3: $F = 59.4$; $df = 3, 92$; $P < 0.001$.



*Fig 1. Mean (\pm SEM) weight loss of blocks after exposure to termites for 3 wk in a 4-way multiple choice test when blocks were previously decayed by *P. chrysosporium* for 3 wk.*



*Fig 2. Mean (\pm SEM) weight loss of blocks after exposure to termites for 3 wk in a 4-way multiple choice test when blocks were previously decayed by *P. chrysosporium* for 8 wk.*

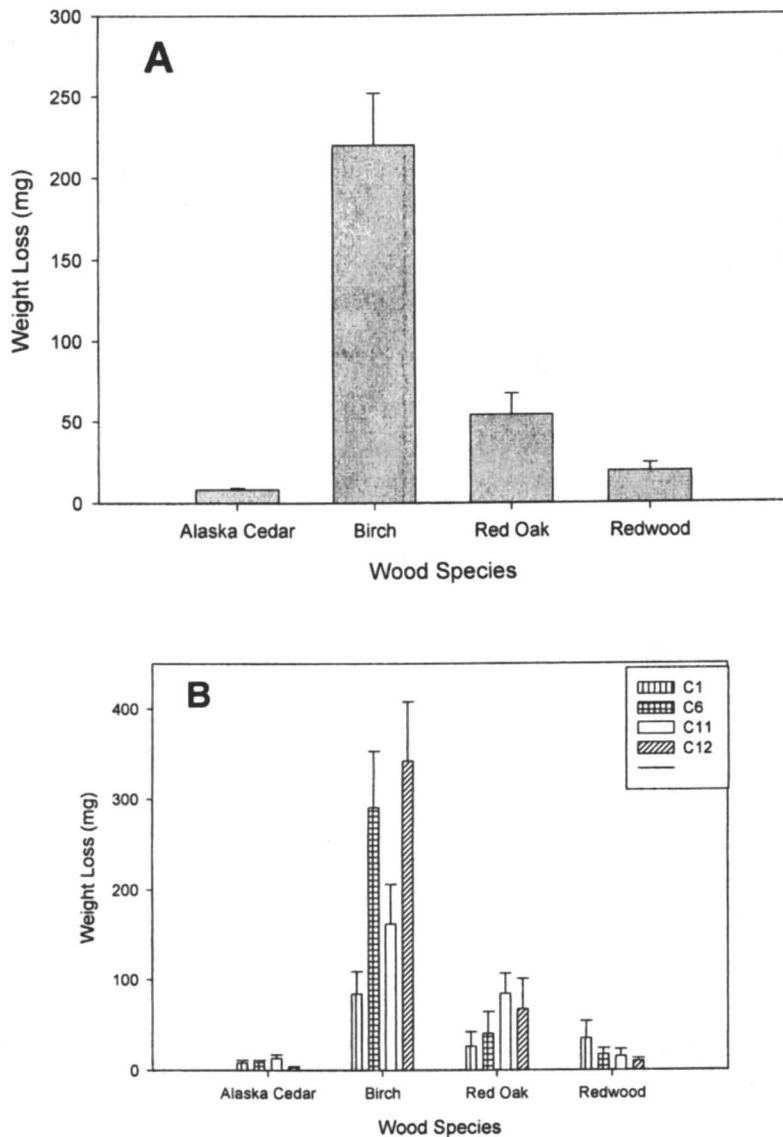


Fig 3. Mean (\pm SEM) weight loss of blocks after exposure to termites for 3 wk in a 4-way multiple choice test when blocks were previously decayed by *P. chrysosporium* for 12 wk. (A) total mean weight loss of blocks. (B) Mean weight loss of blocks for each termite colony.

behavior may have been positively affected by the rate of fungal decay. However, decay by *P. chrysosporium* did not appear to affect the relative feeding preference of Formosan subterranean termites for these four wood species. In contrast, in a previous study, the relative preference of redwood increased when blocks were decayed by *M. troyanus* (4).

The rate of decay by the fungus and the wood consumption of termites on decayed blocks was greater on red oak and birch than on redwood and Alaska yellow cedar. Weight loss due to decay by *P. chrysosporium* was greater on birch than on red oak after 12 wk of decay. Average wood consumption by termites on blocks decayed for 12 wk was also greater on birch than on red oak, indicating that termite feeding behavior may have been positively affected by the rate of fungal decay. Because wood consumption by termites is greater on red oak and birch than on redwood and Alaska yellow cedar in the absence of fungal decay (4), it is difficult to determine whether or not feeding preferences of termites for these four wood species were influenced by decay by *P. chrysosporium*.

In previous research, Formosan subterranean termites showed a significant preference for spruce sawdust inoculated with either *M. troyanus* or *P. chrysosporium* over sawdust without fungus. However, there were differences in how termites responded to spruce blocks decayed by the two fungal species when fungus-inoculated and control blocks were kept in an incubator in a 24 h dark cycle for either 2, 4, 8, or 12 wk before bioassays were conducted. In these experiments, termites showed a significant preference for decayed blocks over control blocks when blocks had been decayed by *P. chrysosporium* for 12 wk, and when blocks had been decayed by *M. troyanus* for only 2 wk. Although termite feeding behavior was affected by *M. troyanus* after only 2 wk of decay, the weight loss of spruce blocks due to decay by *P. chrysosporium* was significantly greater than weight loss due to decay by *M. troyanus* over a 12-week period (8). These results indicated that termites were responding to either differences in the chemicals produced by the fungi or to differences in how the two fungi affected the chemical composition of the wood, rather than merely responding to the rate of decay of the wood by the two fungal species.

There are differences in the process of delignification used by different types of wood decay fungi. White rot fungi, such as *P. chrysosporium*, have enzymes that selectively remove lignin, leaving large concentrations of cellulose intact, whereas brown rot fungi degrade all of the wood carbohydrates, including cellulose (9). The litter rot fungus, *M. troyanus*, contains enzymes that are characteristic of both brown rot and white rot fungi, and has been used in bioremediation projects because it has enzymes that can break down toxic compounds, such as benzene (10). The differences in the processes of decay used by *M. troyanus* and *P. chrysosporium* may explain why there are differences in the behavior of termites towards blocks decayed by these fungi. Termite feeding preferences are presumably affected by changes in the chemical composition of the wood, as well as the rate of decay of the wood.

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Chapter 9

Spectral Considerations of Natural and Laboratory-Simulated Sunlight for Degradation Studies

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A need exists in many disciplines to study and evaluate the effects of sunlight upon various materials. Unfortunately, terrestrial solar radiation is not a constant nor is it always practical to use natural sunlight. Therefore, the use of artificially reproduced solar radiation has been in use at least since ca. 1916 when carbon arc lighting was first used to systematically induce the light fading of dyed textiles. Since that time significant improvements in producing artificial sunlight in the laboratory have been made. This paper will explore some of these technologies and the necessary considerations regarding their proper utilization for chemical degradation studies.

We first need to understand the characteristics of solar radiation, specifically the sunlight that reaches the earth's surface. The electromagnetic radiation emitted by the sun contains a wide range of wavelengths including ionizing radiation such as gamma and X-rays and non-ionizing radiation which includes the ultraviolet (UV), visible (Vis) and infrared (IR). Fortunately, ionizing radiation does not significantly penetrate the atmosphere.

Effects of Atmosphere

The atmosphere absorbs, reflects and scatters solar radiation by numerous mechanisms which are often wavelength dependent. Ultraviolet radiation is in the wavelength range of 100 – 400nm although the stratospheric ozone layer effectively attenuates wavelengths below approximately 300nm (peaking at 250nm but with some absorption out to 350nm). Selective scattering of wavelengths is caused by smoke, fumes, haze and gas molecules the same size or

smaller than the incident wavelength. Scattering is inversely proportional to wavelength and is most efficient for shorter wavelengths. Tropospheric moisture is primarily responsible for Rayleigh light scattering of UV and short wavelength visible light, giving our sky its characteristic blue color when scattering is sufficiently high; when the atmospheric path length is shorter, e.g. solar noon, the sky appears more white due to minimal short wavelength scattering. Conversely, the reddish appearance of the solar disk at sunrise and sunset is due to blue light scattering from the long atmospheric path length. Larger atmospheric particulates and gases scatter longer wavelength visible radiation (Mie scattering). Clouds, dust, and weather conditions can also attenuate UV and visible light.

Non-selective scattering is caused by dust, fog and clouds with particle sizes greater than 10 times the wavelength of the incident light. This scattering is not wavelength dependent; hence clouds appear "white". Because of their high surface reflectivity (albedo), even thin clouds reflect 20-80% of the incident radiation back into space; absorption by even thick clouds is less than 10%.

The total solar radiation incident at ground level consists of both direct and indirect (scattered, diffused or reflected) energy and is called "global radiation"; the UV component comprises only about 6.8% of the total incident radiation at sea level under cloudless conditions. The intensity of sunlight at ground level varies with distance from the equator (latitude), geographic location, atmospheric conditions (moisture, gases and particulates), local albedo, season, cloud cover, altitude, solar zenith angle and target angle of incidence. UV is significantly higher at elevation due to decreased atmospheric path length.

The 23.5° tilt of the earth's axis affects the solar angle of incidence and atmospheric path length and causes seasonal and latitudinal variations in day length. Earth's elliptical solar orbit contributes about 6.6% to variations in radiant energy. Solar sunspot and flare cycles contribute another 1% to variability.

World Distribution of Solar Radiation

Solar radiation has an uneven geographic and chronological distribution throughout the world due to solar azimuth, which varies with latitude and season, elevation and atmospheric conditions including cloud cover and pollution.

The highest levels of solar radiation are primarily found in the belt of 15-35° latitude (North and South). This region averages >3,000 hrs/yr of sunshine and limited cloud coverage. Over 90% of the radiation is direct beam.

The second highest levels are found in the equatorial belt of 0-15° latitude. This high atmospheric humidity and cloudiness results in higher amounts of scattered radiation, particularly in the UV. As seasonal variations in this belt are

limited, solar intensity is distributed relatively uniformly; sunshine is >2,500 hrs/yr.

In the temperate latitudes of 35-45°, scattering of solar radiation increases significantly due to the longer atmospheric path length resulting from latitude and lower solar azimuth. Clouds and atmospheric pollution and particulates result in decreased incident radiation. Above 45° latitude almost half of the incident solar radiation is diffuse scattered sky and not direct beam.

“Standardized” Solar Radiation

The irradiance of the sun falling on the outer atmosphere (Air Mass = 0) when the sun and earth are spaced at a mean distance of 1 Astronomical Unit (AU), 149,597.890km, is called the “solar constant”; currently accepted values range from ca. 1353 – 1367 W/m² over the entire UV/Visible/IR spectrum. A solar simulator delivering this irradiance level is referred to as “one sun”. When optically filtered to deliver the spectral irradiance after passing through an Air Mass of 1.0 (solar noon at the equator) the equivalent irradiance is also often referred to as “one sun”. Some simulators deliver energy over only a portion of the spectrum but may also be referred to in the same way over the delivered wavelength range.

There are several governmental, academic and commercial solar radiation monitoring networks in place but there is no single coordinated global system. Several measurement devices such as broad or narrow band filter photometers as well as spectroradiometers of varying resolution are employed. Commercial worldwide weathering exposure sites typically employ a variety of instruments that measure both direct beam and “all sly” global radiation in the ultraviolet and “total” broadband (UV/Vis/IR) at various exposure angles ranging from near-horizontal to vertical.

Since actual terrestrial global and direct radiation are affected by so many variables, “standard” spectra have been developed which are based on the theoretical air mass attenuation affects on the solar constant. Those developed by the Committee Internationale d’Eclairage (CIE) and ASTM International are most widely used. These are relatively low-resolution approximations and while suitable for general simulation such as visible appearance and infrared heating effects are often inadequate and inappropriate for use in photochemistry, particularly in the UV range, and fail to account for the non-homogeneous distribution of terrestrial solar radiation previously described.

For photobiology and photochemistry studies the match of the simulators’ spectral power distribution (SPD) to terrestrial solar radiation, particularly in the UV, is critically important.

Photochemistry

There are four generalized routes of photochemical degradation:

- Bond dissociation
- Reaction with other species
- Isomerization
- Ionization

These groups are further subdivided into various reactions including photooxidation, hydroperoxide formation and conversion, Norrish Type 1 & II and other carbonyl and carboxyl group reactions, photo-Fries rearrangement, photosubstitution, photoaddition, photoelimination, photodimerization, photocondensation, and others. This paper will not elucidate these mechanisms except to note issues regarding spectral irradiance.

The UV spectrum is further subdivided into three ranges:

- UV-A 315 – 400nm
- UV-B 280 – 315nm
- UV-C <280nm

Wavelengths below about 300nm are severely attenuated by the atmosphere and the UV band comprises only about 6.8% of total terrestrial solar energy. The IR comprises about 40% with the visible comprising the remainder.

According to Planck, the energy in a photon is inversely proportional to wavelength. As a result, low wavelength UV photon energy is critically important in photodegradation as the amount of energy absorbed by a molecule must exceed the bond energy to cause degradation. Bond photodissociation occurs when the molecule is able to absorb radiation of specific wavelength, the source of radiation has the specific wavelength of radiation, and the amount of energy in the absorbed photon is higher than the energy of bond dissociation.

The implication for solar simulation is that the closer the artificial source spectral power distribution is to terrestrial solar radiation the more predictive will be the simulation. Radiation sources that spectrally differ from sunlight, particularly in high energy low wavelength UV, may alter the photochemistry from the normal pathway(s). It should be noted that longer wavelengths often also play a role in photochemical mechanisms in both the species of interest and other reactants in the environment, and that many compounds have more than one chromophore. Additionally, reaction products and intermediates may shift the absorption bands resulting in secondary photoreactive pathways. Alternate mechanisms such as charge transfer complex reactions can also affect spectral photosensitivity. In simulation, a test that does not represent actual full spectrum terrestrial sunlight is a different test than the environmental exposure and may lead to different photochemical pathways. Spectral aging characteristics of

lamps, filters and irradiance control systems should be measured and monitored. The use of calibrated spectroradiometers is highly recommended to verify the performance of the simulator.

Solar Simulators

Solar simulators usually consist of a light source and spectral output modifying filters. The light intensity at the exposure plane (irradiance) may be adjusted through lamp power, the source to specimen distance, attenuating filters or a combination of these.

Deuterium light sources may be used in UV-only simulators but are difficult to optically filter. Several commercial solar simulators based on either short or long arc xenon gas discharge lamps are available. The unfiltered xenon arc source provides an excess of low wavelength UV and IR requiring the use of spectral attenuation filters to more closely match the target spectrum of sunlight. These simulators generally are designed to deliver one of the "standardized" AM0, AM1, AM1.5 or AM2 solar spectra. While suitable for general simulation studies, such as broadband photobiology or photovoltaic studies they lack sufficient match to actual terrestrial solar radiation, particularly in the critical low wavelength UV.

Xenon arc systems

The use of optically filtered high power long arc xenon discharge lamps was pioneered in the 1950's specifically for material degradation studies. Almost five decades later these systems have undergone considerable improvements in spectral output through optical filtering and lamp control technology and are widely used in various artificial weathering and environmental exposure devices. A primary characteristic of these devices is that the optical filtering systems are designed to reproduce, as closely as possible, true measured solar radiation, particularly in the UV and visible, and validated through materials degradation correlation studies with the outdoor environment.

Two forms of high power long arc xenon discharge lamps are commonly used based on lamp cooling technology. These low pressure lamps typically range from 1500 – 15,000 Watts of power making them suitable for irradiating large surface areas or at larger source to specimen distances. The arc temperature can approach 1,000°C and is contained in a sealed quartz envelope. The lamp envelope and spectral filters are cooled by either high volume air flow or a water heat exchange system. A variety of spectral filters have been developed to match actual outdoor sunlight and are typically used in

combination to modify the lamp output in various spectral regions; filters can be flat (Figure 1) or tubular (Figure 2) depending on the system, and additional filters can be employed for specific simulations.

An issue with all xenon lamps is the aging characteristic of the lamp and filter systems. As the xenon arc produces intense UV down to 190nm, the quartz envelope itself undergoes a photodegradation process in which the quartz solarizes and undertakes a cloudy, milky appearance which disproportionately attenuates the UV output. In addition, vaporized electrode material can redeposit on the inside of the envelope decreasing overall light output. As a result, these systems usually employ a light monitoring system of various design which controls power to the lamp to maintain a constant, controlled irradiance level. However, in time, the UV levels cannot be maintained and either filters, lamps or both need replacing (lamp life is typically 1,000 – 4,000 hours depending on type and spectral output). Scientific-grade lamps use purer grades of quartz with fewer impurities than do commercial utility grades.

One undesirable characteristic of all xenon lamps is that they emit large amounts of IR which tend to increase as additional power is supplied to aging lamps to maintain constant UV levels. This may lead to elevated specimen exposure temperatures which could affect rate kinetics, and the use of IR filtering or excess specimen heat removal may be required. Common filtering techniques include circulating water, organic and metallized IR absorbing coatings and hot mirrors.

An advantage of xenon lamps is that power output can be varied over a fairly wide range without altering the spectral power distribution balance. Work continues in advancing optical filtering technology, including the use of gaseous ozone filter systems, to better reproduce solar radiation at all wavelengths.

The electrical power technology that drives the discharge lamps can also have an effect on photochemistry that may not be readily apparent. Drivers that deliver a constant RMS (root mean square) power to the lamp are typically better solar simulators than are pulsed sources which cause the lamp to deliver short high energy spikes which may excite species or exceed activation energies resulting in different photochemistries, even through the total power delivered over a given timeframe by the drivers is the same.

Metal halide systems

Specialty gas discharge short arc sources based on mixed metal and rare earth salts for solar simulation, usually referred to as metal halide - global, (MHG) lamps, are also used in solar simulation. One advantage is the high electrical power to light conversion efficiency making them well suited for very large scale illumination, especially when employed in arrays. These lamps also

Xenochrome 300 Filter Compared to Sunlight

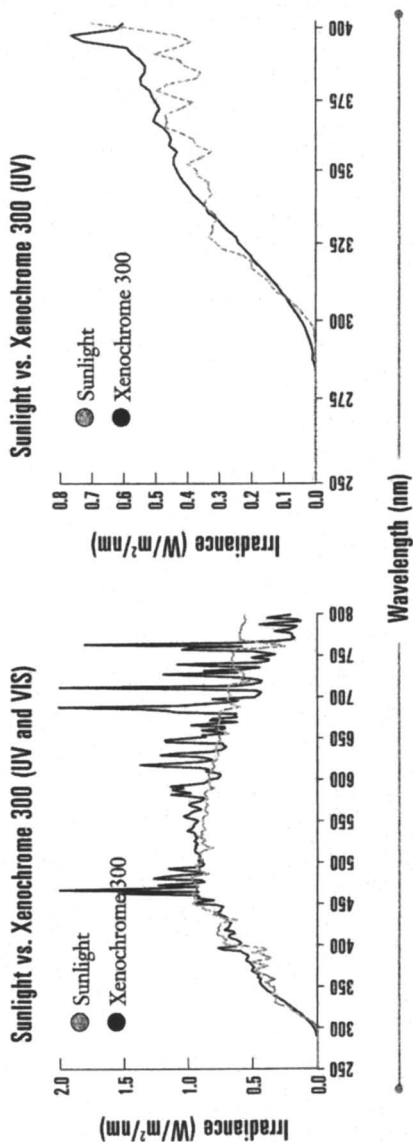


Figure 1. Comparison of air cooled xenon lamp and optical filters to terrestrial sunlight.

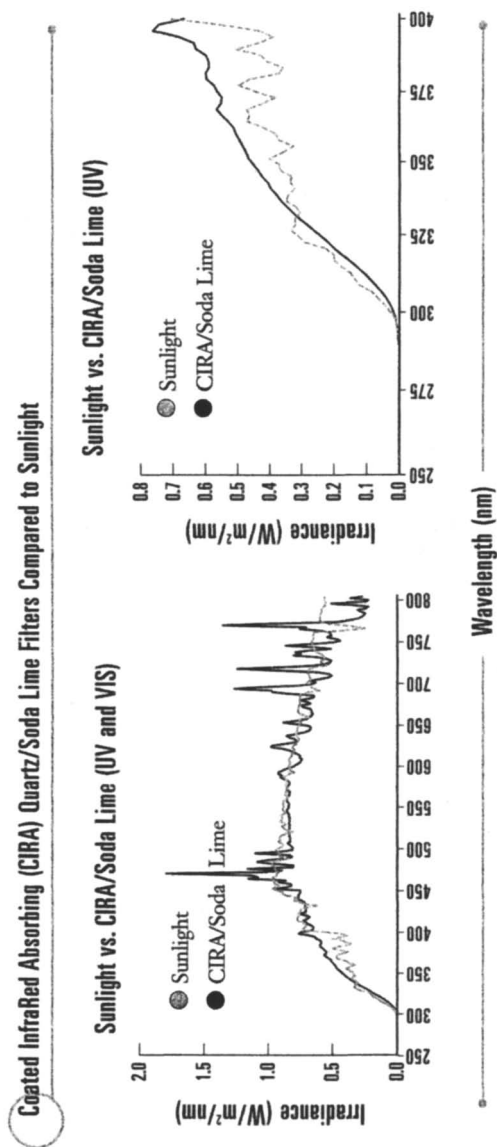


Figure 2. Comparison of water cooled xenon lamp and optical filters to terrestrial sunlight.

require optical filtering (Figure 3) but the large filter sizes required and their limited variety and cost make them unattractive for exacting photochemistry studies.

Unlike xenon, MHG lamps can be operated over only a very narrow power range without seriously affecting the spectral power distribution balance but are often preferred for accurate solar thermal loading studies. The irradiance level is changed by altering the lamp to exposure plane distance. MHG lamps are differentiated from more common commercial MH lamps in that the mixture of salts is formulated for specific spectral output. MHG lamps also require some additional optical filtering to remove excess low wavelength UV and attenuate excess IR.

Fluorescent lamp systems

Systems based on tubular fluorescent lamps have also been developed. Two lamps, the UV-B with a spectral output centered on 313nm and the UV-A with a reasonably good match to sunlight below ca. 340nm are available. The UV-B lamp contains wavelengths well below the spectral cut-on of terrestrial sunlight, has an excess of low wavelength UV, little visible and no IR (Figure 4).

Exposures to the UV-B source may seriously distort photochemistry. The UV-A lamp has been useful in materials durability work where only short wavelength UV-A has been shown to be important, but neither the UV-B nor UV-A lamps are typically used for photochemistry studies. These sources are relatively inexpensive but are not scientific light sources, exhibit considerable lot-to-lot variability and irradiance non-uniformity across their length and have pronounced aging and temperature dependence.

Specimen containers

For laboratory photodegradation studies specimens may need to be contained in bottles or covered dishes. The use of quartz vessels is recommended as laboratory glass or plastic ware have distinct UV absorption properties which can affect the simulation.

Irradiance & quantum yield

During photoradiation, several processes may occur simultaneously, including fluorescence, chemical reaction, intersystem crossing and internal

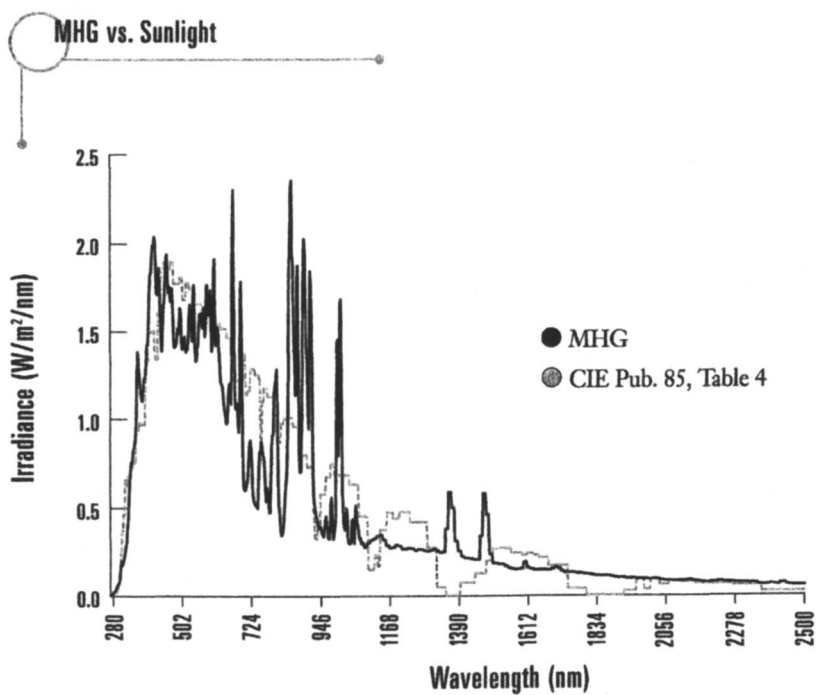


Figure 3. Comparison of Metal Halide Global lamp versus terrestrial sunlight.

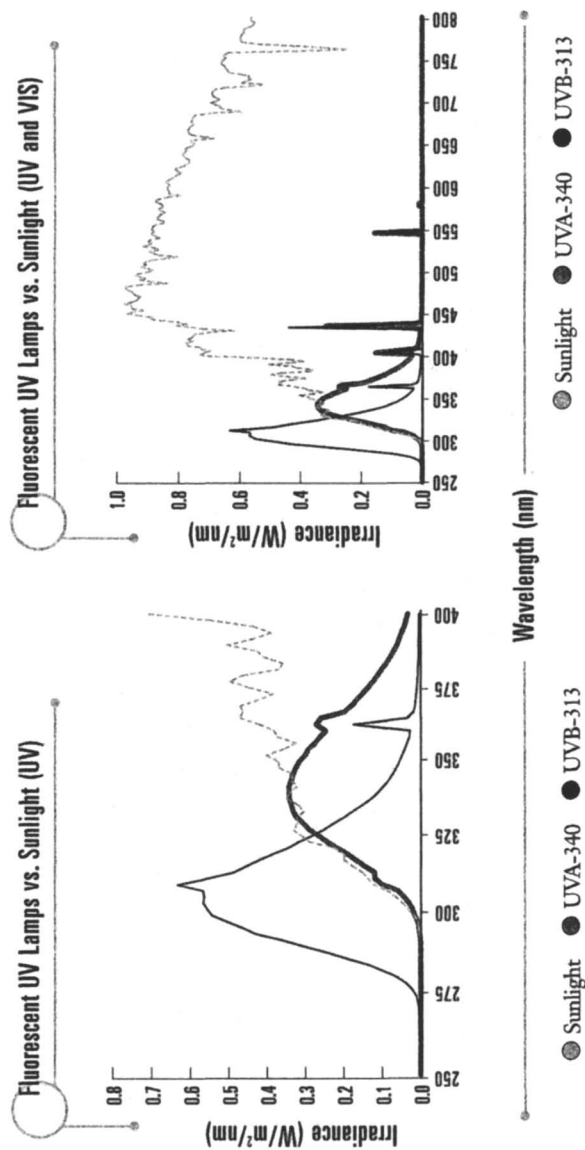


Figure 4. Comparison of fluorescent UV lamps vs. terrestrial sunlight.

conversion. Equations describing the kinetics must be written to account for each change separately:

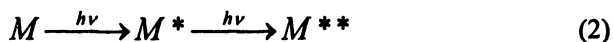
$$\frac{d[P]}{dt} = I_a - k[S] \quad (1)$$

where:

$[P]$	concentration of product
t	time
I_a	absorption of radiation
k	reaction rate constant
$[S]$	concentration of substrate

Photochemical processes are usually complex multistage processes and kinetic equations must include all of these steps because reaction constants usually depend on the parameters that control the rate of the process at each step. In multistage excitation more than one photon is required to make the conversion.

In sequential, biphoton excitation, one photon is first absorbed to form an excited state; if, during the lifetime of the excited state, a new photon is delivered and absorbed, a new higher energy excited state is formed:



The first excited state M^* has a different structure and therefore requires a different energy to promote an electron to a higher excited state. Promotion to the first and second excited states requires different amounts of energy and consequentially different irradiation wavelength (photon energy) or irradiance.

In biphoton, simultaneous excitation, two photons are believed to be absorbed within or near the duration of the first interaction, seemingly simultaneously:



Again, Planck's Law and structure determine the required wavelength of absorption.

Finally, two absorbed photons this time by two different molecules which come into sufficiently close proximity produce either new product or fluoresce:



Multiphoton excitation processes are highly dependent not only on the lifetime of primary excitations but also on the radiation intensity. Increased irradiance increases the probability that multiphoton excitation will occur and cause changes in the degradation mechanism. Therefore it is always important to consider whether increased irradiance may change the original design and outcome of the photochemistry experiment. While high power simulators up to 70 times normal solar radiation values are sometimes used in research, most laboratory and commercial systems are in the range of 1X to 3X terrestrial values.

In general, simulation experiments should model solar radiation intensities and ideally, light/dark cycles if not true diurnal cycles. Especially in the case of free radical chemistries, the provision of dark cycles as opposed to continuous light may provide sufficient recovery time for free radical repopulation and termination reactions. Additionally, for oxidative reactions, dark cycles provide opportunity for oxygen permeation into sample matrices. Environmental exposure tests for organic materials generally show good correlation from average annual or peak solar maximum irradiances, particularly in the UV, to about six to eightfold increases. Irradiances of 10X and above frequently skew the degradation mechanisms and elevated irradiances should be used with appropriate caution.

Summary

Terrestrial solar radiation varies with latitude, season, elevation, cloud cover, atmospheric moisture, gases and particulates, solar and exposure azimuth and time of day. The ultraviolet content can vary proportionately in the direct and diffuse components of solar radiation. Solar simulation for photochemistry and photobiology needs to be particularly accurate in reproducing the UV portion of terrestrial sunlight, both in terms of spectral power distribution and irradiance. Several light sources are available but care needs to be taken in spectral filtering. The reproducibility and repeatability of controlled light sources offers significant advantages over the variable outdoor environment.

Chapter 10

Solar Radiation (UV) Protectants for Microbial Insecticides

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Due to public perception of chemical insecticides' adverse effects and environmental safety, the commercialization and use of highly active and competitive microbial insecticides have gradually increased in the US. However, some disadvantages of microbial insecticides include variable field performance, high production costs, short residual activity, and shelf life among others. Ultraviolet (UV) spectrum from solar radiation inactivates most microbial insecticides. In general, direct exposure of microbial insecticides to solar radiation negatively affects viability and infectivity, mainly due to DNA denaturation and/or generation of highly reactive oxygen radicals. Solar radiation, primarily in the ultraviolet (UV) portion (300 – 400 nm) causes both mutagenic and lethal changes in nucleic acids (1, 2). The main consequence of UV action is the formation of a dimer from adjacent pyrimidines on the same strand, which become unstable (may transfer most of its extra energy by collision to an adjacent molecule) and reactive (formation of new bonds). Thus, the alteration of the DNA blocks its replication, and causes the death of the microorganism (1). Therefore, solar radiation has been the responsible for the rapid loss of activity of *Bacillus thuringiensis*, baculoviruses, microsporidia, and fungi products among many crops (3-5) (Table 1). UV inactivation is especially true when microbial are sprayed on foliage (2). Using aerial application, more pathogens are exposed to UV (on top of leaf surface), whereas using ground application, much entomopathogens are deposited underside of leaves and are less exposed to UV (2, 6). Since UV radiation is always an important inactivating factor for entomopathogenic microorganisms in field, there are many research groups working to improve residual activity after application. The use of adjuvants or improved formulations can address UV inactivation. This review

will discuss the use of adjuvants and formulation ingredients that have been tested as solar radiation protectants for microbial insecticides (7). Also, different approaches for solar radiation protection have been taken by testing many formulation techniques such as spray drying, freeze drying, fluidized bed dryer, and pan granulation (7).

Table 1. Inactivation of microbial entomopathogens after 4 hours to simulated solar radiation exposure (4).

<i>Entomopathogen type</i>	<i>Name</i>	<i>Loss of activity (%)</i>
Bacteria	<i>Bacillus thuringiensis</i>	54
Fungi	<i>Nomurea rileyi</i>	87
Virus	<i>Entomopox virus</i>	82
Baculovirus	Nucleopolyhedrovirus	92
Baculovirus	<i>Cytoplasmic polyhedrovirus</i>	92
Microsporidia	<i>Vairimorpha necatrix</i>	96

Bacterial Insecticides: Effect of Solar Radiation and UV Protectants

Bioinsecticidal products that are applied in a manner similar to chemical insecticides, are more easily accepted by growers worldwide. The microbial control agent that has been commercialized and used most widely is *Bacillus thuringiensis* (Bt). Bt produces an insecticidal protein (crystal) toxin during sporulation. Both, spores and crystals, are responsible for the insecticidal activity. Bt does not multiply in the insect but causes a toxicosis. Specifically, and depending upon the insect, Bt crystals contain proteins that might cause pore formation in cells of midgut of insect larvae, which acts as a stomach poison. Bt is used similarly to chemicals, in an inundative application in field and forest. The production process (by fermentation) is rigidly controlled and is not expensive. Bt's acceptance is due to its activity against agriculturally and medically important insect pest species. Unfortunately, Bt-based insecticides are inactivated by solar radiation (8, 9). Some results indicate that the mechanisms of inactivation by UV radiation, includes the presence of chromophores that induce the tryptophan oxidation and destruction (9).

Adjuvants

Screening for commercially available sunlight screens to protect Bt against inactivation by sunlight, Morris (6) reported that Uvinul DS49® (sodium 2'2 – dihydroxy-4,4'-dimethoxy-5-sulfo), at 1% in combination with Erio Acid Red XB100® at 0.1% plus molasses, produced the higher residual activity values in field (Table 2). Laboratory assays showed that the combination of screens plus film forming surfactants PVP® and CMV®, were the most effective coating material and increased the half life of Bt spores from 0.16 to 3.34 hours after exposure to simulated UV radiation (Table 3). Some natural edible polymers are film-forming materials as well. For example, pregelatinized cornstarch, after being mixed in water, produces an insoluble film on a leaf surface upon drying (10). Using the retrogradation technique, other edible ingredients, such as potato starch and corn flour, can be gelatinized similarly to cornstarch to produce similar film-forming products (10) (Table 3).

Table 2. Phagostimulants, dyes and optical brighteners reported as solar protectants

<i>Ingredient</i>	<i>Entomopathogen</i>	<i>Used as</i>	<i>Cited by</i>
Molasses, Coax® and Gustol® ^a	<i>Bacillus thuringiensis</i> , HzSNPV	Adjuvant	(11, 58)
Molasses ^a	<i>Bacillus thuringiensis</i>	Adjuvant	(7)
Erio Acid Red XB100® + Uvinul DS49®	<i>Bacillus thuringiensis</i>	Adjuvant	(6)
Congo red	<i>Bacillus thuringiensis</i>	Granules	(10)
Malachite green ^b	<i>Bacillus thuringiensis</i>	Granules	(18)
Congo red	LdMNPV	Adjuvant	(60)
Congo red	HzSNPV	Adjuvant	(67)
Melanin	<i>Bacillus thuringiensis</i>	Adjuvant	(26)
Melanin	<i>Bacillus thuringiensis</i>	Expressed in Bt	(25)
Tinopal LPW ^c	<i>Beauveria bassiana</i>	Adjuvant	(38)
Optical brighteners	Baculoviruses	Adjuvant	(61, 66)

^a Phagostimulants

^b Formulated with nixtamalized corn flour

^c Optical brightener

For the microbial insecticides having a peroral mode of action (bacteria, viruses, and microsporidia), both phagostimulants and UV protectants can be used to increase efficacy and residual activity. Commercial adjuvants such as molasses, Coax® and Gustol® acted as phagostimulants and UV protectants to Bt (7, 11) (Table 2). The use of the pregelatinized cornstarch Mirasperse®, mixed with powdered sugar as an additive, improved rainfastness and residual activity (12).

Table 3. Film forming material reported as solar protectants for microbial agents.

<i>Ingredient</i>	<i>Entomopathogen</i>	<i>Used as</i>	<i>Cited by</i>
PVP® and CMV®	<i>Bacillus thuringiensis</i>	Adjuvant	(6)
Mirasperse® Cornstarch ^a	<i>Bacillus thuringiensis</i>	Adjuvant	(12)
Flours ^a	<i>Bacillus thuringiensis</i>	Adjuvant	(10)
Gluten ^a	<i>Bacillus thuringiensis</i>	Adjuvant	(13)
Polymer from Sandoz®	<i>Bacillus thuringiensis</i>	Adjuvant	(14)
Lignin ^b	Several microbial entomopathogens	Adjuvant	(23)
Clay emulsion	<i>Beauveria bassiana</i>	Adjuvant	(38)
Calcium alginate	<i>Beauveria bassiana</i>	Granules	(41)
Calcium minerals (CaF ₂) ^c	Baculovirus	Crystals	(69)
Kaolin	<i>Beauveria bassiana</i> <i>Metarhizium anisopliae</i>	Powder	(42)

^a Pregelatinized

^b Mixed with salts to induce cross-linking

^c Mixed with salts to induce heterocoagulation

Corn flour and pregelatinized cornstarch, in combination with congo red or activated carbon as UV-protectants and Coax was tested as phagostimulant on cabbage for diamond-back moth, *Plutella xylostella*, control with Bt (12). Pregelatinized corn flour, which is less expensive, compared with pregelatinized cornstarch, was effective in protecting Bt from solar radiation at a rate of 0.5% w/v (10) (Table 3).

Casein, gluten, and corn flour/sucrose were tested as adjuvants for Bt, at 0.5%, 1% and 2% w/v respectively, and the percentage of original activity remaining (OAR), after 8 h of artificial sunlight exposure, was 55, 86 and 99%, respectively (13) (Table 3).

Recently, Sandoz (Novartis) applied for a patent of a carrier intended for bacteria (such as Bt) or baculovirus (either nucleopolyhedroviruses, granuloviruses or non-occluded viruses). This granular formulation was made using a polymer and an organic agent as a solar protector. The polymer works as a matrix for the active ingredient and solar protectant (14) (Table 3). Final formulation, depending on the active ingredient, may contain 30-80% of the active ingredient, 1-15% of the polymer and 5-60% of the solar protectant agent. The formulation may have polymer and co-polymer combinations, which may contain preferably high weight molecules, such as styrene, malic anhydride copolymer and carboxy polyacrylic acid, with an adequate final pH between 5.5 to 7.0. Solar protectants might consist of a solar reflective agent (titanium dioxide) and a light absorbing agent (black carbon). Polymer properties makes it capable to retain the solar protectant, to be insoluble in water (spraying tank), but soluble in the midgut insect, where Bt and baculovirus are activated.

In addition, ingredients such as acriflavin, methyl green and rhodamine B's cationic chromophores provided good protection following UV- radiation exposure of Bt crystals. Acriflavin was the best solar protectant (15) (Table 4).

Table 4. Other organic compounds reported as solar protectants

<i>Ingredient</i>	<i>Entomopathogen</i>	<i>Used as</i>	<i>Cited by</i>
Acriflavin	<i>Bacillus thuringiensis</i>	Adjuvant	(15)
Paraffinic oils	<i>Metarhizium anisopliae</i>	Adjuvant	(37)
Emulsifiable oils	<i>Beauveria bassiana</i>	Adjuvant	(29)
Liquid-flowable material (Sandoz)	<i>HzSNPV</i>	Adjuvant	(50)
Antioxidant (Pr-gallate)	<i>HzSNPV</i>	Adjuvant	(62)
Enzyme (catalase)	<i>HzSNPV</i>	Adjuvant	(52)
Aminoacid (tryptophan)	<i>HzSNPV</i>	Adjuvant	(62)

Granules

Granular formulations are ideal for control of insect larvae that feed in the leaf axils, where granules remained. In general, natural polymers can be

formulated to produce a UV-protection matrix in which the active ingredient of microbial pesticides stays until contacted by the host. For many polymers such as starch, gluten, or pregelatinized flours, formulation processes involve a gelatinization to induce polymer retrogradation, in which the sugars of the polymer chains associate, giving an insoluble particle (16) (Table 5).

Table 5. Natural polymers reported as solar protectants

<i>Ingredient</i>	<i>Entomopathogen</i>	<i>Used as</i>	<i>Cited by</i>
Cornstarch ^a	<i>Bacillus thuringiensis</i>	Granules	(16, 17)
Cornstarch ^a	<i>HzNPV</i>	Granules	(67)
Cornstarch	<i>Beauveria bassiana</i> and <i>Metarhizium anisopliae</i>	Granules	(43)
Cornstarch	<i>Bacillus thuringiensis</i>	Powder	(20)
Flours ^a	<i>Bacillus thuringiensis</i>	Granules	(12, 18, 19)
Flours ^a	<i>SfMNPV</i>	Granules	(68)
Flours ^a	<i>Bacillus thuringiensis</i>	Powder	(20)
Gelatin, pectin, chitin ^b	<i>Bacillus thuringiensis</i>	Granules	(21)
Biopolymer	<i>Serratia entomophila</i>	Granules	(22)
Lignin ^c	<i>AfMNPV</i>	Powder	(70)
Lignin ^c	<i>Bacillus thuringiensis</i>	Powder	(24)

^a Pregelatinized

^b Insecticide-like substance

^c Mixed with a compound to induce cross-linking (23).

Using a mixture of congo red (as UV protectant) and Coax (as phagostimulant) into a starch matrix in granular-starch based formulation, Bt was evaluated in the field for control of *Ostrinia nubilalis* (European corn borer) larvae. In these test, granular formulated treated plots, significantly reduced tunneling due *O. nubilalis* (17). Results of testing *B. thuringiensis* granular formulations, made with nixtamalized corn flour and extract of *guishe* (a subproduct of *Agave lechuguilla* processing) for the control of *S. frugiperda* malachite green, showed the highest UV radiation protection over congo red and activated carbon (18) (Table 2).

The addition of sunscreen dyes to granular formulation does not always improve solar protection of Bt. Granular formulations without dyes, made with corn flour, cottonseed flour, and sugars, also protected Bt from solar radiation

inactivation (19) (Table 5). Similar results were reported with powder formulation, where malachite green did not improve the solar protection in corn flour microgranular formulations (20). Other natural polymers that have protected Bt from UV radiation, when tested as granules, were gelatin and pectin (21) (Table 5). By definition, polymers are molecules formed by chemically bonding multiple copies of one or more monomer molecules, either in a linear chain or in a branching pattern. As well as providing UV protection, gelatin and pectin also improved palatability to *T. ni* larvae (21).

Biopolymer (polymer formed of biological-produced monomers and enzymatic activity) matrix has been used for other bacteria for UV protection. *Serratia entomophila* can be formulated in biopolymer-based granules and applied directly to grass for New Zealand grass grub (*Costelytra zealandica*) control. *S. entomophila* persisted in soil at a level that caused infection of the grass grub population (10^4 UFC/g of soil) (22) (Table 5).

Powders

Since the required application volume is different among crops and application techniques, adjuvants may not be suitable for commercialization. Spray-dried formulation may improve compatibility with application techniques.

Bt formulations were tested using spray dried corn flour as matrix formulation with positive results (20) (Table 5). Spray dried lignin has also been used as matrix for microencapsulated formulations. The use of sodium or potassium lignate salts in solution, mixed with CaCl_2 , results in a polymer retrogradation as well, where the Ca^{++} form cross links with the lignin radicals and Cl^- precipitates when the ion is removed by the calcium (23) (Table 5). Microencapsulated formulations with lignin alone or lignin plus corn flour, showed a higher UV protection to Bt in laboratory and field tests, compared with flour-based formulations. In these tests, *Trichoplusia ni* (cabbage looper) neonates fed on treated cotton (for laboratory tests) or cabbage (field tests) (24). Microencapsulated lignin-based formulations also showed higher persistence after artificial rain (24) (Table 5).

Genetic engineering

In an attempt to improve the residual activity of Bt var. *kurstaki* after solar radiation, a mutant of var. *kurstaki* was generated by UV irradiation of the culture. The mutant strain produced a dark-brown pigment, which was characterized as melanin. The mutant strain was more resistant to UV light than the parent strain, and possessed greater insecticidal activity against *Plutella*

xylostella (diamondback moth) larvae (25). Similarly, the residual activity of Bt var. *israelensis*, applied for mosquito control, was enhanced by using melanin as a UV protectant (26) (Table 3).

Fungi Insecticides and Solar Radiation Protectants

The fungal insect pathogens *Beauveria bassiana*, *Metarhizium anisopliae* and *Paecilomyces farinosus* can be effective biocontrol agents. Temperature, relative humidity, and UV are important factors, because they can reduce the conidia stability and delay germination (27-29) (Table 4). Studies of environmental factors that most affect the survival of *Entomophaga grylli* conidia demonstrated that artificial solar radiation (xenon lamps, at several intensities, time and distance) was the most damaging conidia factor, compared with temperature and moisture (30). This study revealed that at low exposure of 3 Langleys (irradiance measure where $\text{mW/cm}^2 = 0.01433$ Langleys) decreased the conidia viability, but 20 Langleys reduced 100% viability. Exposed conidia showed loss of vacuoles, cell wall lysis (30). In 1995, Similarly, Smits *et al.* (31) testing the germination of *P. fumosoroseus* conidia after exposing to artificial UV-B and natural solar radiations, found that artificial UV-B was more detrimental compared with natural solar radiation. They discussed that the contribution of UV-B from natural solar radiation depended on many factors, including place and time of experiment, meteorological conditions, substrate slant or direction (31). *Metarhizium anisopliae* can be completely inactivated after a few hours exposure to simulated solar radiation. Modeling the lost of viability when *Paecilomyces fumosoroseus* conidia were exposed to UV-B showed a significant reduction after an average of 50 min exposure at 5000 J/m^2 (32). Braga *et al.* (33) determined the inactivation of three *M. anisopliae* strains after exposure to full-spectrum simulated sunlight, UV-A (320-400 nm), and UV-B (280-320 nm). The strains showed variation in tolerance to UV-A, UV-B, and full-spectrum sunlight, where UV-A had a higher negative effect on conidia stability compared with UV-B (32). Moreover, Braga *et al.* (33) observed that conidia in advanced germination phase were more sensitive to UV-B, compared with the dormant conidia, whereas UV-A was the most harmful to *M. anisopliae* conidia. When *P. fumosoroseus* conidia were tested for germination, survival, and infectivity toward *Spodoptera frugiperda* (armyworm) larvae after exposure to radiation, results showed that visible and near infrared radiation were less harmful than UV, and that UV-B was more detrimental than UV-A (34).

Pigmented conidia from different fungi were exposed to simulated sunlight, and it was shown that black conidia were significantly more stable than were light colored conidia (35). In addition, dry conidia were significantly more resistant to UV than were wetted conidia (35). In addition, differences in UV-B

susceptibility have been observed among *M. anisopliae* strains. Miller *et al.* (36) found a relationship among isozyme profiles for catalase-peroxidase, glutathione reductase, and superoxide dismutase and UV-B resistant strain level. They proposed that UV exposure may affect negatively the *M. anisopliae* growth due to the lack of ability to handle oxidative stress.

Adjuvants

B. bassiana conidia are hyaline and are rapidly inactivated by solar radiation (35). Though various oils have been used to inhibit conidial dehydration, and paraffinic oils shown to increase the residual activity of fungi after solar radiation exposure in laboratory and field tests (37) (Table 2). Alves *et al.* (29), tested emulsifiable oils at 10% V/V in water and found that different oils improved conidial tolerance to simulated UV radiation. Seven out of 10 oils tested significantly increased the conidial viability after 6 h exposure to simulated solar radiation (29) (Table 4).

The use of oil in fungal formulations has had a good acceptance. Moore *et al.* (37), testing UV stability of *M. flavoviridae* conidia, reported that oil formulations in combination with chemical solar blockers protected better form UV compared with water formulations, specially under dry conditions. Inglis *et al.* (38) reported that *B. bassiana* conidia in water, applied on coverslips or on *Agropyron cristatum* (wheatgrass) leaves, and exposed to UV-B radiation, lost more than 95% viability after 15 min exposure, but conidia in oil lost 74% viability after 60 min exposure (Table 2). In contrast, they found that in field, oil did not protect *B. bassiana* against UV-B light, which might have been the result of a poor spraying technique, or to oil absorption by leaves (38). The addition of two water-compatible ingredients, tested as adjuvants (an optical brightener, Tinopal LPW and a clay emulsion) significantly increased the conidia residual activity on wheatgrass leaves (38) (Table 3). Jenkins *et al.* (39) reported same biocontrol level of desert locust, *Schistocerca gregaria* using either water- or oil-based formulations with solar protectants with *M. flavoviridae*

Wright and Chandler (40) tested *B. bassiana* conidial formulations containing cotton products (Konsume®) as phagostimulants, a pheromone and Nufilm 17® as solar protectant, and stiker. They applied the formulation in field on non-cotton plants and compared the boll weevil attraction. They observed that non-cotton treated plants effectively attracted to boll weevil compared with the untreated control (94 vs 4). In addition, they applied the formulation on re-growth cotton plants and examined adults captured. They found a 60.1% of *B. bassiana* infected boll weevil in treated plots versus 44% on untreated control (40).

Sunlight blockers such as clays, starches or carbon may be preferable because they are less expensive, environmentally innocuous and/or biodegradable (20, 38).

The use of oil-based emulsions, containing lignin (23) and ZnO as solar protectants and glycerol to increase water activity of *B. bassiana* (27) improved its residual activity. After application on tomato in fields in these formulations provided greater UV protection as compared with a powder (dry conidia and diatomaceous earth) and a commercial *B. bassiana* emulsion (Mycotrol ES) against *Bemisia tabaci* (whitefly) (Behle *et al.*, unpublished data).

Granules

Different approaches have been developed to protect entomopathogenic fungi against damaging solar radiation under field conditions. Granular formulations are a good choice to provide protection for fungal inocula, especially for soil and plants with leaf axils where granules can remain. Matrix ingredients provide solar protection to different degrees. Calcium alginate-based granules with *B. bassiana* were reported for aphid control in cereals (41). Using the described retrogradation technique (16), *B. bassiana* and *M. anisopliae* were formulated in a biopolymer-based granule, and good results were obtained (more details on the nature of the results) (22, 42) (Table 5).

Inglis *et al.* (43) studied the efficacy of *B. bassiana* conidia against grasshopper nymphs (*Melanoplus sanguinipes*) among two formulations (oil and water) and two bait substrates (lettuce and bran). They observed higher mortality after ingested conidia in oil than in water, and no differences among the baits tested. Interestingly, they found that if nymphs were superficially sterilized before bait ingestion, a higher incidence (33-82%), showed faster onset of mycosis and more active conidia were recovered from feces. They conclude that the efficacy of the bait relies on the extent to which nymphs become surface-contaminated during ingestion (43). Similarly, Caudwell and Gatehouse (44) produced rigid, hard and highly expanded maize-starch extrudate contact baits, using starch extrusion technology, to evaluate the uptake of entomopathogen fungi by contact rather than ingestion by *S. gregaria*. Locust accepted cornstarch bait formulation, and that fungi infected by contact rather than ingestion (44). The fungi formulation efficacy improvement using peanut oil as phagostimulant was also demonstrated by Bextine and Thorvilson (45). They produced granular formulations as alginate pellets in an effort to increase the efficacy to control the fire ant *Sonolepsis invicta* in field. They mixed *B. bassiana* mycelia with sodium alginate, calcium gluconate, wheat bran and an orange UV-reflective dye, to produce a granule pellet. Treatments consisting of no pellet pellets with peanut oil coating or without oil, inserted into *S. invicta* mounds. Results

demonstrated that the addition of peanut oil reduced ant activity in 80% of the treated mounds, whereas no-oil formulation, resulting in an ant activity reduction in only 40% of the treated mounds (45). Other type of granulation, with *Trichoderma harzianum* to control the soilborn plant pathogens, and *B. bassiana* for insect control pest, were prepared using alginate pellets with wheat bran (46). Granules were prepared by mixing alginate and wheat bran with mycelia and conidia and allowed pellets for air drying. Before full drying, they soaked the pellets in 25% polyethylene glycol (PEG) solution for up to 24 h and allowed to continue drying. Production of *T. harzianum* conidia treated with PEG slowed down the first days but recovered in one week, and enhanced hyphal extension of the fungus in soil. In contrast, *B. bassiana* conidia development from PEG treated pellets was significantly more rapid, but did not generate extensive mycelial growth (46). Authors discuss that water availability might be the factor for this fungi growth and developing effects (46).

Powders

Kaolin-based powder formulations of *M. anisopliae* or *B. bassiana* were tested on soybean as biological control agents for soybean stink bugs, *Nezara viridula*, *Piezodorus guildinii*, and *Euschistus heros* in a field trial (41). The relative humidity was the main factor for occurrence and prevalence of both fungi on stink bug species, where *M. anisopliae* provided more effective control when compared with *B. bassiana* (41) (Table 3).

In general, blastosporas, produced by liquid fermentation, are more susceptible to environmental factors (high temperature, low humidity and solar radiation) compared with conidia. In an attempt to provide a protective coat to *B. bassiana* blastosporas, (47) tested clay-coating method. They found that the clay coated blastosporas survived better in the soil, compared with the uncoated blastosporas (47). Studdert and Kaya used the same technique, but using *B. bassiana* conidia, for the control of *Spodoptera exigua* (48). The use of clay coated conidia did not increased the *S. exigua* mortality but allowed the conidia to germinate, demonstrating that this technique can be used for formulation purposes (48). Alginate and cornstarch, in combination with dry mycelium of *M. anisopliae* and *B. bassiana* were used for formulations with or without oil, to improve survival and conidia production after sunlight exposure (42). Results demonstrated that despite fungi survived in cornstarch or cornstarch-oil formulations better than in alginate stored at 22°C, fungi in alginate formulations survived better than the others after exposure to artificial solar radiation using xenon lamps of 300 W (42).

The use of spray-dried formulation with *B. bassiana*, using lignin among other ingredients demonstrated that *B. bassiana* lost 50% viability after 180 min

artificial solar radiation exposure (xenon light), whereas unformulated conidia lost 50% of their activity after 60 min and 71% after 90 min exposures. Lignin-only formulation showed the highest insecticidal activity against *Epilachna varivestis* (Mexican bean beetle) and *T. ni* in cotton leaf disk bioassays (Behle *et al.*, unpublished data).

UV Protectants Reported for Viral Insecticides

Inactivation of baculoviruses due to solar radiation is an important limiting factor for commercialization. One of the first studies of the negative effect of solar radiation on nucleopolyhedroviruses (NPV) was conducted by Gudauskas and Canerday (49). They found that the NPV from *Helicoverpa zea* (*H_zSNPV*), was inactivated after 5 min, whereas the NPV from *T. ni* (*T_nSNPV*) was negatively affected after 5 min exposure of artificial solar radiation at 5.08 cm from the light source, using a Spectroline 2537-A Lamp. The lost of activity after exposure time at the same distance was 58% after 1 min, 90% after 2 min and 96% after 3-4 min. The lost of activity after 5 min exposure was 100% at 5.03 cm, 96% at 10.06 cm, 77% at 20.12 cm and 32% at 40.24 cm (49). In another study, direct exposure of viral bioinsecticides to simulated UV radiation (using fluorescent lamps which provided 1.5 mw/cm² at a peak of 365 nm) resulted in inactivation of a standard acetone-dried preparation of *H_zSNPV* diseased larvae mixed with 20% lactose after 4-h exposure) (50). UV radiation can interact with water to generate reactive oxygen species that damage virus. Samples of *H_zSNPV* were more sensitive to inactivation by artificial sunlight in an aqueous suspension than as a dry powder. This observation indicated that the presence of free water could adversely affect the virus activity after solar radiation exposure (51). Laboratory studies, testing different UV-light lamps to determine the inactivation level of NPVs from *H_zSNPV* and *S_eMNPV* (*Spodoptera exigua*) showed that, in general, higher inactivation occurred with exposure to UV-B radiation for both viruses (52). Moreover, *H_zSNPV* was more sensitive to UV radiation than was *S_eMNPV* (52, 53). The baculovirus occlusion body (OB) helps to maintain stability against the environmental factors. In laboratory tests, when samples of non-occluded and occluded *Anagrapha falcifera* nucleopolyhedrosis virus (*A_fMNPV*) were exposed to xenon light, non-occluded samples lost insecticidal activity faster than did samples of occluded virus (54). In field tests, *H_zSNPV* persistence was significantly affected by virus concentration (i.e., inactivation was inversely related to virus concentration) (52). To maintain persistence, insecticidal virus must be deposited in a habitat or microhabitat that has some protection from sunlight, which is the main limiting factor for NPV survival on exposed surfaces (55). After *Anticarsia gemmatilis* NPV (*A_gMNPV*) was sprayed on the upper

surfaces of soybean leaves, it lost 60% or more activity within two days, whereas *AgMNPV* that was sprayed on the underside of the foliage lost 13% or less activity in the same period of time (56). Solar radiation was one of the most important factors affecting epizootics of *S. frugiperda* NPV (*SfMNPV*) in corn (57).

Adjuvants

Commercial adjuvants were tested for *HzSNPV* activity improvement and Coax® and Gustol® did not provide UV protection for the *HzSNPV* baculovirus (58) (Table 2). They compared inactivation of a standard acetone-dried preparation of *HzSNPV* diseased larvae mixed with 20% lactose (IMC-01); a commercial wettable powder (SAN-240-WP, Sandoz), and a water-based formulation (SAN-2450-WDC, Sandoz). They added a liquid-flowable material from Sandoz-Wander Inc., in the final spray-tank, at 10% V/V. San-240-WP formulation without adjuvant protected *HzSNPV* activity similarly than the formulations with adjuvants, after 4 h exposure to simulated solar radiation (98.4, 113.1 and 108.8 % OAR, respectively; whereas IMC-01 and no-adjuvant SAN-240-WDC lost significantly activity (9.1 and 39.0 % OAR, respectively). The use of a liquid-flowable material enhanced the *HzSNPV* activity 2-11 times after artificial sunlight exposure (50) (Table 4).

Shapiro and Robertson (59, 60) evaluated 79 dyes as solar radiation protectants for gypsy moth, *Lymantria dispar* nucleopolyhedrovirus (*LdMNPV*). Virus was mixed with the dye and exposed 60 min to a set of two 275-W, high intensity, mercury sun lamps with built in aluminium reflectors. Samples were placed at a vertical distance of 50 cm from the light bulb (intensity). Congo red was the only dye tested that fully retain *LdMNPV* activity after artificial solar radiation exposure. Solar protectants absorbed at 280-320 nm (UV-B), as well as 320-400 nm (UV-A) (Table 3).

Highly reactive oxygen radicals can form when UV interacts with water leading to inactivation of microbial insecticides (61). Antioxidants and oxidative enzymes can prevent or minimize the generation of radicals. Three antioxidants; Pr-gallate, ascorbic acid, and phenylthiocarbamide, acted as UV protectants to *HzSNPV*. Pr-gallate (at 0.2 mg/mL) was the most effective antioxidant tested (Table 4) (62). Similarly, catalase provided significantly greater UV protection than superoxide dismutase or peroxidase (62) (Table 4). Among several aromatic amino acids tested, tryptophan provided significantly greater protection to *HzSNPV* from solar radiation, and also acted as a UV-B absorber (63) (Table 4).

Whereas optical brighteners have been demonstrated to provide UV protection to other microbial entomopathogens (Table 3) (64, 65), their costs are

a limiting factor for application to crops with low profit margins. In addition, several optical brighteners are viral enhancers for specific entomopathogenic virus. Insecticidal activity was more than 1000-fold higher for *LdMNPV* and *SfMNPV*, when tested against *L. dispar* and *S. frugiperda* (64- 66). Fluorescent brightener 28 (Calcofluor M2R or Tinopal LPW) protect *LdMNPV* and *AfMNPV*, but only enhanced activity of *LdMNPV* against *L. dispar* (66).

Granules

Starch-granular formulations have also been tested as baculovirus solar radiation protectants. For example, four different UV protectants were tested in starch-encapsulated formulations with *HzSNPV*. Two UV protectants, congo red and activated carbon (Type RB), were the best in protecting baculovirus from artificial UV exposure (almost 60% activity after exposure versus 37% and 32% for Tinopal CBS and Shade®, respectively (67) (Table 3). In contrast, malachite green provided greater UV radiation protection than congo red in granular formulations made with nixtamalized corn flour (18) (Table 3). Field tests against *Spodoptera frugiperda* in corn using *SfMNPV* formulated with nixtamalized corn flour demonstrated that persistence of viral infection was greater among treatments with granular application than those with aqueous spray application (68) (Table 5).

Powders

Minerals milled as fine or ultra-fine particles can be used as UV protectants since some of them are not adversely affected by ionization and free radical formation caused by UV-A and UV-B. Fine crystals of CaF_2 form an irreversible coating of the occlusion body (OB) by heterocoagulation, whereas atapulgit showed a lower coating degree. Both minerals provided UV-protection when used as baculovirus coatings (69) (Table 2).

Microencapsulation was first used for *HzSNPV* formulation as wettable powders using different ingredients and a spray dry processing. Spray dried formulations maintained residual activity after artificial and natural solar radiation exposure of the *HzSNPV* (50). Low cost ingredients, such as lignin, corn flour, or sugar could be components in commercial formulation for baculovirus (24, 70) (Table 5). A lignin-based spray-dried formulation, using a CaCl_2 for lignin cross-linking, was used as matrix material for spray drying (60). In laboratory tests, *AfMNPV* retained full activity after 8-h artificial or 4-h natural solar exposure, in a lignin plus pregelatinized corn flour-based formulation (70). Similarly, field tests demonstrated that lignin-based spray-

dried formulations extended the residual activity of *AcMNPV* and *AjMNPV* by protecting the virus from inactivation by sunlight, in several field tests at different US-locations. Results demonstrated that all formulations maintained more than the 50% of original activity after 24-h exposure, whereas unformulated virus did not (71) (Table 5).

Conclusion

Since the 1980's, studies have demonstrated the adverse effect of solar (UV) radiation upon the effectiveness of microbial insecticides. Microorganisms' residual activity in field can be improved with selected solar protectants. To determine the treatment strategies necessary to protect microbial insecticides, many chemicals and natural ingredients have been tested as adjuvants or as matrices in a formulation. The selection of appropriate UV protectants will contribute to efficacious microbial insecticides with higher persistence in field, compared with unformulated products. By increasing their reliability as microbial insecticides, grower acceptance will increase, and used in combination with phytosanitary techniques, will be useful and compatible to integrated pest management programs.

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Chapter 11

Analysis of Chemical Mixture Experiments: A Statistical Brew

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Chemical mixture experiments have distinct characteristics that can preclude the use of traditional statistical analysis techniques. Mixture experimentation often poses unique exploratory questions that can be answered efficiently and economically with non-traditional statistical techniques. General statistical guidelines stress the importance of design, preliminary studies, action levels of variables, graphics, and appropriate statistical testing. Fractional factorial and Simplex designs are just two of many statistical tools that are useful for analyses of mixture experiments.

Introduction

Traditional confirmatory statistical methods are not always the best option for analysis of chemical mixture experiments. A mixture experiment is defined as one where the response is dependent upon the relative proportion of ingredients comprising the mixture rather than the absolute amount of different ingredients that influence a response (*I*). Mixture analysis data can't be analyzed using conventional statistical techniques. Traditional methods assume independent results for each ingredient, an assumption which is violated by the use of relative mixtures.

Mixture experiments pose a unique set of exploratory questions that can be

answered efficiently and optimally with non-traditional statistical designs. Often, questions are asked such as: what is the best combination of ingredients for a product, what are the range of optimal concentrations of those combined ingredients, which are the most important components driving a reaction, and at which concentration of an ingredient does further benefit of a response cease?

Statistical Guidelines for Conducting Mixture Experiments

Having a good experimental design before conducting research and gathering data is essential for optimizing experimental resources and obtaining top-quality results. One unique aspect of mixture experiments is that there can be large numbers of variables being simultaneously examined, along with all of the interactions of these variables. To keep focused on an experimental question, it is recommended to define an "Area of Interest" for the dependent response, or measured variable, as well as the independent controlled, or treatment, variables. Finding action levels and sufficient ranges of variables that encompass the response effects of interest will save valuable experimental time and dollars. Action levels are educated estimates or best guesses of the numeric values of the range of the independent variables that have an effect on the response variable. Surrounding the action level with a sufficient range, both lower and upper, ensures that the response variable effects of interest are taken into account. Time and effort is saved by not running experiments with levels of ingredients that are impractical.

Operating Characteristic Curve

In hypothesis testing experiments, one wants a high probability of avoiding both false positive and false negative conclusions, i.e. Type I and Type II errors (2, 3). A Type I error occurs when a true null hypothesis is rejected, that is, we assume two treatments are different when they really are the same. Most experimenters select an alpha level that controls against excessive Type I errors. A Type II error occurs when a false null hypothesis is accepted, that is, we assume two treatments are the same when they really are different. An experiment without sufficient replication risks being unable to discern treatment differences which really exist, increasing the probability of Type II error. Use of an Operating Characteristic Curve (Figure 1) can be used to determine the minimum replication necessary for testing treatment effects as well as for determining which ranges of an independent variable should be examined in order to focus on a particular area of interest while controlling for Type II errors

(4, 5). The Y-axis is the probability that a measured response from a sample will be falsely accepted as true (Type II error).

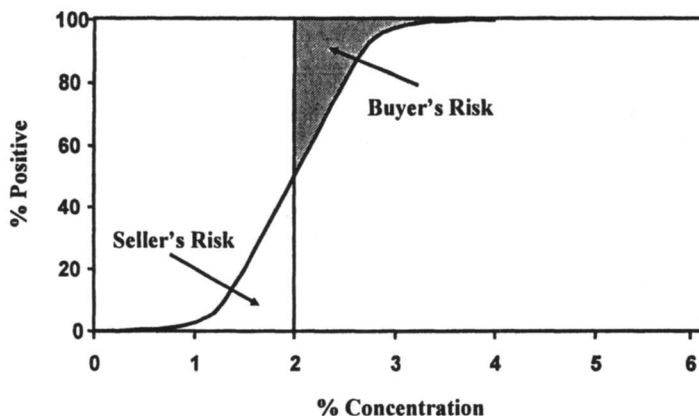


Figure 1. Operating Characteristic Curve.

The buyer's risk area (denoted by diagonal line fill) indicates that for concentrations greater than 2%, there is a higher probability of assuming no treatment effect differences when they really do exist (Type II error). A buyer's risk is the probability of accepting a bad lot. The seller's risk area (denoted by dot fill) indicates that for concentrations between 1 and 2%, there is a probability of assuming unequal treatment effects when they really are the same (Type I error). A seller's risk is the probability of rejecting a good lot.

Graphing and Curve-Fitting

After one obtains preliminary data, it is always a good idea to graph the results. Using explanatory graphs with interpretable parameters gives credence to the saying that one picture is worth 10^3 words.

Some hypothetical data is plotted in Figure 2 where X represents a concentration value (ppb) of a single ingredient in a mixture and Y is a measured response.

One can fit a variety of curves to the data, so which is the correct one to use? Let us examine two curves as shown in Figures 3 and 4. The first is a quadratic response curve of the form $Y = a + b \cdot X + c \cdot X^2$.

The second is a logistic dose response curve of the form $Y = a + b/[1 + (X/c)^d]$.

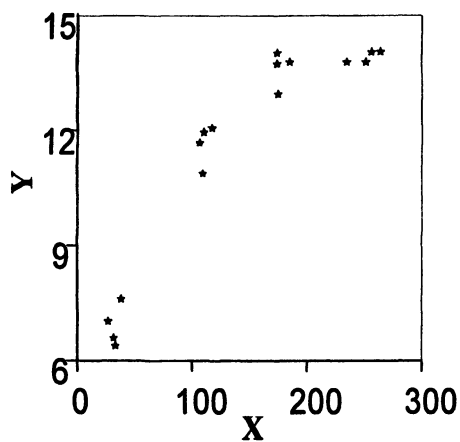


Figure 2. Plot of hypothetical concentration data (X) against a response (Y).

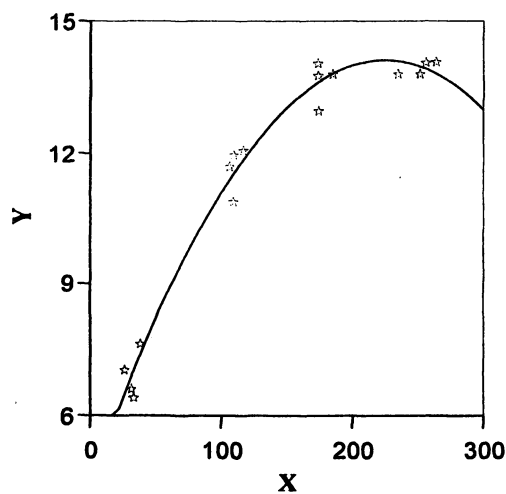


Figure 3. Quadratic response curve fit to hypothetical data.

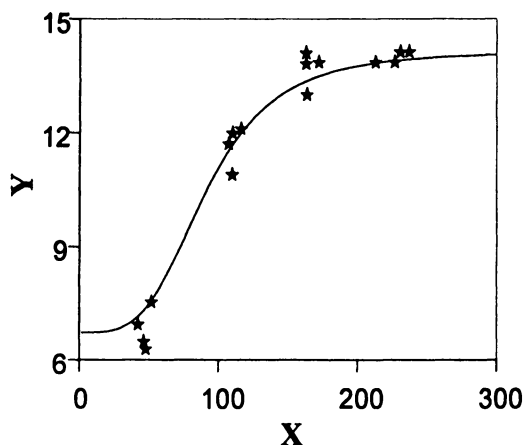


Figure 4. Logistic growth curve fit to hypothetical data.

Both curves fit the hypothetical data equally well, as measured by R^2 values, and one may be inclined to choose the less complex quadratic over the logistic model. The quadratic curve seems simpler than the logistic, but the parameters (a, b, and c) are not all biologically interpretable. Notice that the quadratic curve declines rapidly for X values greater than 300, which is probably unrealistic. Care must be taken when extrapolating curves beyond the range of your data. The logistic curve does have biologically interpretable parameters (a, b, c, and d). For both curves, the a-parameter represents the initial level of Y when no treatment has been applied (i.e. the concentration level of an ingredient is zero). The b and c parameters of the quadratic have no particular interpretation. For the logistic curve: the b-parameter is the asymptote of Y minus the initial level a, the c-parameter is the X value at the inflection point (i.e. the concentration at $Y = a + b/2$), and the d-parameter is indicative of the slope at the inflection point ($d = [4*c/b]*\text{slope at } c$). The parameters of the logistic function represent meaningful biological results that may be of interest to the experimenter (6).

A good rule of thumb in experimentation is that no more than 20 percent of one's resources, including time, should be spent on a first experiment. If an experimental study is unknowingly examining a local vs. a global phenomenon, or fails to find any treatment differences, one is more efficient if a preliminary study had been performed.

Two Traditional Statistical Approaches

Analysis of Variance

Analysis of variance (ANOVA) is used in a hypothesis testing experimental approach where means of discrete or categorical treatment variables are compared to determine if overall treatment effects are equal (3). ANOVA is not designed for analysis of continuous variables, such as concentrations or for determining where differences between variables might be occurring. ANOVA is a confirmatory analysis that answers the question: Over a set of discrete treatments, are there differences between those treatments, or do they all have the same mean effect? One can examine differently behaving treatments that have the same mean values while differing markedly in their scale of responses to a set of stimuli.

Use of ANOVA techniques for determining ingredient differences becomes time consuming and costly when more than 3 independent variables (ingredients) are examined. Obtaining an adequate number of replicates, satisfying homogeneity of variance assumptions, proper handling of subsamples and pseudo replicates, and using full-factorial, balanced models are some of the stumbling blocks inherent in this conventional analysis technique for mixture experiments. Higher order interactions in factorial ANOVAs are difficult to interpret and shed no light on the form of the relationship between factors.

Regression

Regression techniques are more useful in examining mixture experimentation results in that the functional form of a relationship between a response variable and a set of continuous variables, such as concentration through time, can be examined. Regression analysis does not take a hypothesis testing approach, but is more of an exploratory method. It is visually informative in determining how a set of independent variables (treatments/ingredients) relates to a measured response (dependent variable). In order to obtain a useful equation that is representative of the relationship between a response variable and a set of controlled, continuous variables, one must ensure that sufficient data is collected and sampled over the range of interest of the experiment.

While more information about the functional relationship between continuous variables can be obtained from regression techniques than from ANOVA, there are some pitfalls associated with this analytic approach. One must always determine if an obtained equation is representative of the raw data

as well as determine if the raw data is representative of the underlying experimental relationship. Local maxima and minima effects can mask a larger, global association.

Proportional mixtures, treatments that sum to 100% and multicollinearity are all issues that are unique to mixture experiments and pose problems for traditional regression techniques (1, 3). If multicollinearity exists (treatments are not independent), one has data which is correlated with each other, as in a mixture experiment. This presents problems for regression analysis because one cannot vary one ingredient without varying the relative proportion of the other ingredients that sum to 100 percent of the product. The result is imprecise standardized regression coefficients and a resultant equation that does not adequately represent the underlying product composition response (3).

Alternatives to Traditional Statistical Approaches

Fractional Factorial ANOVA

When examining a large number of factors, a fractional factorial design is a good alternative to traditional factorial ANOVAs for exploratory experiments. Like traditional ANOVA, a fractional factorial uses hypothesis testing on means but in an exploratory way. When there are lots of variables of interest but insufficient replication, and no interest in all possible variable interactions (including higher order interactions), this design is similar to a preliminary study. A fractional factorial can be used to screen for those variables/ingredients that have impact on the response variable under study.

As an example, 4 components (independent variables) will be examined at 3 levels each in an experiment in media optimization for lactic acid production. The components are an ammonia nitrogen source, a phosphate source, sugar, and yeast. The goal is to obtain an optimum level combination of the 4 factor constituents in a mixture for lactic acid production. An examination of 4 factors at 3 levels each would dictate 3^4 , or 81, possible combinations. A traditional, full factorial ANOVA would require at least 3 replications from different batches of the mixture constituents at each level. Furthermore, there are equipment, lab personnel, and time limitations in performing the experiment. They are only able to run a maximum of 20 sample combinations per day in their laboratory. The researchers would like to use this experiment for determining a subset of the components that are influential in lactic acid production, and to ascertain the most effective concentrations of these components. They are not very interested in the 3 or 4 way interactions of the components, and would only like to examine some of the possible 2 way interactions. The researchers decide to perform a

fractional factorial design that incorporates component main effects and only some of the 2 way interactions. To further reduce complexity, they decide to use minimum and maximum known concentration values of the components instead of the 3 levels originally planned. The researchers have narrowed their area of interest of the 4 factors from 3 to 2 levels each for a total of 2^4 , or 16, possible combinations. They are able to obtain their replications from 3 different batches of product combinations with 3 days of actual laboratory work.

The researchers have found some startling results from their fractional factorial analysis. The range they were using for phosphate level was too high and could be scaled back by half. The sugar by phosphate 2 way interaction was the driving force in lactic acid production whereas levels of nitrogen and yeast were not as important. They can now hone their experiment and run a new, more refined model within the new area of interest. Since the sugar by phosphate interaction was discovered to be important to the lactic acid mixture, a new study with 3 levels each of sugar and phosphate can be conducted as a full factorial design. A fractional factorial design is generally used as a preliminary, refining study, to be followed by more in-depth experimentation.

The Simplex Method

The Simplex method is a nontraditional alternative to regression. It is used for experiments that are concerned with proportions of components where the ingredients sum to 100 percent (I). Regression is not a viable analysis alternative for this situation since least-squares derived regression coefficients will be imprecise and unstable. Multicollinearity problems occur due to related, overlapping independent variables (the component proportions).

Proportion mixture experiments usually have an end product such as a recipe for food, or a pesticide or herbicide. The product can be composed of chemicals or foods and spices. Properties of interest such as efficacy and stability over time, or optimal ingredient concentration, are measured. All component ingredients must sum to 100 percent and the only items that are varied are the product ingredient proportions. The end product is not dependent on the amount of the mixture.

A Simplex region is a constrained spatial area of interest for all of the variable components in the mixture. Proportions mixtures always have at least two basic constraints (I):

- $0 \leq X_i \leq 1$ for $i=1, 2, \dots, n$ where n =number of ingredients in the mixture
- $\sum X_i = 1$ for all i from 1 to n

Figure 5 is an example of a constrained Simplex space for two ingredients, X1 and X2, in a mixture.

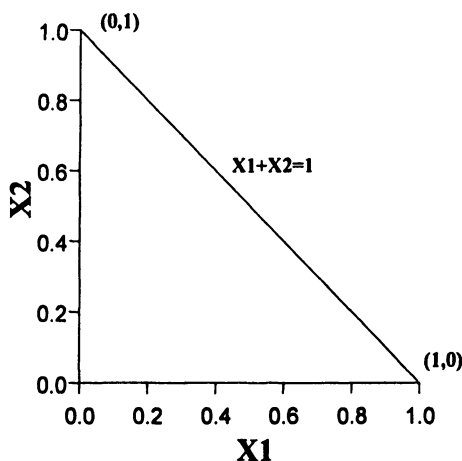


Figure 5. Simplex space for 2 ingredients with 2 minimal constraints.

Model Choices and Adequacy

Like regression, the choice of a model for approximating the surface over the Simplex region is a consideration. Model designs should be representative of the sampling scheme, fit the raw data, and adequately reflect the underlying relationship among the variables. For ease of interpretation and parsimony, it is best to try and fit lower-order models first, such as the 1st degree model $Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \dots + \beta_n X_n$, instead of a more complicated, higher-order model like the full quadratic in 2 variables 2nd degree model $Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_1^2 + \beta_4 X_2^2 + \beta_5 X_1 X_2$. Least-square methods for linear relationships have the advantage of easy to obtain coefficient of determination, or R^2 , values, residuals are easy to examine, and the software usually produces parameter variance estimates. Nonlinear methods, including those for logistic models, are useful for binary responses or count data. Error terms for parameter variance estimates are based on a maximum likelihood approach and it is more difficult to obtain curve fit statistics.

Additional constraints in a Simplex method further reduce the area of interest from a full Simplex space. The constraints come from additional information about the possible range of the ingredients. For example, some new information obtained from a fractional factorial design with two ingredients showed that the optimal concentration of an ingredient X_1 is 3%. We can restrict the Simplex space with the constraint: $0.01 \leq X_1 \leq 0.05$. Since the sum of the ingredients totals 100% of the mixture, a consistent range for X_2 would be: $0.95 \leq X_2 \leq 0.99$.

Experimental Considerations

There are many experimental considerations that go into the planning of mixture experiments. Statistical analyses should be just as carefully planned to ensure the best use of lab time and to obtain the most accurate research results. Conventional statistical analysis is not always appropriate, economical, efficient, nor optimal for analyzing mixture experiments. Alternative analyses are available that are more suitable for addressing research objectives, especially if traditional analyses present assumptions that the researcher cannot satisfy.

Fractional factorial designs and the Simplex method are two examples that display the availability and existence of alternative statistical analyses that are especially suited for analyzing chemical mixture experiments. Statistics is not a static science and one way in which new methodology arises is for statisticians to work with experimentalists to discover what is needed. A close association with a statistician during the planning stage of research can aid in determining the best experimental designs and analyses for individual studies.

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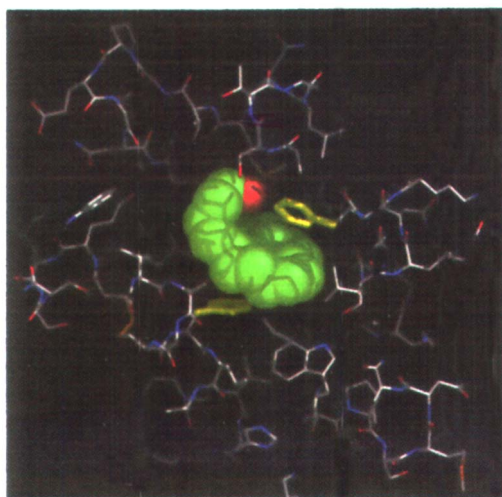


Plate 4.3. Structure of BmorPBP-bombykol complex highlighting bombykol in the binding cavity sandwiched by two phenyl alanine residues.

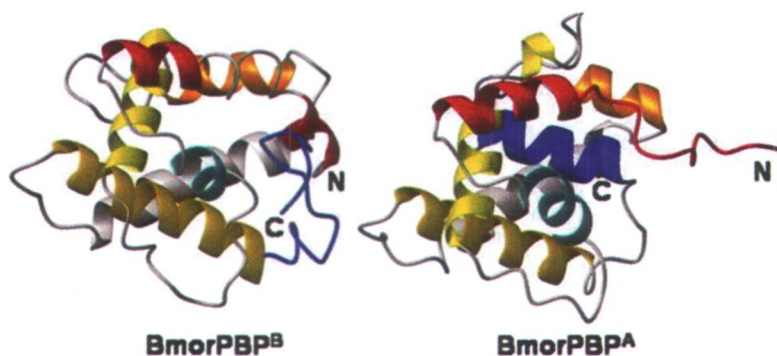


Plate 4.4. Structures of the BmorPBP, at the pH of the sensillar lymph (BmorPBP^B) and the acid form (BmorPBP^A). An unstructured conformation in the C-terminal sequence of the protein (C in BmorPBP^B), rearranges at lower pH to form a regular α -helix (C in BmorPBP^A) that occupies the pheromone-binding pocket thus "ejecting" the pheromone to the receptor. Note that the N-terminus (N), which formed as α -helix in BmorPBP^B, is unfolded in BmorPBP^A.

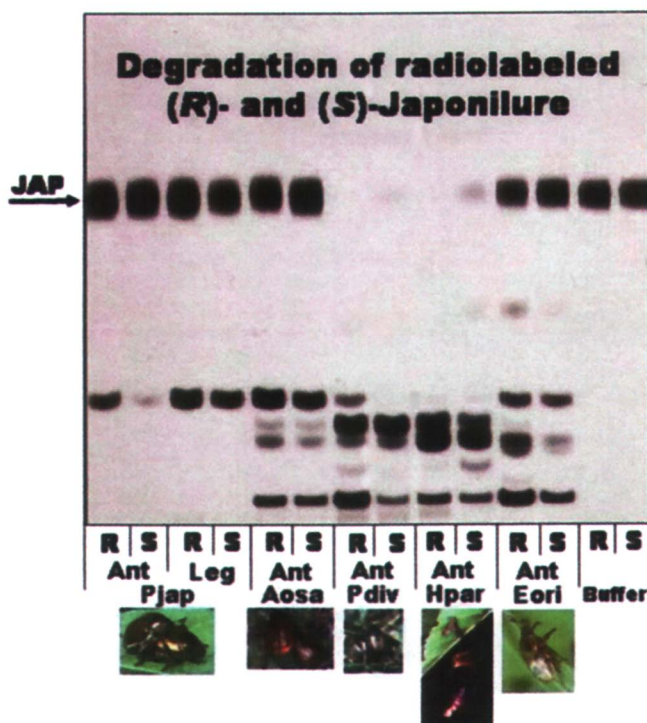


Plate 4.6. TLC plate showing degradation of (R)- and (S)-japonilure by esterases from legs (Leg) and antennae (ANT) of the Japanese beetle (Pjap), A. osakana (Aosa), P. diversa (Pdiv), H. parallela (Hpar), and E. orientalis (Eori). Note the slower degradation of the behavioral antagonist, the (S)- enantiomer, by the sensilar esterase(s), but not with enzyme(s) from legs. Neither (R)- nor (S)-japonilure is degraded in control experiments (buffer).

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