Phytochemicals for Pest Control

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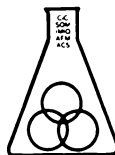
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Foreword

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Before a symposium-based book is put under contract, the proposed table of contents is reviewed for appropriateness to the topic and for comprehensiveness of the collection. Some papers are excluded at this point, and others are added to round out the scope of the volume. In addition, a draft of each paper is peer-reviewed prior to final acceptance or rejection. This anonymous review process is supervised by the organizer(s) of the symposium, who become the editor(s) of the book. The authors then revise their papers according to the recommendations of both the reviewers and the editors, prepare camera-ready copy, and submit the final papers to the editors, who check that all necessary revisions have been made.

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

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Preface

RESEARCH ON NATURAL PRODUCTS has been increasingly reported in the public press in recent years. Contributing to this trend are the compounds or processes themselves, which have profound biological implications, particularly for medicine, nutrition, and agriculture. The significance of these reports is readily grasped by the lay public, which in turn enhances both further expectations and some degree of support. This new surge of natural-products research has been fueled both by the need to provide environmentally and therapeutically safe agents and by the development of increasingly sophisticated techniques and instrumentation. The recent rise of combinatorial chemistry has been fueled by the expanding availability of lead compounds with described biological activities, most of which are of natural-product origin.

It is noteworthy that these agents are perhaps most often isolated from plants or associated microorganisms in the humid tropics, where the competition to thrive and even surive is intense. Therefore, there is the expectation that those compounds may be especially fit to compete.

We have had a continuing interest in biologically active agents that protect plants from pests. Following this interest, we organized conferences on these topics in 1984, 1987, and 1992. Books based on the presentations at those meetings have been compiled and published.

This volume is based on presentations given at the 1995 International Chemical Congress of Pacific Basin Societies, held in Honolulu, Hawaii, December 17–25, 1995. The book includes sections on identification and utilization of biologically active natural products, novel natural products with applications for pest management, structure–activity studies of natural-product pest-control agents, and biologically active proteins and peptides affecting insects.

As the development of diverse uses for these and succeeding agents continues to evolve, populations, animals, and crops will increasingly be protected from a broad spectrum of pests, insects, and diseases by more selective technologies. We hope that this book will contribute to the understanding and the subsequent adoption of additional criteria and research strategies for the control of pests. This volume will be of interest to industry, the academic and federal research sectors, and the agencies responsible for regulation of pest-control agents.

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Chapter 1

New Applications for Phytochemical Pest-Control Agents

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Natural products from plants continue to be the subject of novel and straightforward applications as pest control agents. It is noteworthy that these agents are perhaps most often isolated from plants or associated microorganisms that grow in the humid tropics where the competition to survive and even thrive is intense. Therefore, there is the expectation that those compounds may be especially fit to compete.

This chapter surveys and highlights presentations given at the December 1995 International Chemical Congress of Pacific Basin Societies held in Honolulu, which have now been compiled as a book consisting of sections on identification and utilization of biologically active natural products, novel natural products with applications for pest management, structure-activity studies of natural products pest control agents, and biologically active proteins and peptides affecting insects.

As the development of diverse uses of these and succeeding agents continue to evolve, populations, animals, and crops will increasingly be protected from a broad spectrum of pests by more selective technologies. These agents may also serve as lead compounds for the development of economically superior agents against pests.

For thousands of years, people have recognized that plants contained substances that could promote health and cure illnesses, and they observed that some plants resisted pests better than others. Through trial and error, people discovered the applications of these substances and developed procedures for their extraction and use. With the advent of modern chemistry, the structures of many of these biologically active agents became known, and the systematic studies of natural products that protected plants from pests became a recognized activity within the field of chemistry.

Microbes associated with plants, perhaps because they often evolved with them, have also been a source of chemicals with these biological activities.

For a period of time, the use of natural products for the protection of plants waned with the increased use of large-scale synthesis and production of synthetic chemical compounds. During recent years, there has been a growing recognition that many of these synthetic compounds display a variety of adverse effects, and their use is often circumscribed. The need to control pests is even greater today, and attention is being refocused on natural products because they often have a reduced impact on the host and the environment, they may have desired specificity, and they may serve as prototype models for industrial innovation. We have had a continuing interest in biologically active agents that protect plants from pests. Naturally-occurring pesticides have been a common source of inspiration to the chemical industry, which has often been able to develop compounds with superior properties for agriculture based on these natural structures. Several examples are presented in this volume, such as the synthetic pyrethroid insecticides based on plant-derived pyrethrins or the more recent insecticidal halogenated pyrroles derived from the microbial metabolite, dioxapyrrolomycin. Additional examples of the use of natural templates in pesticide development include new methoxyacrylate fungicides derived from the fungal metabolite, strobilurin, and a chemically modified version of the microbial toxin, avermectin, with much enhanced toxicity to lepidopterous species.There is every reason to believe that this approach to agrochemical innovation will continue to be fruitful with special emphasis on products with high levels of safety and low environment impact. Following this interest, we have organized several symposia and special conferences on these topics, and books based on the presentations at meetings have been compiled and published.

This introductory chapter is largely based on presentations given in a symposium "Phytochemical Pest Control Agents" that was a part of the PACIFICHEM 95 conference held in Honolulu, Hawaii in December 1995. This introductory chapter is organized into four sections. Each includes several reports of plants or microbes that possess chemicals with significant biological activities and discusses their interactive relationships. They are (A.) Identification and utilization of biologically active agents from woody plants, (B.) Novel natural products from plants and microbes with applications for pest management, (C.) Structure-Activity studies of natural pest control agents, and (D.) Biologically active proteins and peptides affecting insects.

We are moving into a new era in which crops, animals and even the public, will be protected from a broad spectrum of pests, including plants, insects, and diseases, by more selective technologies. Naturally occurring toxic proteins are of intense interest because of the development of genetic engineering methodologies that allow their expression in many important crop plants. Transgenic plants with protection against insects, bacteria and viruses are now being commercialized. The discovery of new genes with protective products is urgently needed for the development of future generations of transgenic crop varieties. Although it currently stretches the bounds of genetic engineering to manipulate the production of non-proteinaceous secondary plant metabolites for enhanced pest protection,

there is good reason for optimism that this approach will open broad additional avenues for pest-resistant crop varieties in the future. Knowledge of secondary plant protection chemicals will then become even more significant. We believe that this book will contribute to the understanding and the subsequent adoption of additional criteria and research strategies for the control of pests based on natural products and the chemical basis of agroecology and would therefore be of interest to industry, the academic and federal research sectors, and the agencies responsible for regulation of natural products.

Identification and Utilization of Biologically Active Agents from Woody Plants

Hostettmann *(1)* has isolated a variety of antifungal, molluscicidal, and larvicidal agents from a number of tropical plants. They are a rich source of novel natural products with a variety of different bioactivities. Extracts of tropical plants have been screened against *Candida albicans* and the plant pathogenic fungus *Cladosporium cucumerinum* using bioautography on TLC plates. They have also been tested against *Biomphalaria glabrata* snails, the intermediate hosts responsible for the transmission of the widespread tropica parasitic disease shistosomiasis. Finally, the extracts have been screened against larvae of *Aedes aegypti*, the vector of yellow fever.

Once an activity has been revealed, isolation of pure bioactive compounds from the plant extracts is performed using bioactivity-guided fractionation procedures. For this means, combinations of different modern separation techniques are employed including liquid-liquid partition chromatography. LC-MS and LC-UV (photodiode array detection) permit a rapid detection of plant metabolites in crude plant extracts, enable identification of new and known compounds in the plant under study and provide a good idea of the structure of the metabolite in question.

Screening of wood extractives from tropical timber species in the mahogany (Meliaceae) and African walnut (Olacaceae) families against pestiferous lepidopterans such as the tobacco cutworm (*Spodoptera litura*) and the variegated cutworm (*Peridroma saucia*) as reported by Isman *(2)* have indicated the presence of insecticidal and growth inhibitory factors in several genera. The Meliaceae, which includes the well-known neem tree *(Azadirachta indica)*, is phytochemically characterized by the presence of limonoid triterpenes, many of which are biologically active against insects. In most cases, specific limonoids are responsible for this bioactivity, but in one genus (*Aglaia*) the insecticidal principles are modified benzofurans. Recently isolated insecticidal principals from *Trichilia, Cedrela* and related genera have been identified. The Olacaceae have not been chemically characterized to date. The results of screening members of this pantropical family are given and the putative active principles discussed. Bark, woodwaste and sawdust from certain commercially harvested timber species could be exploited for their biologically-active constituents, and some extracts from these forest waste products have potential for development as botanical insecticides *(2)*.

Arnason and co-workers *(3)* have studied some neotropical species including Meliaceae, Piperaceae and Lepidobotryceae that are sources of useful insecticidal and anti-parasitic compounds. Limonoids with potent insect anti-feedant and

antimalarial properties have been isolated from *Swietenia humulis* and *Cedrela* spp. Over a dozen derivatives of one of these compounds, gendunin, have been prepared to determine structure activity relationships and functionalities required for activity. A group of novel spiro-triterpenoids with insect anti-feedant activity have been isolated from the Lepidobotryceae. Several new neotropical *Piper* spp. have been identified with potent insecticidal properties.

A number of phenolics and flavonoids have been isolated from *Acer* and *Pinus* species *(4)*. Extracts of native tree species have been screened against larvae of forest tent caterpillar *Malacosoma disstria* Hbn. and gypsy moth *Lymantria dispar* L. When activity was found, a bioassay-guided isolation of active constituents from the plant extracts was carried out. The isolation, purification and structural elucidation of some new and known phenolics and flavonoids from *Acer* and *Pinus* species was reported. The effects of extracts, fractions and pure compounds from *Acer* and *Pinus* species against larvae of forest tent caterpillar and gypsy moth were extensive.

Vascular plants, through the phenylpropanoid pathway, afford an array of plant defense compounds which provide protection against predators, bacteria, viruses, and fungi, e.g., the insecticidal lignan, haedoxan A, and the *Drosophila* larva growth inhibitor, epimagnolin A. Others, such as lignins and suberins, also have protective roles as physical barriers to opportunistic pathogens. Curiously, the biochemical pathways to this rich source of lignan metabolites is only now being unraveled. The biosynthesis of the pine, forsythia, and sesame lignans giving the furanofuran, furano, dibenzylbutane, and dibenzylbutyrolactone skeleta, and how these metabolic events are induced preferentially compared to lignin, suberin, and flavonoid formation is described. The novel enzymatic steps involved in obtaining transgenic organisms with altered lignan-producing abilities are being elucidated *(5)*.

Potent insecticidal components against *Nilaparvata lugens* Stal were isolated from the *Ginko biloba* (L.) leaves by chromatographic techniques and characterized by spectral analysis as bilobalide, and gingkolides A and B. These compounds were highly effective against the susceptible strain as well as three strains of *N. lugens* resistant to diazinon, fenobucarb and carbofuran. Their toxic effect was comparable to the widely used fenobucarb and carbofuran. In addition, these compounds revealed low toxicity to mice (LD_{50}>500 mg/kg) and caused no mutagenicity when tested against four strains of *Salmonella typhyimurium*. As naturally occurring insecticides, *G. biloba*-derived materials could be useful as new control agents against *N. lugens (6)*.

Novel Natural Products from Plants and Microbes with Applications for Pest Management

Since their discovery in 1982, the annonaceous acetogenins have become one of the most rapidly growing classes of bioactive natural products. Chemically, they are C-32 or C-34 long chain fatty acids which have been combined with a 2-propanol unit at C-2 to form a gamma-lactone. Biogenetically, double bonds along the fatty acid chain seem to epoxidize and cyclize to form one, two, or three tetrahydrofuran rings, often with flanking hydroxyls; other functional groups include hydroxyls,

acetoxyls, carbonyls, and double bonds. Biologically, they are among the most potent of the known inhibitors of complex I (NADH:ubiquione oxidoreductase) in mitochondrial electron transport systems and of the plasma membrane NADH:oxidase, which is characteristic of cancerous cells; these actions seem to induce apoptosis (programmed cell death), perhaps as a consequence of ATP deprivation.   Applications as pesticides and antitumor agents hold excellent potential, especially in the thwarting of resistance mechanisms which require an ATP-dependent efflux from cells *(7)*.

In response to pathogen attack, plants synthesize a diverse array of secondary metabolites. These metabolites, phytoalexins, are part of the plant's defense mechanism. Phytoalexins from brassicas have an indole ring and at least one sulfur atom as a common structural feature.   Despite their close biogenetic relationship, these phytoalexins possess significantly different structures. Such structural differences suggest that brassica phytoalexins have different biological activity.

Recently, Pedras et al. *(8)* have investigated the biotransformation of the phytoalexin brassinin by the blackleg fungus (*Leptosphaeria maculans*, asexual stage *Phoma lingam*), the causative agent of the blackleg disease of brassicas. They now compared the toxicity of three additional phytoalexins of brassicas, brassicanal A, brassilexin, and camalexin, to the blackleg fungus. Pedras et al. *(8)* have also studied the bioactivity of these phytoalexins on brassica pathogens, as well as results of their biotransformation by isolates of the blackleg fungus, and the interaction of these phytoalexins with the blackleg phytotoxins, and a possible mechanism of action for brassinin, have been suggested.

A number of compounds isolated from microbial agents have been identified that have significant biological activities. The spinosyns are a group of new macrolide pest control agents isolated from a new species of actinomyce, *Saccharopolyspora spinosa*. This organism produces at least 10 active and structurally related factors which are unrelated to any known chemistry. The organism has been optimized for the production of two factors, spinosyn A and spinosyn D, which together constitute the insecticide spinosad. Spinosad is currently being developed as a pest control agent for cotton and vegetables. DeAmicis et al. *(9)* have elucidated the structure, physical properties and biological activity of the spinosyns, their pseuoaglycones and aglycones. The results of SAR studies on insects and mites, the field activity of the spinosyns on insects and mites, and results from environmental and mammalian safety studies have been determined.

Entomopathogenic   nematodes   in   the   genera   *Steinernema*   and *Heterorhabditis* have become commercially available against a wide-range of soil inhabiting insects. These nematodes function as vectors transmitting their symbiotic bacterium *Xenorhabdus* and *Photorhabdus* into the host haemocoel. The bacterium multiplies, producing toxins that kill the host. Both of these bacteria produce antimicrobial agents that inhibit a broad range of bacteria, yeasts and some fungi. In addition to the use of entomopathogenic nematodes as soil insecticides, they are capable of suppressing the population of plant-parasitic nematodes, thus reducing their damage to plant roots *(10)*.

The identification of novel microbial metabolites for use as biological insecticides is an area of research with significant importance to agriculture due to the number of currently registered insecticides under review by regulatory agencies. A novel microbial metabolite produced by *Bacillus thuringiensis* has been identified that enhances the activity of a biological insecticide against a number of agriculturally important insects. This small water soluble molecule was found to synergize the activity of *Bacillus thuringiensis* delta-endotoxins against lepidopterans including beet armyworm (*Spodoptera exigua*) and tobacco budworm (*Heliothis virescens*). However, the compound has essentially no insecticidal activity without the delta-endotoxins. The isolation, structure determination, insecticidal activity and field trial results of this microbial metabolite are being studied *(11)*.

In addition to its practical implications for the direct or indirect development of new commercial pest management technologies, an understanding of the chemical interactions between organisms is an important element of agroecology. The competition among and between microorganisms, plants and insects is often chemically-based and the outcome of these myriad competitions has profound effects on the fertility and productivity of agricultural productions systems. The maintenance of a favorable balance of forces with minimal exogenous chemical inputs is the major goal of biointensive pest management. The kind of studies and information represented in this volume provides an important background for understanding the complex world of interspecific competition and chemical ecology that surrounds us.

Non-selective weed control has become a significant aspect of weed management worldwide. CGI-350 is a natural product produced by *Pseudomonas syringae* with excellent activity as a non-selective post-emergent herbicide. The mode of action of this compound involves the inhibition of glutamine synthetase which in turn may lead to the inhibition of photorespiration. All weeds and crops tested to date have been found to be sensitive to CGI-350. Initial symptoms of water soaking appear within 48 hours of application with apparent desiccation and necrosis of the foliage beginning with 72 hours. Maximum injury and death occurs within 6 to 10 days after application. Field rates with the current formulation are 1.0 kg ai/ha for many broadleaf and grass weeds *(12)*.

Two strains of *P. aureofaciens*, TX-1 and TX-2, were isolated from a turfgrass soil sample from Texas. Preliminary field tests showed that the TX-1 bacterial extract is active against *Sclerotinia homoeocarpa* (dollar spot pathogen) when applied as a foliar spray at Hancock Turfgrass Research Center at MSU. In vitro tests indicated that TX-1 antibiotic was effective against a variety of turfgrass fungal pathogens. TX-1 was fermented in A-9 medium, and the active component was isolated and assayed for a variety of turfgrass fungal pathogens. The identification and detailed bioassays of this novel antifungal compound, spartifungin, will be discussed *(13)*.

Structure-Activity Studies of Natural Pest Control Agents

Chemical investigations on the interaction between organisms have led to the isolation and identification of biologically active natural products which have served as leads to the discovery and development of commercialized agrochemicals. Miyakado et al. *(14)* discuss how natural products such as physostigmine led to the carbamates and pyrethrum led to the pyrethroids. They give important requirments for leads including stability, the understanding of the mode of action or specificity, and activity under practical conditions. They also examined the evolution of commercial compounds from pheromones, juvenile hormones, plant allelochemicals, and microbial compounds.

Matsuo and Miyamoto *(15)* described their extensive efforts in structure modification of alcohol and acid moieties of natural pyrethrins that have led to a number of synthetic pyrethroids with diversified characteristics. They proposed that the structures of pyrethrins I and II could be divided into five moieties. Alterations led to commercial compounds such as *allethrin, tetramethrin, chrysanthemic acid, permethrin, cyphenothrin,* and *fenvalerate.* Because of these extensive modifications, the structural resemblance between the original natural pyrethrins and the modern synthetic pyrethroids is not obvious. However, these synthetic pyrethroids have generally favorable toxicological features and environmentally compatible properties.

Kuhn et al. *(16)* have studied dioxapyrrolomycin, a naturally occuring fermentation product that was found to have moderate activity against a variety of agronomically important insect pests. Dioxapyrrolomycin has been used as a template for a synthesis program that has led to a new class of selective insect control agents, the 2-arylpyrroles. It has been established that these compounds are propesticides, requiring metabolic activation in the target organism, whereupon they act as uncouplers of mitochondrial oxidative phosphorylation, rapidly depleting the organism of ATP *(17)*.

C.-T. Hsu *(18)* and co-workers at the Rohm and Haas Company arrived at an effective larvicide, RH-5992 [Benzoic acid, 3,5-dimethyl-,1-(1,1-dimethylethyl)-2-(ethylbenzoyl) hydrazide], through a structure-activity study. This compound is a non-steroidal ecdysone agonist that evolved during structure-activity studies from a class of 1,2-diacyl-1- substituted hydrazines that were discovered to have insecticidal activity.

Another example of the evolution of effective compounds from structure-activity studies is that of the avermectins and milbemycins *(19)*. In 1976, scientists at Merck and Co., Inc. discovered a complex of eight closely related natural products, subsequently named avermectins, in a culture of *Streptomyces avermitilis* MA-4680 (NRRL8165) originating from an isolate by the Kitasato Institute from a soil sample collected at Kawana, Ito City, Shizuoka Prefecture, Japan. They are among *the most potent anthelmintic, insecticidal and acaricidal compounds known.* The avermectins are structurally related to another group of natural products, the milbemycins, the first examples of which were described by Japanese workers. Avermectin B_1, under the non-proprietary name abamectin, is widely used as an agricultural miticide and its 22,23-dihydro derivative, ivermectin, is used world-wide

as a broad spectrum parasiticide in animals and in man. More recently, a new avermectin, doramectin, has been prepared by directed biosynthesis and developed for similar use in animals. Three milbemycins are used for animal health, milbemycin D, milbemycin oxime and moxidectin. This report describes some of the structure-activity relationships between the avermectins and milbemycins and an intensive synthetic program on 4"-deoxy-4"-epiamino avermectins culminating in two novel compounds in development, emamectin benzoate as an agricultural insecticide and eprinomectin as a broad spectrum endectocide for use in animals. Mode of action studies have revealed a glutamate-gated chloride channel from *C. elegans* that is proposed to be the target site for both avermectins and milbemycins.

Another biorational approach that has met with success is the development of herbicides from fungal metabolites *(20)*. Host-specific toxins (HSTs) can be utilized to design selective herbicides because of their extremely potent specific phytotoxicity that originates from their characteristic structural features. This research on HSTs has led to a biorational approach to develop selective herbicides, based on bioorganic chemical experiments starting with fungal metabolites. As a lead compound to express a minimum selective phytotoxic effect against Japanese pear cultivers, (4R,5S)-4-(N-acetylphenylalanoyl)oxy-5,6-epoxy-5-methyl-2(E)-hexenoate was selected from structure-toxicity relationships using natural and synthetic analogs of AK-, AF- and ACT-toxins produced by *Alternaria* species.

An example of the development of an effective fungicide by structure modifications was that of the macrolide soraphen A_1 alpha, which was isolated from the myxobacterium *Sorangium cellulosum*. It shows potent and broad fungicidal activity. The molecule was derivatized at various positions and many compounds were found with excellent fungicidal activity, some more potent than soraphen A itself. The fungicidal activity of soraphen A derivatized at positions 2,5,11, and 12 is described in this report *(21)*.

Structure-activity studies have also been accomplished with proteins. Ellar *(22) reported that the bacterium Bacillus thuringiensis produces novel and highly specific insecticidal protein toxins (delta-endotoxins) grouped into two families - Cry and Cyt toxins - by sequence similarity.* Both types of delta-endotoxin bind to insect-specific receptors on the surface of midgut epithelial cells and insert into the cell membrane to form leakage channels that result in cell death by colloid osmotic lysis. The protein nature of these toxins coupled with genetic engineering offers great potential for pesticide development and has allowed them to be expressed in plants as systemic biopesticides. The X-ray structure of the first Cry toxin revealed putative membrane insertion and receptor binding domains whose functions have been explored by intensive mutagenesis. The structure of the first Cyt toxin is entirely different from the Cry toxins and current genetic attempts to define structure-activity relationships for these toxins is reported. The potential of these pesticides has been further enhanced by the recent cloning and sequencing of two Cry toxin receptors. These receptors are transmembrane proteins exposed on the lumen surface of midgut epithelial cells. The structure of these receptors and their role in toxin recognition, the mechanisms of toxin insertion into membranes and cytolytic pore formation has also been studied.

Biologically Active Proteins and Peptides Affecting Insects

This section reports recent research on hormone metabolism which may eventually lead to an alternative approach to designing agents that disrupt insect systems. Three of the reports investigated results of studies with neuropeptides. Analogs are being designed that may disrupt hormonal processes.

Rayne and O'Shea *(23)* found that the peptide adipokinetic hormones (AKHs), are important regulators of metabolism in many insects. In the locust, *Schistocerca gregaria*, AKHs are produced by glandular cells of major neuroendocrine centres called the corpora cardiaca (CC). They have determined the temporal order and the intracellular location of AKH precursor processing events, and have documented the enzymatic steps in precursor to product conversion. In addition, they have used NMR spectroscopy to determine the solution structure of the AKH precursor in hopes of identifying features of this protein which govern processing endopeptidase specificity. This work provides a basis for the design of specific processing enzyme inhibitors to be used as lead compounds for new insecticides.

Masler *(24)* reported that neuropeptides are involved in essentially all physiological processes in insects. Control of hemolymph neuropeptide titer and attenuation of the neuropeptide signal are two key elements in these processes. He and co-workers have discovered specific proteolytic activities in the hemolymph and in neural membrane preparations from a lepidopteran species. They appear to have roles in the catabolism of biologically active insect neuropeptides. The characteristics of specific endopeptidase and aminopeptidase activities, and enzymatic activity during development, are described. Evidence for the presence of multiple similar, but not identical, enzyme activities has been obtained. The roles of the various enzymes examined are considered as leads to biologically-based pest control agents.

Nachman and co-workers *(25)* also have investigated the importance of neuropeptides to the maintenance of critical physiological processes in insects. Nevertheless, insect neuropeptides in and of themselves hold little promise as insect control agents because of their susceptibility to enzymatic degradation in the target insect, lability under environmental conditions, and inability to pass through the hydrophobic insect cuticle. The removal of the peptide nature (i.e. the constituent amide bonds) of insect neuropeptides represents a strategy that could overcome these limitations. Active non-peptide, peptidomimetic agonists and/or antagonists may hold promise for future utilization in pest insect control strategies. They have investigated the enzymatic degradation by insect angiotensin-converting enzyme (ACE) of the insect kinin, insectatachykinin and pyrokinin neuropeptide families, and the synthesis of active analogs that are resistant to degradation. Conformationally-restricted analogs of the myosuppressin and insectatachykinin families shed light on the active conformation adopted at the receptor site. Utilization of this information for the development of peptidomimetic analogs is discussed. The first nonpeptide analog of an insect neuropeptide both mimics the biological activity and binds to a locust oviduct receptor site of the myosuppressin peptide family.

Kubo and co-workers *(26)* have investigated tyrosinase inhibitors from plants as alternative insect control agents. Since tyrosinase is one of the key enzymes in the insect molting process, its inhibitors may ultimately provide clues to control insect pests. Bioassay guided fractionation has led to the isolation of a number of tyrosinase inhibitors. For example, 2-hydroxy-4-methoxybenzaldehyde has been characterized as the principal tyrosinase inhibitor from various East African medicinal plants. It inhibited the oxidation of L-3,4-dihydroxyphenylalanine (L-DOPA) by tyrosinase. Two buddlenoids (flavonoids from a Bolivian medicinal plant), and anacardic acids and cardols from cashew fruit also inhibit this oxidation.

M. Eldefrawi *(27)* reported that cholinergic receptors are cell membrane receptors that regulate cell function when activated by acetylcholine. Death from organophosphate and carbamate anticholinesterases results from overstimulation of cholinergic receptors in nerve and muscle. There are two types of acetylcholine receptors: nicotinic and muscarinic receptors. The former are ionotropic, having multiple heteromeric subunit structure and when activated open cation channels. Muscarinic receptors are metabotropic, consist of a single subunit and are linked to cellular catalytic effectors via G-proteins. In mammals, there is large subtype diversity of these receptors with different molecular and pharmacological properties, but little is known about diversity in insects. There are similarities in drug specificities between insect neuronal nicotinic receptors and mammalian brain alpha[7] receptor subtypes and between insect muscarinic receptors and mammalian M_3 receptor subtypes. Insect nicotinic receptors are molecular targets for nicotinoid and neonicotinoid insecticides. Cholinergic receptor diversity amongst insects can be the basis for discovery of species-selective insecticides.

Nakanishi et al. *(28)* have also investigated the nicotinic acetylcholine receptor in search of alternative agents that disrupt insect systems. The venom of the Egyptian wasp *Philanthus triangulum* (philanthotoxin-433) is a polyamine amide with butyryl/tyrosyl/spermine moieties. It is a noncompetitive inhibitor of the nicotinic acetylcholine receptors (nAChR) and various glutamate receptors (GluR). Over 100 analogs have been synthesized for structure-activity relations (SAR) and various other purposes. Preliminary photocross-linking results, coupled with SAR, have led to a putative model representing the binding of philanthotoxins in channels gated by nAChR. Recent studies aimed towards clarifying the tertiary structural interaction of PhTX and the nACh-R will be presented. These include: further photoaffinity binding, assays with analogs designed to elucidate their mode of entry into the receptor, affinity chromatography/tandem MS to increase sensitivity of protein sequencing, solid state ^{19}F-NMR of fluorinated PhTXs and nAChR complexes.

In summary, these reports constitute a sampling of natural products recently isolated from plants and microbes that have profound implications for medicine, nutrition, and agriculture. The great interest in natural products research has been fueled by the needs to provide environmentally and therapeutically safe agents and by the development of increasingly sophisticated techniques and instrumentation. Biologically active natural products will play an increasingly important role in providing the means to protect our health and to feed and generally sustain the rapidly increasing world population.

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Chapter 2

Strategy for the Isolation and Analysis of Antifungal, Molluscicidal, and Larvicidal Agents from Tropical Plants

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Tropical plants have been investigated for antifungal, larvicidal and molluscicidal properties. Bioautography on TLC plates against the yeast *Candida albicans* and the plant pathogenic fungus *Cladosporium cucumerinum* was used in the screening for antifungal activity. Larvicidal activity was assessed against *Aedes aegypti*, the vector of yellow fever. The schistosomiasis transmitting snail *Biomphalaria glabrata* was used for detecting molluscicidal properties. Tests are briefly described and illustrated by a selection of recently discovered active compounds. Finally the contribution of hyphenated techniques such as HPLC-UV and HPLC-MS to the targeted isolation of promising molecules is discussed.

The plant kingdom represents an enormous reservoir of new molecules to be discovered. Tropical plants, which grow under climatic conditions favouring microbial or insect attack have developed a great variety of defense molecules. They constitute therefore a particularly rich source of substances which can find an application, directly or as lead compounds, for the development of new drugs or pest control agents. At the same time, preparations from tropical plants are likely to play a significant role in the future for the vector control of some of the main tropical diseases such as schistosomiasis, malaria or yellow fever. Systematic screening of tropical plants for antifungal, larvicidal and molluscicidal properties resulted in the isolation in our laboratories of several promising active molecules.

Fungicidal Activity

Diseases caused by fungi bring phenomenal losses in agriculture. At the same time, the growing occurrence of systemic mycoses, in particular candidiasis, cryptoccosis and aspergillosis, as a consequence of the spread of AIDS and the increasing use of immunosuppressive drugs is a major concern of public health. Broad programs aimed at new antifungal agents are being undertaken in the fields of pharmaceutical and agrochemical research. Such programs include plant metabolites as it has been demonstrated that plant-derived compounds may offer potential leads for novel agents against fungal infections, in particular systemic mycoses (*1*). Two bioautographic assays against *Cladosporium cucumerinum* and *Candida albicans*, respectively, are

currently used in our laboratories for the screening of plant extracts. Bioautographic assays on TLC plates are ideally suited for the screening of plant extracts and subsequent activity-guided fractionation, since they permit a direct localization of active compounds within a complex matrix. *Cladosporium cucumerinum* is a spore producing fungus which has been employed for more than 25 years for the detection of antifungal substances (*2*). This fungus infests plants of the family Cucurbitaceae and is well suited for the detection of substances active against plant pathogenic fungi. The test procedure involves chromatography of an extract, a fraction or a pure compound on a silicagel TLC plate and then spraying with a suspension of spores of the fungus in a nutrient medium. After an incubation period of three days, the active substances appear as white spots on a grey background of normal spore growth. This bioassay is quick, simple and safe since the fungus is not pathogenic to humans.

Direct bioautography is not possible with yeasts such as *Candida albicans*. To overcome this problem, a bioautographic agar overlay assay has been developed (*3*). Inoculated agar medium is poured onto a developed TLC plate and allowed to solidify. After overnight incubation, zones of inhibition are visualized by the detection of dehydrogenase activity with a tetrazolium salt (MTT) which is converted by the enzyme into a formazan derivative. Active compounds appear as clear spots against a purple background. Even though the agar overlay test is somewhat more labor intensive compared with the straightforward procedure with *C. cucumerinum* and requires a minimum of microbiological expertise for maintenance of yeast cultures and *ad hoc* preparation of the inoculum, the assay can be readily set up in a chemistry department. As yeasts do not produce spores, working with weakly pathogenic *C. albicans* strains requires only minimal precautionary measures. A selection of antifungal substances, recently discovered in our laboratories, is presented below.

Eriosema tuberosum (Leguminosae) is a Chinese medicinal herb used in Yunnan province to treat diarrhoea, orchitis, hydrophobia and as a detoxifying drug. The
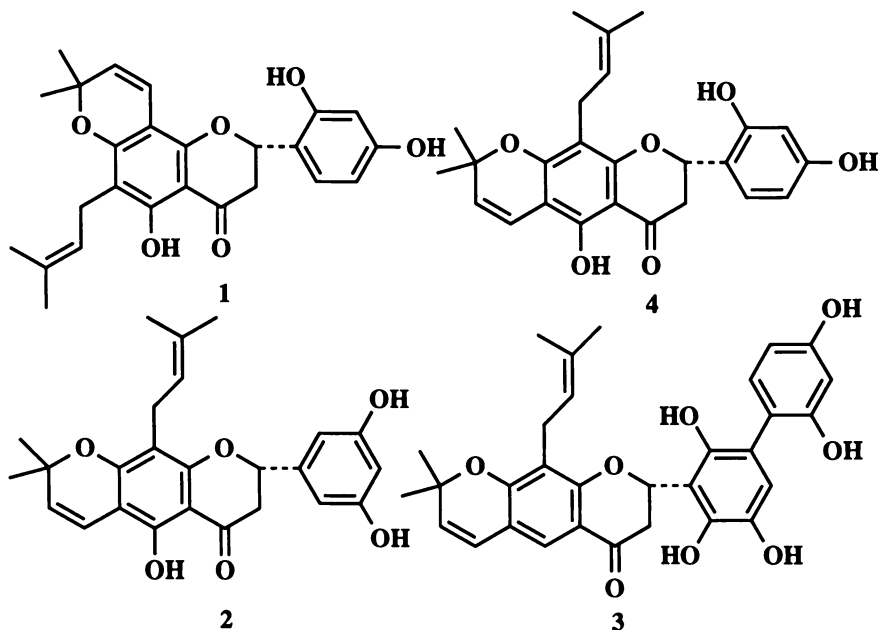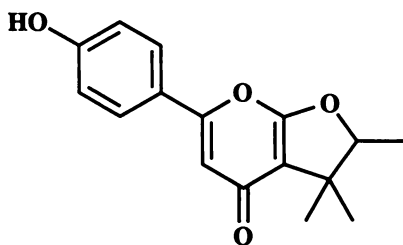
dichloromethane extract of the roots exhibited antifungal activity against both *C. cucumerinum* and *C. albicans*. Bioassay-guided fractionation carried out by a combination of open column chromatography (CC) on silica gel, medium pressure liquid chromatography (MPLC) on RP-18 and gel filtration on Sephadex LH-20, led to the isolation of the flavanones eriosemanones A-C (**1-3**) and flemichin D (**4**), together with the isoflavone eriosemanone D (**5**). Eriosemanones A-D were new compounds. The minimum amounts of these flavonoids required to inhibit *C. cucumerinum* and *C. albicans* growth on TLC plates were 5 and 1 μg, respectively. For comparison, the reference compound propiconazole was active at 0.1 μg towards *C. cucumerinum*, while miconazole inhibited the growth of *C. albicans* at 0.001 μg (*4*).
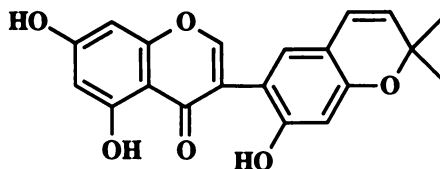

5

Hypericum brasiliense (Guttiferae) is a herbaceous plant of South and Southeast Brazil. Stems and roots were extracted successively with petrol and dichloromethane. Bioautography of the dichloromethane extract revealed antifungal activity against *C. cucumerinum*. Gel filtration of this extract over Sephadex LH-20 allowed the isolation of a new γ-pyrone (**6**). The xanthones **7-9** were obtained by a combination of CC on silica gel and gel filtration on Sephadex LH-20. The minimum quantities of **6-9** required to inhibit growth of the fungus in this assay were 3, 0.25, 3, and 3 μg, respectively. Simple xanthones were not previously known for their antifungal properties and the excellent inhibition for **7** justifies a more extensive search in this class of compounds for potential leads (*5*).
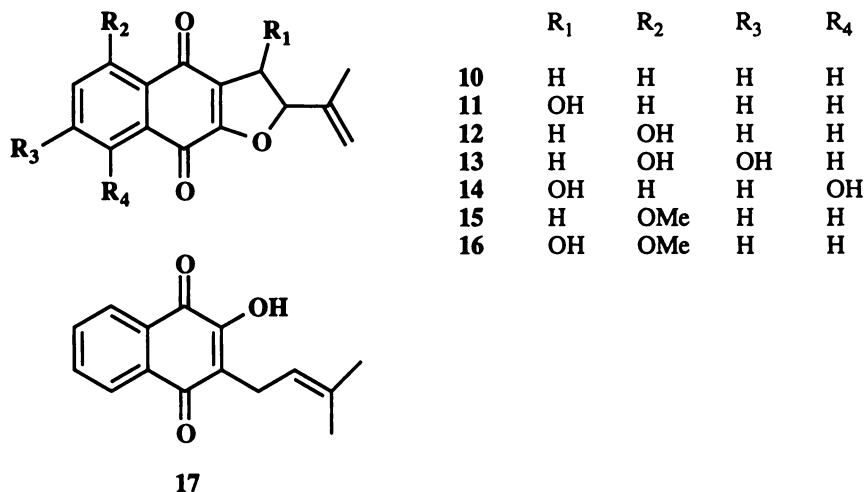

6
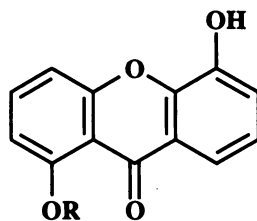
7 R = H
8 R = OCH$_3$
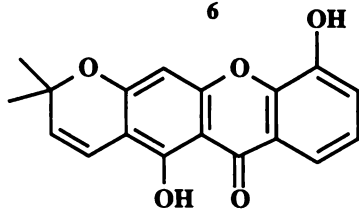
9

Newbouldia laevis (Bignoniaceae) is a shrub growing in West Africa which is widely used in traditional medicine. Fractionation of the dichloromethane extract of the roots by a combination of CC on silica gel, gel filtration on Sephadex LH-20 and MPLC on RP-18 afforded eight naphthoquinones with strong antifungal properties (**10-17**). Two of them, 3-hydroxy-5-methoxydehydroiso-α-lapachone (**16**) and 5,7-dihydroxydehydroiso-α-lapachone (**13**) were new. Most active was compound **14** with minimum inhibitory quantities of 0.01 μg and 0.1 μg against *C. cucumerinum* and *C. albicans*, respectively (Gafner, S.; Wolfender, J.L.; Nanga, M.; Stoeckli-Evans, H.; Hostettmann, K. *Phytochemistry* **1996**, in press.).

	R_1	R_2	R_3	R_4
10	H	H	H	H
11	OH	H	H	H
12	H	OH	H	H
13	H	OH	OH	H
14	OH	H	H	OH
15	H	OMe	H	H
16	OH	OMe	H	H

17

Cosmos caudatus (Asteraceae) is a flowering herbaceous plant native of tropical America. The dichloromethane extract of the root exhibited strong antifungal activity against *C. cucumerinum* and *C. albicans*. HPLC analyses with diode array detection revealed the presence of phenylpropanoids. Activity guided fractionation afforded a hydroxy-eugenol (**18**) and four coniferyl alcohol derivatives (**19-22**), one of which, **22**, was a novel compound. Compounds **18** and **20** were antifungal against *C. cucumerinum* at 0.1 μg, **19**, **21** and **22** at 1, 0.5 and 5 μg, respectively. Only **19-21** were active against *C. albicans*, at 5 μg, suggesting that the epoxy moiety is an important structural element (*6*).

18

19 R = R_3
20 R = R_1
21 R = R_2

22
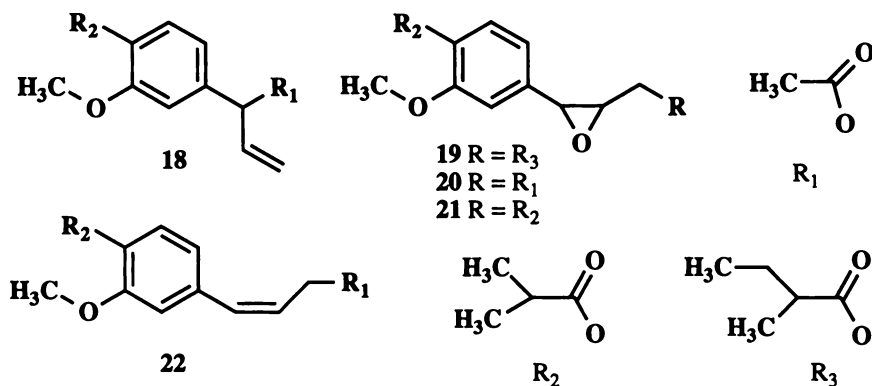
A lipophilic whole plant crude extract of *Valeriana capense* (Valerianaceae) collected in Malawi exhibited activity against *C. cucumerinum*. Fractionation by a combination of CC on silicagel, liquid-solid extraction and semipreparative HPLC on RP-18 provided the active compound valtrate (**23**), together with several inactive valepotriates. Valepotriates are common constituents of *Valeriana* species, such as *V. officinalis*, but the antifungal properties of valtrate had not been previously noticed. Valtrate inhibited the growth of *C. cucumerinum* at 1 μg. High structural specificity was observed, since the isomer isovaltrate, as well as didrovaltrate were completely inactive. In a dilution assay using solid media, the minimal inhibitory concentrations of **23** were 10 μg/ml against *C. albicans* and *Aspergillus fumigatus*, and 20 μg/ml against *Trichophyton mentagrophytes*. As valtrate exhibited noteworthy activity against *C. cucumerinum*, *in vitro*, it was tested *in vivo* against other plant pathogenic fungi. Valtrate was active against *Cercospora arachiodicola*, a pathogenic fungus for the peanut plant, *Erysiphe graminis*, a fungus which infests barley plants and *Venturia inequalis*. Since the activity of **23** against *E. graminis* was comparable to that of the commercial product Calixin, valtrate could become of interest as an agricultural fungicide (*7*).


**23**
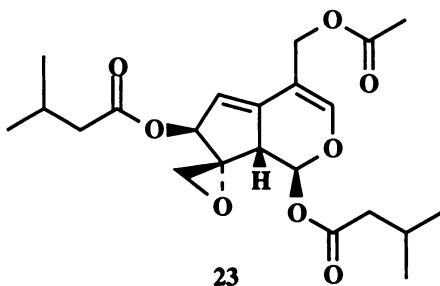
Larvicidal Activity

Mosquitoes, in particular species of *Anopheles*, *Aedes* and *Culex*, are important vectors of tropical diseases: *Anopheles* spp. are responsible for the transmission of malaria which still remains endemic in more than 100 countries and affects 250 million people in the world; *Aedes* spp., and most notably *A. aegypti*, transmit diseases caused by arboviruses (*arthropod borne virus*) such as yellow fever and dengue fever. While yellow fever has been reasonably brought under control with the development of a vaccine, there is no vaccine available yet against dengue fever. The current strategy postulated by the W.H.O. for the control of this disease is to destroy the vector. The majority of mosquitoes hunt and feed at night and /or live near and in settlements, where they cannot be reached by sprayed insecticides. The ideal control method is thus the systematic treatment of their breeding places with larvicidal agents. Plants can provide lead compounds for the development of new larvicidal agents. At the same time, plant-derived preparations can represent an alternative to the use of synthetic pesticides, cheap and readily available to the affected population. A simple bench-top assay has been recently included in our screening assays and crude plant extracts are now systematically tested for larvicidal properties (*8*). The testing procedure involves second instar larvae of *A. aegypti*. The eggs of *A. aegypti* are easy to handle and can be stored in a controlled atmosphere (26-28°C, 70-80% rel. humidity) for up to six month. Larvae hatch readily when put into tap water and incubated for 24 h. The assay consists of exposing approximately 20 larvae to various dilutions of the extracts, previously solubilized in DMSO. Mortality is evaluated with the naked eye after 30 min. and 24 h. A sample is considered active when all larvae have been killed after 24 hours.

In the course of this screening, a few plant species with strong larvicidal activity have emerged. *Diplolophium buchanani*, a shrub of the family Apiaceae is one of them.

The dichloromethane leaf extract of this species endemic to Malawi, showed potent larvicidal and fungicidal properties. Activity guided fractionation carried out mostly by centrifugal partition chromatography (CPC) resulted in the isolation of the phenylpropanoids myristicin (**24**), elemicin (**25**), trans-isoelemicin (**26**), together with the furanocoumarin oxypeucedanin (**27**). Myristicin and oxypeucedanin (LC_{100} 25 mg/l) were larvicidal at concentrations similar to that of the reference compound β-asarone (LC_{100} 16 mg/l). Compounds **24-27** also exhibited antifungal properties against *C. cucumerinum*. Oxypeucedanin in particular was still active at 1 μg (*9*).
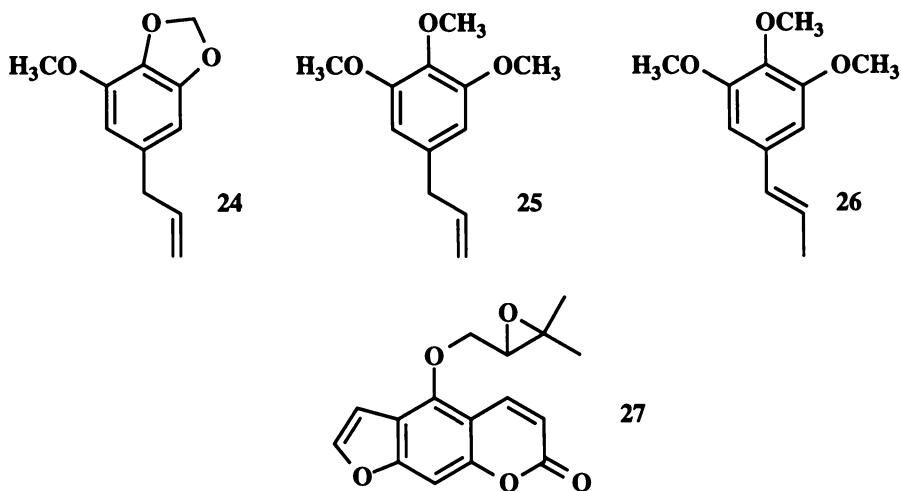

Another interesting species is *Melantheria albinervia* (Asteraceae) collected in Zimbabwe, the lipophilic root extract of which exhibited significant larvicidal activity. Bioassay-guided fractionation carried out by a combination of CC on silicagel and low pressure liquid chromatography afforded the larvicidal diterpenes ent-kaur-16-en-19-oic acid (**28**) and 9(11),16-kauradien-19-oic acid (**29**) which had LC_{100} of 62.5 and 250 mg/l, respectively (*10*).
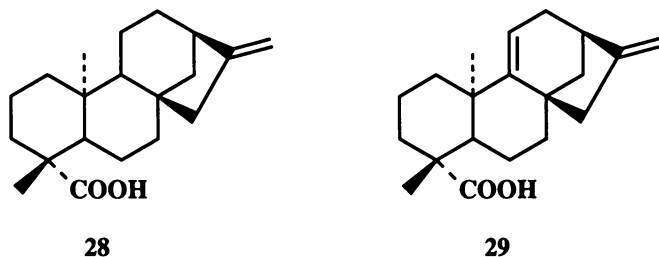
One of the most potent plants discovered so far is *Annona purpurea* (Annonaceae). Fractionation of the larvicidal dichloromethane extract of *A. purpurea* resulted in the isolation of four active acetogenins including the new compounds purpureacin 1 (**30**) and purpureacin 2 (**31**). Most active were rolliniastatin (**32**) (LC_{100} 0.2 mg/l) and bullatacin (**33**) (LC_{100} 0.3 mg/l) (*11*). Acetogenins, however, are known to exhibit general toxicity, which could impede their use as larvicidal agents. Testing of more than 130 crude plant extracts showed that less than 10% of them were active in

this assay (8). Interestingly, no correlation was found between larvicidal and insecticidal activities. Lipophilic extracts of *Psorospermum febrifugum* (LC_{100} 1.25 mg/l) and *Annona purpurea* (LC_{100} 10 mg/l) were the most active. Plant extracts with selective larvicidal potential include *Rogeria adenophylla* and *Sesamum indicum* (Pedaliaceae) root dichloromethane extracts. The small percentage of active extracts in this test confirms its relatively high specificity. Moreover, activity can be reliably followed during fractionation to isolate defined active compounds. It may also be noticed that only a few extracts exhibit strong activity, worth investigating, when one keeps in mind the potency of known insecticides such as pyrethroids (LC_{100} 0.31 mg/l) or the synthetic compound lindan (LC_{100} 0.0031 mg/l) in this assay. However the simplicity of the test, the need of new and easier biodegradable agents and the prospect of using plant derived preparations for the local control of dengue and yellow fevers fully justifies a broad scale screening of plant extracts.

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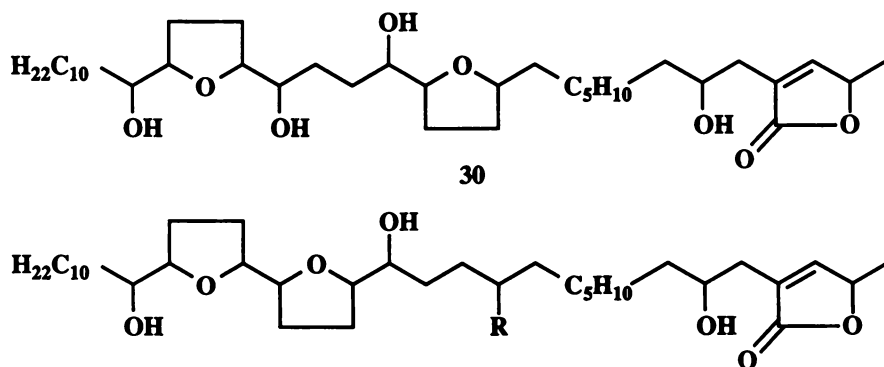
|	R	C(15)/C(16)	C(16)/C(19)	C(19)/C(20)	C(20)C(23)	C(23)/C(24)
31	OH					
32	H	threo	cis	threo	cis	erythro
33	H	threo	trans	threo	trans	erythro

Molluscicidal Activity

Schistosomiasis, commonly known as bilharzia, is a parasitic disease caused by threadworms of the genus *Schistosoma* and is endemic throughout South America, Africa and the Far East. It affects more than 250 million people in over 76 countries. The reproductive cycle of schistosomes involves a stage implicating aquatic snails of the genera *Biomphalaria* and *Bulinus*, in which the parasite multiplies into cercariae. These cercariae, after leaving the snails, can penetrate the skin of humans who come into contact with contaminated water. Once through the skin, they change gradually into the mature trematodes known as schistosomes. The schistosomes mate and lay eggs which are carried away with faeces or urine. As eggs reach water, they produce miracidia, which locate snails of the appropriate species and the cycle begins again (*12*). One way to attack the problem of schistosomiasis is to destroy the carrier snails and thus remove a link in the life cycle. This may be achieved with the aid of synthetic products such as Bayluscide (2,5'-dichloro-4'-nitrosalicylanilide) or, alternatively, with molluscicides from plant sources. The use of molluscicidal plants growing abundantly in areas where

schistosomiasis is endemic is a simple, inexpensive and appropriate technology for local control of the snail vector and may become in the near future a useful complement for the control of this disease (*13*). Schistosomiasis-transmitting snails of the species *Biomphalaria glabrata* are being used in our laboratories for screening plant extracts. The assay consists of exposing two snails to a solution containing the sample to be tested. Extracts are usually tested at an initial concentration of 400 mg/l. Snails are examined after 24 hours under a binocular microscope and mortality is assessed by noting the absence of heart beats.

Among the plants which are of greatest interest are those which contain large quantities of saponins. Saponins possess high toxicity towards cold blooded organisms including snails; they are often present in large amounts in plants; and owing to sufficient water solubility, their extraction and application is simple and does not require any sophisticated apparatus or specially trained people. 'Endod' (*Phytolacca dodecandra*, Phytolaccaceae) is one of the first plants to have been extensively studied (*14*). The berries contain triterpenoid saponins with high molluscicidal activities, and promising field trials have been undertaken in Ethiopia. *P. dodecandra* has however a limited geographical distribution and has to be cultivated if required for use in other countries. *Swartzia madagascariensis* (Leguminosae), on the other hand is very common in many regions of Africa. This tree which bears large fruits has been selected for one of our studies. Phytochemical investigation showed that molluscicidal activity of the fruits was due to the saponin content of the plant. Four active glucuronides of oleanolic acid and gypsogenin (34-37) were isolated from the aqueous extract. Compound **34** presented the highest activity against *B. glabrata* snails with a LC_{100} of 3 mg/l, an activity which is comparable to that of synthetic molluscicides (*15*). Two field trials were performed in Tanzania in order to test the molluscicidal activity of *S. madagascariensis* in a natural habitat. The results of the trials showed that an initial molluscicide concentration of not less than 100 mg/l was reached. The densities of aquatic snails dropped to zero one week after a single application of *S. madagascariensis* water extract. The snails were observed only at low densities and never reached the initial density during the short-term and long-term follow-up period of five months (*16*).

	R_1	R_2
34	CH_3	H
35	CHO	H
36	CH_3	Glc
37	CHO	Glc
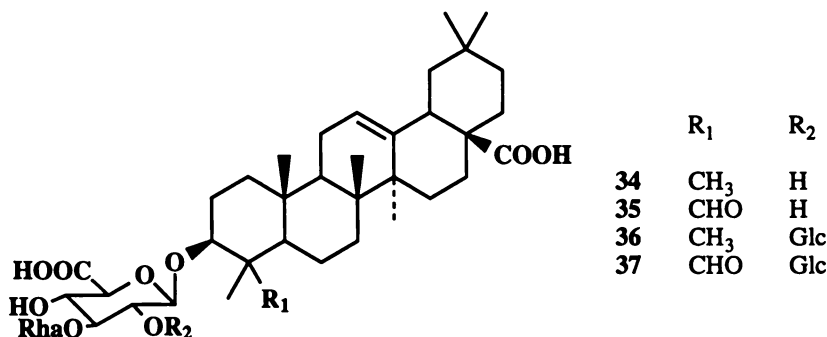
Another legume also retained our attention. *Tetrapleura tetraptera* is a large tree growing throughout the rain forest belt of West Africa. Because of the strong molluscicidal properties exhibited by the fruits of *T. tetraptera* in laboratory experiments, field trials have been carried out and this tree is now considered to be a promising plant for the local control of schistosomiasis. In a reinvestigation of the plant constituents, three new active saponins (**38-40**) were isolated together with the previously described aridanin (**41**) by a combination of CC on silicagel, MPLC on RP-8, droplet countercurrent chromatography (DCCC) and gel filtration on Sephadex LH-

20. These compounds, which are responsible for the molluscicidal activity of the fruits, are *N*-acetylglycosides, with either oleanolic acid or echinocystic acid as aglycone (*17*). Glycosides **39** and **40** are among the most powerful natural molluscicides and have potencies similar to those isolated from *P. dodecandra* and *S. madagascariensis*. A long study, over 2-year period, was carried out in the area of Ife-Ife (Nigeria). The density of snails was reduced by a factor of 30 during the first weeks after application, and the transmission sites were kept free from cercariae for a minimum of 1 month after application of molluscicide (*18*).

	R_1	R_2	R_3
38	OH	H	H
39	H	H	Gal
40	H	Glc	H
41	H	H	H
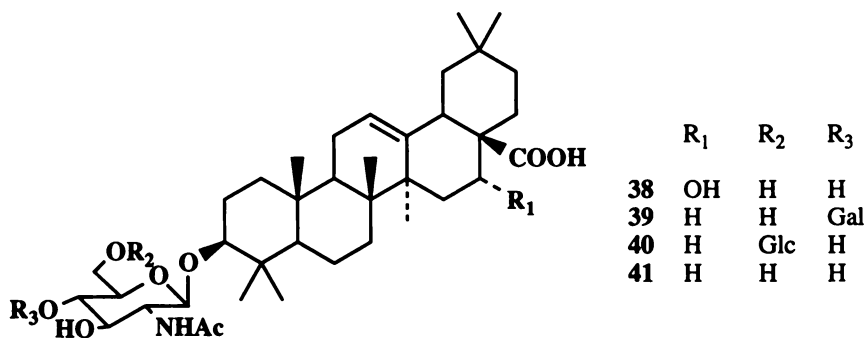
Our screening program is not confined to African species. Systematic efforts are also being devoted to Asian and South American plant species. Indeed, while some very promising African species have so far emerged from research programs aimed at the discovery of plant molluscicides, there is still an urgent need for efficacious plants in several Asian and American countries. *Sapindus rarak* (Sapindaceae) occurs naturally on the islands of Southeast Asia and produces fruits that are not used for food purposes. From the molluscicidal methanol extract of *S. rarak* fruits collected in Indonesia, four saponins (**42-45**), among them three *O*-acetylglycosides, have been obtained through activity guided fractionation. The four saponins exhibited comparable molluscicidal activity with LC_{100} of 6.25 (**42, 43 and 45**) and 12.5 mg/l (**44**) (*19*). Compounds **44-45** were later also isolated from *S. saponaria*, a South American species producing large quantities of fruits which are used by poor people for laundry. *S. saponaria* grows notably in regions of Brazil where schistosomiasis is endemic. It has been known for a long time to possess molluscicidal properties (*20*).

An efficient analytical method is necessary for the quantitative determination of saponins in plant material from different strains and geographical locations. Quantitative analysis is also important for the monitoring of extraction procedures in order to maximize the yield of saponins for the most effective application to infected sites. Lastly, information on the content of active saponins in treated water is essential for biodegradation and toxicological studies. Separations by HPLC have previously been carried out with detection at 206 nm owing to the poor absorption of saponins at higher wavelengths. Consequently there are limitations concerning the solvents and gradients that can be used and sensitivity remains low. One solution to this problem is the introduction of a chromophore in the saponins which enables UV detection at 254 nm. As the majority of the active saponins such as those from *P. dodecandra, S. madagascariensis* and *T. tetraptera* possess a free carboxyl group at the C-28 position, derivatization can be carried out at this function. Encouraging results have been obtained by derivatization of the saponins with 4-bromophenacyl bromide in presence of a crown ether. Quantitative analysis of a derivatized extract of *P. dodecandra*, for example, can be achieved by HPLC on RP-18, using a gradient of acetonitrile in water and detection at 254 nm (*21*). Alternatively, a mode of detection such as evaporative light scattering or

mass spectrometry, better suited to saponins than UV can be used. HPLC-MS, which is assuming increasing importance for the analysis of saponins, is discussed below.

	R_1	R_2	R_3
42	OAc	OAc	H
43	OAc	H	OAc
44	OH	OAc	H
45	OH	OH	H
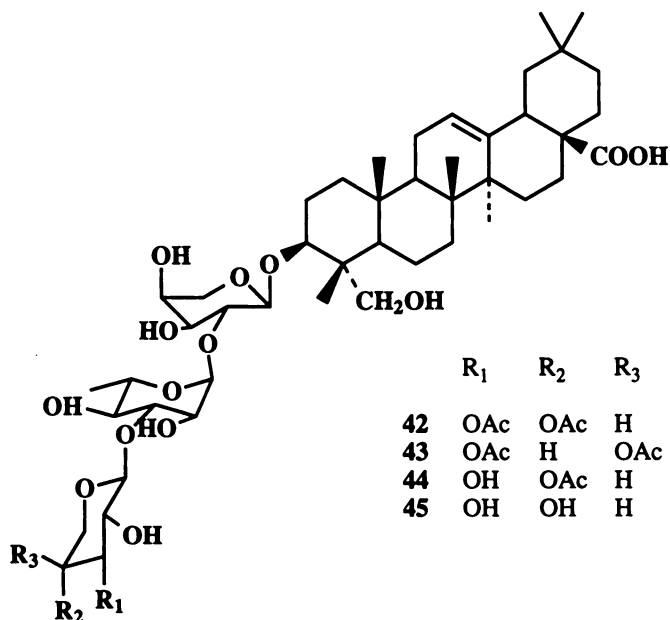
Liquid Chromatography Coupled with Mass Spectrometry (HPLC-MS) and Diode Array Detection (HPLC-UV)

When searching for biologically active compounds, biological screening of extracts followed by activity-guided fractionation is the standard strategy. In this procedure, crude extracts and subsequent fractions are biologically evaluated and those continuing to exhibit activity are further purified until active principles are obtained. A drawback, however, is that the bioassay-guided fractionation strategy can lead to the frequent re-isolation of known metabolites which is time and money consuming. Chromatographic analysis of plant extracts and crude fractions concurrently with biological screening allows recognition of known metabolites at the earliest stage of separation and, consequently, enables targeted isolation of new constituents. During the last decade, hyphenated techniques have been developed which are able to provide at the same time efficient separation of the metabolites, selectivity and sensitivity of detection, and valuable structural information on-line. Mass spectrometry provides a type of 'universal' LC detection and gives important structural information on-line (molecular weight and fragments). By coupling HPLC with both mass spectrometry (HPLC-MS) and UV diode array detection (HPLC-UV) a large amount of data can be obtained about the constituents of a plant extract before starting any isolation work. Indeed UV spectra give useful complementary information (type of chromophore or pattern of substitution) to that obtained with HPLC-MS (22).

Such an approach was used for the investigation of *Swertia calycina*, a small Gentianaceae species from Rwanda. The dichloromethane extract of *S. calycina* showed a strong inhibition of *C. cucumerinum*. HPLC-UV and HPLC-Thermospray-MS (HPLC-TSP-MS) analyses revealed the presence of three main compounds: a bitter principle, a xanthone and a quinonic derivative with a MW of 188 (Figure 1).
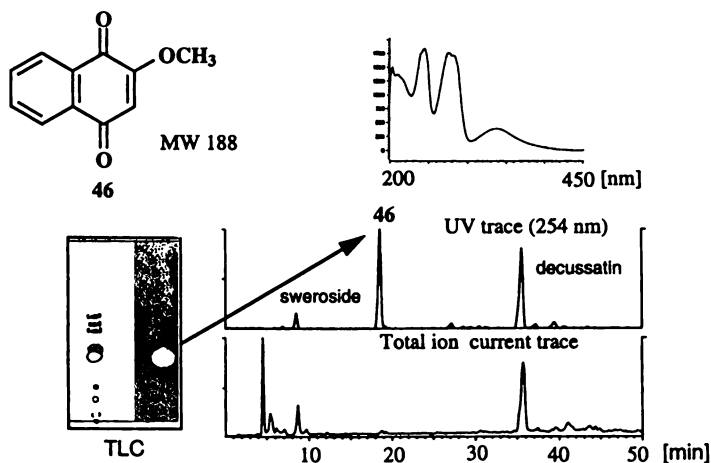
Figure 1. HPLC-TSP-MS and HPLC-UV analyses of the CH$_2$Cl$_2$ extract of *S. calycina*. (RP-18, MeCN-H$_2$O 5:95 -> 65:35, 0.05% TFA, positive ion mode, source 280°C, vaporizer 100°C). A TLC plate with UV (left) or bioautographic (right) detection is also shown. (Adapted from ref. 23).
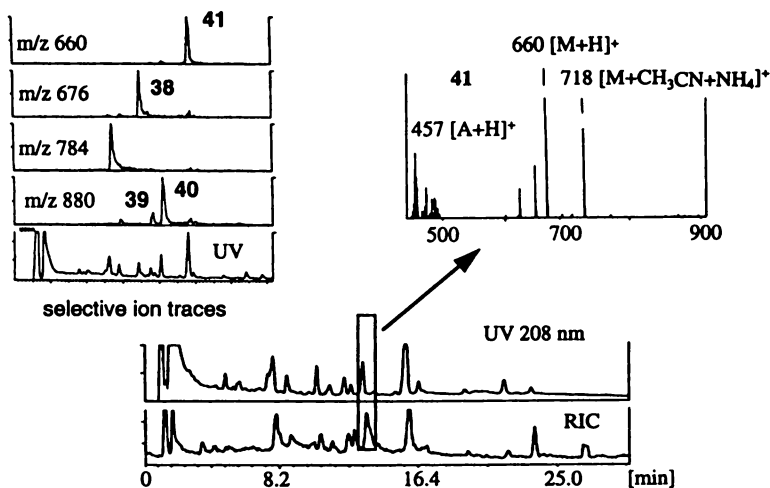

Figure 2. HPLC-TSP-MS of the saponins contained in a MeOH extract of *T. tetraptera* fruits (RP-18, MeCN-H$_2$O 3:7 -> 8:2 positive ion mode, source 270°C, vaporizer 100°C). (Adapted from ref. 24).

Comparison of on-line data with a data bank allowed identification of the bitter principle as sweroside and the xanthone as decussatin. As these have no antifungal properties the strong activity of the dichloromethane extract was attributed to the naphthoquinone, a class of compound which is known to have strong bactericidal and fungicidal properties. This compound has been selectively isolated and identified as 2-methoxy-1,4-naphthoquinone (**46**). Interestingly, quinones were previously not known to occur in Gentianaceae. The minimum quantities of **46** required to inhibit the growth of *C. cucumerinum* and *C. albicans* on TLC plates were 0.1 and 0.4 µg, respectively (*23*).

HPLC-MS is particularly well suited to the analysis of saponins. This technique provides sensitive and selective detection without requiring any derivatization and can be thus used for quantitative determination. At the same time, highly valuable structural information, concerning in particular the glycosylation pattern can be obtained. As an example, the HPLC-TSP-MS analysis of the saponins contained in an extract of the fruits of *Tetrapleura tetraptera* is shown in Figure 2 (*24*). The TSP total ion current trace (mass range 450-900 u) coincided well with the UV trace at 208 nm. Each saponin could also be selectively revealed by display of selected ion traces. The TSP spectra acquired on-line for each saponin displayed strong [M+H]$^+$ quasi-molecular peaks together with adduct species, such as [M+CH$_3$CN+NH$_4$]$^+$. Fragmentation of the sugar moiety was observed and peaks corresponding to the loss of one (mono- and diglycosides) and then two sugars (diglycosides) were present, thus giving information on the number, the nature and the sequence of the sugar components, as well as the molecular weight of the aglycone. In a similar manner, HPLC-MS has been successfully applied to the analysis of saponins in several molluscicidal plants, *S. madagascariensis* and *P. dodecandra* in particular.

Conclusion

Natural products will continue to function as very important lead compounds. In the future, the combination of simple biological assays (enabling rapid screening of plant extracts) with powerful hyphenated analytical techniques will provide, at an increasing rate, new active compounds with novel structures. The ultimate success of such research will however depend on the effective collaboration between chemists, biologists and pharmacologists. Only if such a collaboration exists will promising molecules be further developed and find application as drugs or pest control agents.

Acknowledgments

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Chapter 3

Tropical Timber Species as Sources of Botanical Insecticides

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Screening of wood extractives from tropical timber species and other trees against pestiferous lepidopterans such as the tobacco cutworm, *Spodoptera litura* and the variegated cutworm, *Peridroma saucia*, indicates the presence of insecticidal and growth inhibitory factors in certain genera. The mahogany family (Meliaceae) includes several important timber species in the genera *Swietenia, Khaya, Cedrela* and *Entandophramgma*, but also includes the Indian neem tree, *Azadirachta indica*, already well-known as a source of potent botanical insecticides. Strong bioactivity against insects has been observed in extracts from species of *Aglaia, Trichilia* and *Chisocheton*. In most cases, limonoid triterpenes, characteristic secondary metabolites of the Meliaceae, are responsible for bioactivity, but in the case of *Aglaia*, the insecticidal principles are modified benzofurans. Extractives from several genera of the African walnut family (Olacaceae) have potent bioactivity as larval growth inhibitors, but this family has had sparse chemical characterization to date. Extractives from the Dipterocarpaceae, the most important family of timber species in tropical Asia, were essentially inactive in our screening program. However, bark, woodwaste and sawdust from certain commercially harvested timber species could be exploited for their biologically-active constituents.

Increasing political and consumer pressures aimed at reductions in the usage of synthetic chemical insecticides as crop protectants are creating new opportunities for "alternative" pest control products. Among these alternatives are microbial insecticides, botanical insecticides, and insect growth regulators, of either natural or synthetic origin. Botanical insecticides, relegated to near trivial market status after the introduction of synthetic insecticides in the 1940s (viz. organochlorines, organophosphates, carbamates, pyrethroids), are enjoying renewed interest from both

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the scientific and agricultural communities.   There is general agreement that tropical forests represent vast unexploited storehouses of potentially useful natural chemicals.

According to Wink (*1*) roughly 30,000 plant secondary metabolites have been reported (to 1993), but as probably less than ten percent of terrestrial plants have been phytochemically analyzed, the actual number of secondary metabolites likely exceeds 100,000. Floral species diversity reaches its' greatest density in tropical rain forests, where as many as 200 species of trees alone may occur per hectare (*2*). It is not surprising then, that tropical rainforests are often the focus of exploration for pharmaceuticals and other useful chemicals (*3-6*).

At present, only five botanical insecticides are approved for use in the United States, but all of these are of tropical origin. Pyrethrum, rotenone and sabadilla are obtained from milling and/or extraction of herbaceous plants, ryania insecticide represents the ground stemwood of the tree *Ryania speciosa* (Flacourtiaceae), and neem is an extract of the seeds of *Azadirachta indica* (*7*). As these botanical insecticides are essentially plant extracts with varying stages of refinement, but rarely exceeding 25% active ingredient(s) by weight, an important consideration in the commercial development of a new botanical insecticide is the ongoing availability of the raw commodity. If the desired insecticidal principles occur in the plant in especially low concentrations, then tonne quantities may be required for primary extraction.

For reasons of availability and cost, industrial byproducts and wastes are attractive sources of plant material from which extracts can be made. Seeds, obtained as byproducts of the fruit juice industry, are one potentially useful source of starting material for botanical insecticides (*7*). Wood wastes from the forest industry are another. We have previously demonstrated that crude extracts of wood and/or bark from tropical trees can have strong inhibitory effects on insect growth (*6, 8-9*). In most commercial harvesting of timber, bark is stripped off the bole of the tree at the site of felling, and the crown of the tree is similarly left in the forest. As a result, at least 20% of the biomass of the tree may be discarded before the timber is processed. At the sawmill, about 30% of the log is converted to "waste" (primarily as sawdust) during processing (*10*). However, efficient forest products companies are recognizing the potential of this "waste" and it is starting to find uses in non-lumber products, such as particle-board, fiber-board, or, in North America, as decorative mulches for the landscape industry. Gums, resins and essential oils emanating from cut wood may also eventually find uses as crop protectants. For example, crude resins from certain timber species in the family Dipterocarpaceae from Indonesia are insecticidal to termites (*11*).

Insect Bioassays

Where the goal of screening plants is to discover new pesticides, there has been much discussion as to the most appropriate bioassay organism and endpoint. Many multinational chemical companies use a battery of *in vitro*, or even cell-free

bioassays, targeting specific enzymes, receptors or biochemical pathways. While such bioassays require very low amounts of test material and have a high throughput, they have not proven reliable at predicting efficacy under real-world (i.e. field) conditions (*12*). Brine shrimp, *Artemia salina*, are used in several laboratories as a general screen for bioactivity of plant consitutents, and in guiding fractionation of extracts and isolation of active principles (*13*). They have been especially useful in the isolation of acetogenins from the family Annonaceae, compounds that have a wide range of bioactivities, including insecticidal action (*14*). However, toxicity to brine shrimp is not generally a good predictor of toxicity to insects (*15*). Other investigators have used mosquito larvae (generally *Aedes aegypti*) as a screening organism; many of the convenient features of the brine shrimp bioassay carry over to bioassays with mosquito larvae (*16-17*). If the goal of the research is the development of a mosquito abatement product, then the merit of this bioassay is obvious. On the other hand, if the goal is a broad spectrum insecticide, or at least a product efficacious against phytophagous insects (viz. pests of agriculture and forestry), then the mosquito larvae bioassay is probably a poor predictor. Plant extracts and natural products that are highly active against mosquito larvae often prove to have weak activity, if any, against phytophagous insects (*16,18*).

For our research program, aimed at the discovery and development of botanical insecticides, we routinely use two species of noctuid caterpillars for screening crude plant extracts and for guiding extract fractionation and isolation of active compounds. These are the tobacco cutworm (=Asian armyworm), *Spodoptera litura*, and the variegated cutworm, *Peridroma saucia*. Both insects are highly polyphagous crop pests. They are robust species and therefore conservative models; compounds that are effective against these species are likely to be effective against other lepidopterans, if not a wider range of insect pests. However, it is quite possible to miss important bioactivity by screening with noctuids alone. For example, Ahn et al. (*19*), discovered that gingkolides, sesquiterpenes from *Gingko biloba*, are potent insecticides against the brown planthopper (*Nilaparvata lugens*), even though they are essentially inactive against the armyworm *S. litura*.

Our basic bioassay is based on the addition of crude methanolic plant extracts to an artificial medium on which neonate larvae are allowed to feed for ten days at 25°C and then weighed (for examples, see *8-9*). We screen crude extracts at 1000 ppm fresh weight (0.1%); pure compounds isolated by us or provided by others are screened at 50 ppm. EC50 values are obtained by interpolation from dose-response relationships based on four or five concentrations of extracts or pure compounds. Note that in this bioassay, growth inhibition can arise from either behavioral inhibition (i.e. an antifeedant effect) or a post-ingestive physiological effect; the bioassay does not discriminate between these possibilities. A rapid bioassay has recently been developed to expedite the isolation of insect antifeedants from plant extracts (*20*). This is a reasonable strategy, given that every compound originally isolated due to its antifeedant properties that we have investigated has been subsequently shown to have other physiological actions on insects (*21*).

Plants Investigated

Several different strategies can be used in the search for bioactivity in plants (3). We have used two in our research program: screening species within a taxonomic group (plant family) from which bioactive species are well known or have been discovered, and random screening of common commercial timber species in certain tropical countries.

The Mahogany Family (Meliaceae). The mahogany family has proven to be a profitable taxon to investigate in the search for natural products bioactive against insects. Members of this family are chemically characterized by the production of a class of triterpenoids called limonoids, many of which have demonstrated bioactivity against insects (22). The most potent of these is azadirachtin, the major insecticidal principle in seeds of the Indian neem tree, *Azadirachta indica*. Refined neem seed extracts have been commercialized in North America as the newest botanical insecticide, although the use of neem in India for pest control dates back thousands of years (for a complete review, see 23). It is worth noting that azadirachtin was first isolated, in 1968, on the basis of its outstanding antifeedant effect on the desert locust, *Schistocerca gregaria*, and it remains the most potent antifeedant for that insect yet discovered. It was only later observed that azadirachtin is also a potent natural insect growth regulator, acting largely through interference with the release of molting hormones. On the other hand, the structural requirements for this growth-regulating activity are quite specific; species producing these compounds are restricted to *Azadirachta* and the closely related genus *Melia* (24). Most of the limonoids isolated from the Meliaceae are far less active in insects (25).

This plant family is also of interest to us because it includes some economically important timber species: the true mahoganies (*Swietenia* species), the African "mahoganies" (*Khaya* species), and the tropical "cedars" (*Cedrela* and *Toona* species). To date, in collaboration with the laboratories of Drs. Towers and Arnason, we have examined over 100 species in the Meliaceae, representing about one-half of the recognized genera (6, 26-27). From our screening studies, and based on reports by other investigators, it is obvious that there are a number of genera in the family that are highly active against insects.

We have focussed much of our effort on three genera: *Aglaia, Chisocheton,* and *Trichilia* (8-9, 28). More than a third of the 19 *Aglaia* species screened were significantly active in our bioassay, as were half of the dozen *Trichilia* species and 4 of 6 *Chisocheton* species. With over 230 recognized species of *Trichilia* and perhaps 150 species of *Aglaia*, the probability of finding additional bioactive species appears high.

In collaboration with our colleagues in Thailand, we isolated the insecticidal principle of *A. odorata* and identified it as the highly substituted benzofuran, rocaglamide (29). Additional insecticidal analogues have been isolated from the same species (30). Rocaglamide is the most potent natural insecticide we have discovered to date (EC_{50} [*Spodoptera*] = 0.33 ppm), and the only one comparable in potency to azadirachtin (EC_{50} [*Spodoptera*] = 0.24 ppm)(also see 31). Table I indicates the bioactivity of crude extracts from some of the more potent species of

Meliaceae we have examined, based on the diet incorporation/larval growth bioassay using *Spodoptera* larvae.

Table I. Bioactivity of Crude Extracts from Selected Species of the Meliaceae

Species	Tissue	Country	EC_{50} (ppm) (95% c.i.)
Aglaia odorata Lour.	twig	Thailand	28 (11-68)
Chisocheton microcarpus Koord. & Val.	leaf	Indonesia	25 (2-317)
Dysoxylum gaudichaudianum (Juss.) Miq.	leaf	Indonesia	159 (15-1673)
Trichilia americana (S. & M.)Pennington	wood	Costa Rica	14 (5-44)
Trichilia connaroides (Wright & Arn.).	bark	India	185 (118-252)

Extracts from most of the species shown in Table I have EC_{50} values of less than 100 ppm, comparing favorably with values for the major commercial botanical insecticides, pyrethrum (98 ppm) and rotenone (163 ppm). The *Dysoxylum* extract is also noteworthy in that it causes molting disruption when applied topically to nymphs of the milkweed bug, *Oncopeltus fasciatus* (unpubl. data). This species may possess a natural insect growth regulator acting in a manner similar to that of azadirachtin.

Commercial timber species of the Meliaceae have only modest bioactivity, at least according to our preliminary screening studies. A bark extract of *Swietenia mahogani* was found to be active (at 2000 ppm) against the variegated cutworm (*6*), and foliar extracts also proved to be active against this insect (*26*). Seed extracts of *S. humilis*, containing fissinolide-type limonoids, are inhibitory to larval growth of the yellow mealworm beetle, *Tenebrio molitor* (*32*). Wood and bark extracts of spanish cedar, *Cedrela odorata*, are only moderately active against the variegated cutworm, but the limonoid gedunin can be readily isolated in large quantities from this species. While gedunin is not appreciably active against *P. saucia* and *S. litura*, it is toxic to the European corn borer, *Ostrinia nubilalis* (*33*), as well as to aphids and earwigs (Arnason *et al.*, this volume). This again points out the need to evaluate plant extracts and putative active principles against a spectrum of pest insects.

Apart from neem, the only other species currently used in the production of a botanical insecticide on a commercial scale are *Melia toosendan* and *M. azedarach* (common name = chinaberry). Some authorities consider the former species to be synonymous with the latter. From the bark of these trees a botanical insecticide is manufactured in the Peoples' Republic of China. The active ingredients (3% by weight) in the bark extract are amoorastatin-type limonoids, dominated by the compound toosendanin (*7*). Pure toosendanin is moderately active in bioassays with *P. saucia*, having an EC_{50} value of 78 ppm. However, a refined bark extract containing only 60% toosendanin was almost twice as active (EC50 = 41 ppm)(*34*).

The African Walnut Family (Olacaceae). Random screening of wood samples collected in Malaysia resulted in the serendipitous discovery of considerable larval growth inhibitory activity in extracts of *Ochanostachys amentacea*, a large member of the family Olacaceae. This pantropical family of trees and woody shrubs includes a few species recognized as minor use timbers. We obtained plant material from eleven species in this family, five of which have considerable bioactivity against *S. litura* in our bioassay (Table II).

Table II. Bioactivity of Crude Extracts From Selected Species of the Olacaceae

Species	Tissue	Country	EC50 (ppm)(95% c.i.)
Coula edulis Baill.	Bark	Nigeria	393 (117-669)
Malania oleifera Chun & Lee	bark	P.R. China	149 (25-868)
Minquartia guianensis Aubl.	Wood	Costa Rica	88 (7-166)
Ochanostachys amentacea Mast.	Wood	Malaysia	96 (12-727)
Schoepfia schreberi Gmel.	Wood	Costa Rica	402 (122-684)

Of these, the most active extract is that from the wood of *Minquartia guianensis*, sometimes called black manwood. This species grows to 100 feet in height and "...is highly valued for its durability and strength" (*35*). Interestingly, the stembark of this species is used by indigenous peoples in South America as a medicine and fish poison; these bioactivities may be attributable to the cytotoxic polyacetylene, minquartic acid (*36-37*). However, in our investigation, the bark extract was essentially inactive at 1000 ppm, whereas the wood extract was active. The following other species of Olacaceae have also been screened:

- *Citronella sauveolens* (Bl.) Howard
- *Scorodocarpus borneenesis* (Baill.) Becc.
- *Strombosia ceylanica* Gardn.
- *Strombosia javanica* Bl.
- *Strombosia philippinensis* (Baill.) Rolfe
- *Ximenia americana* L.

Among these species, twig extracts of *C. sauveolens* and *S. ceylanica* showed moderate activity: 74% and 86% inhibition of *S. litura* larval growth relative to controls at a dietary concentration of 1000 ppm.

Random Screening of Tropical Timber Species. Another approach to finding bioactive species suitable for exploitation is to investigate commercially-harvested timbers. In December 1992, samples of cut lumber representing twelve tree species were obtained from a sawmill near Tawau, Sabah (Borneo), Malaysia. Samples representing a further seven species were cut from logs imported from Indonesia to

Vancouver by a forest products company. Methanolic extracts were screened against the variegated cutworm (*P. saucia*) at 1000 ppm as described above.

Extracts from 18 of the species, including some of the major timber species of southeast Asia, were inactive at the screening concentration. Those species are shown in Table III. However, a crude extract from one of the wood samples collected in Sabah proved to be exceptionally active (EC_{50} = 4 ppm). Additional samples of this species collected elsewhere in Malaysia were all found to be highly active. We have recently isolated a series of active principles from this species, and elucidation of their structures is nearing completion. We anticipate a patent application to protect this novel finding and therefore the identity of this species is not revealed in the present paper.

Table III. Tropical Timber Species Producing Inactive Wood Extracts

Local Name	Common Name	Family	Genus-Species
Agathis	Agathis	Araucariaceae	*Agathis philippinensis*
Merbau	Moluccan ironwood	Caesalpiniaceae	*Intsia palembanica*
Keruing	Wood oil tree	Dipterocarpaceae	*Dipterocarpus* spp.
Kapur	Camphor tree	Dipterocarpaceae	*Dryobalanops lanceolata*
Selangan	Yellow seraya	Dipterocarpaceae	*Shorea hopeifolia*
Bangkirai	Bangkirai	Dipterocarpaceae	*Shorea laevis*
Red seraya	Red seraya	Dipterocarpaceae	*Shorea* spp.
Markabank	Meranti merah	Dipterocarpaceae	*Shorea* spp.
Lampung	White meranti	Dipterocarpaceae	*Shorea* spp.
Kamar		Dipterocarpaceae	*Shorea* spp.
Serayaminak	Seraya	Dipterocarpaceae	*Shorea* spp.
Kenuar		Dipterocarpaceae	*Shorea* spp.
Kayu malam	East Indian ebony	Ebonaceae	*Diospyros* spp.
Belian	Borneo ironwood	Lauraceae	*Eusideroxylon zwageri*
Acacia	Acacia	Mimosaceae	*Acacia mangium*
Obah Suluk		Myrtaceae	*Eugenia* spp.
Kayu cina		Podocarpaceae	*Podocarpus rumphii*
Kelempayan	Laran tree	Rubiaceae	*Anthocephalus cadamba*

Comparison of Extracts to Existing Botanical Insecticides.

To properly assess the potential of the plant extracts we have investigated, it is necessary to compare their bioactivities to those of commercial botanical insecticides. Table IV indicates the potency of some botanical insecticides in the *Spodoptera* larval growth bioassay.

Table IV. Bioactivity of Commercial Botanical Insecticides

Insecticide	% a.i.*	EC50 (ppm)(95% c.i.)
neem (refined seed extract)	25	0.2 (0.05-0.90)
pyrethrum (oleoresin)	20	98 (8-1460)
rotenone	95	163 (3-14000)
ryania	0.05	117 (41-337)

*a.i. = active ingredient

When the concentration of active ingredients is taken into consideration, the most active insecticides are neem and ryania. Pyrethrum is significantly less active than these two, and rotenone, though reputed to be a stomach poison effective against chewing insects, is least effective. These results corroborate those obtained using a similar bioassay (albeit at a single concentration) with the tobacco budworm, *Heliothis virescens*, as the test species (*38*). On the other hand, pyrethrum and rotenone are acute toxins and measuring inhibition of larval growth from exposure to low dietary concentrations may not realistically reflect the potency of these materials as pest control agents.

Nonetheless, we take encouragement from the observation that several of our more active crude methanolic extracts have bioactivity comparable to that of the commercial materials (i.e. EC_{50} values < 100 ppm). Methanol is a useful solvent for preliminary screening of plant material in that it can extract compounds representing a wide polarity range. However, once an active principle is identified, it should be possible to use other solvents and/or methods (e.g. supercritical fluid extraction, microwave-assisted extraction, steam distillation) capable of optimizing the yield of the desired compound(s). Overall, our philosophy in developing new botanical insect control products is to keep processing of plant material to a minimum. Any steps needed to refine initial plant extracts can drive up the final price of the product considerably, as is the case for neem insecticides. On the other end of the spectrum is ryania insecticide, which basically consists of finely-milled stemwood of *Ryania speciosa* (50% by weight) and an inert diluent such as clay (50%). Soft methanol extraction (i.e. overnight at room temperature) of wood or bark normally yields from 3-10% extractable material by weight. Therefore, even a simple extraction concentrates the insecticidal principles 10-30 fold.

Summary

Preliminary screening studies indicate that certain tropical timber species and related tree species have potential as leads for the development of new botanical insecticides. Even our cursory examination of the families Meliaceae and Olacaceae have revealed species from which extracts of wood, bark or foliage possess potent bioactivity against our conservative insect models. In one case, we have discovered a new group of insecticidal natural products, the *Aglaia* benzofurans, that are

comparable in toxicity to azadirachtin. Whether sufficient natural biomass of *Aglaia* exists to permit sustainable harvest remains to be seen; we are also pursuing total synthesis of these compounds.

We have identified some species of trees (e.g. *Trichilia, Minquartia*), that while not sufficiently common to be harvested for timber, may be so for extraction of high value phytochemicals. We have not found striking bioactivity in extracts from the commercial timber species within the Meliaceae (e.g. *Cedrela, Swietenia, Khaya, Entandrophragma*), but we have not exhaustively investigated these genera either. Further investigation of these trees, especially where large quantities of wood waste are being generated, is warranted.

Finally, excepting one species, our random examination of some economically important timber species of southeast Asia did not reveal any useful bioactivity, although more concentrated extracts, e.g. essential oils, from these species could prove to have useful bioactivity against specific insects. On the other hand, screening of only 19 species led to the discovery of a species producing a potent insecticidal extract. Random screening of tropical timber species from other regions, irrespective of plant family, may yet prove to be a worthwhile search strategy.

It must be remembered that identifying sources with useful biological activity is only the starting point in the long process of developing a botanical pest management product. Important practical issues are the ongoing availability of the natural resource, adequate biomass (usually tonne quantities) to justify extraction, the feasibility of extraction near the harvest site, and the stability of the extract in storage after preparation. Regulatory agencies are becoming more sympathetic toward the chemical complexities of botanical preparations and the difficulty in fully characterizing such preparations, but the necessity to identify and standardize at least the putative active ingredients remains. Finally, natural products can, under certain circumstances, pose appreciable health and safety risks, and therefore, all botanical preparations proposed for use in pest management must be subjected to acute and subacute toxicological evaluation at the minimum, as well as tests for phytotoxicity.

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Chapter 4

Efficacy of Botanicals from the Meliaceae and Piperaceae

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Efficacy trials were undertaken with Meliaceae and Piperaceae botanicals against corn leaf aphid and European earwigs. Piperaceae botanicals were most active aginst earwigs because of their knockdown effect while Meliaceae botanicals were more effective against corn leaf aphids in field trials. New tropical American botanicals were found to have comparable efficacy to established Asian and African botanicals. In trials with European corn borer or mosquito larvae, the efficacy was improved by the addition of an all natural synergist, dillapiol, or the repellent, linalol.

Botanical pesticides have received renewed interest because of poor public opinion of synthetics and the increase in organic growing practices (1). Besides their natural origin, botanicals are valued for their low environmental persistance and alternate modes of action, although their instability and lower efficacy compared to synthetics are barriers to development. However, the recent registration of a neem product for food crops by W.R. Grace (2) suggests there is commercial potential for a new type of botanical with an antifeedant and growth reducing mode of action.

While active phytochemicals from new botanicals remain important leads for the development of new synthetic pesticides, with clear commercial advantages (3), we provide some results here on an alternative approach through the development of standardized extracts. Botanical extracts have different biological characteristics from pure compounds. Mixtures may be more effective due to synergism or joint action of actives with different mechanisms. For example Berenbaum et al (4) found that a mixture of 6 furanocoumarins was more effective than an equimolar amount of pure xanthotoxin in reducing growth of *Spodoptera* larva. In addition, the rate at which resistance can develop in recurrently treated pest populations is slower in mixtures than pure insecticides. Isman's group (5) has recently

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shown that peach aphid lines selected with pure azadirachtin, developed a 9 fold resistance to this subtstance over 40 generations, but a parallel line selected with neem seed extracts containing azadirachtin and other materials did not develop significant resistance.

Efficacy and synergy trials with advanced botanicals.

Our phytochemical program on neotropical botanicals has led to the development of a number of new botanical extracts (see companion paper by Durst et al) (Table I, Figure 1). Examination of the neotropical botanicals as insecticides has been restricted to a few species of Lepidoptera and mosquito larvae (6,7,8). In the current study their efficacy was compared with some established Asian and African botanicals in field trials with the corn leaf aphid, and laboratory trials with European earwigs, which are a relatively recent pest of gardens in North America.

In a second part of the study, we have attempted to improve the efficacy and reduce the instablity of these botanicals with two potential synergists. The first of these is the natural source polysubstrate monooxygenase (PSMO) inhibitor dillapiol (8), as a replacement for synthetic piperonyl butoxide (PBO) which is conventionally used in some botanical preparations, but reduces product appeal for organic growers because it is not a natural product. The second agent used in mixtures was the repellent, linalol, found in many aromatic plants and an effective insecticide against the aphid *Rhopalosiphum padi* (9). It was employed as a complementary material to the antifeedant and growth reducing activity of the selected Meliaceae and Piperaceae botanicals.

Effect of Piperaceae and Meliaceae extracts on European earwigs.

European earwigs *Forficula auricularia* L. (Dermaptera: Forficulidae) were collected from corn fields and transferred to the laboratory several days prior to the beginning of this experiment. Within the laboratory, earwigs were maintained on a diet of apples and carrots.

Extracts were in general optimized for the extraction for active secondary metabolites. The *Trichilia hirta* and *T. glabra* extracts were prepared from EtOAc/BuOH, *Cedrela odorata* extracts in toluene and Piperaceae extracts in hexane/EtOAc.

Formulations were prepared on the day of the experiment. Diced apples were allowed to soak in all formulations for a period of 1 hour, blotted dry and allowed to stand for 1 hour, prior to the addition of the insects. Emulsifiable concentrate formulations were prepared as follows (extracts were omitted from carrier controls):

Atlox 3403	34 mg
Atlox 3404	40 mg
extract	50 mg
solvent (xylene)	1 mL

Formulations were diluted in distilled water to produce the various ready to use formulations.

The extracts tested against earwigs all produced significant growth inhibition at 125 ppm and above (Table II). The *Piper* extracts were slightly more effective than the Meliacaeae extracts, with *P. retrofractum* extracts showing the highest growth inhibition. The rating scale showed the greatest effect with *Piper* extracts which is not surprising because of their well known knockdown effect. At 500 ppm and above all insects died in the *Piper* extracts while the Meliaceae extracts were toxic at 1000 ppm. There is little in the way of comparative data for this species which has become a relatively recent pest.

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rocaglamide
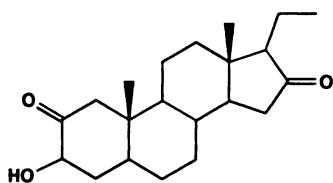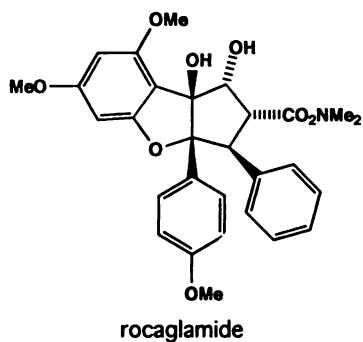
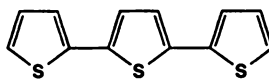3-hydroxypregnane-2,16-dione
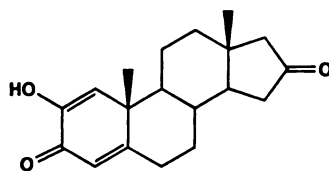
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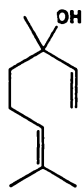
linalol

α-terthienyl

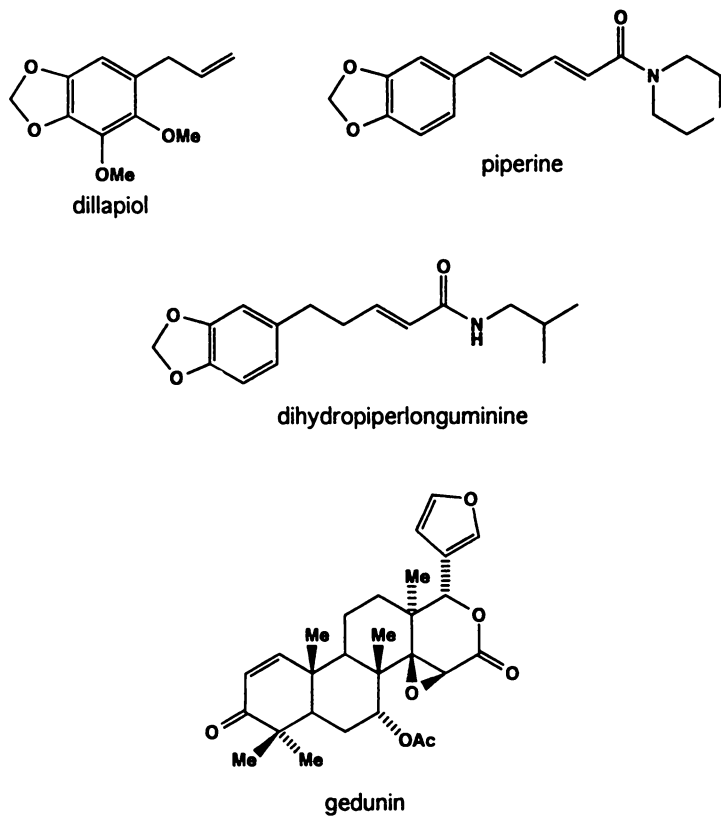Figure 1. Structures of active principles of botanicals used in the present study.

dillapiol

piperine

dihydropiperlonguminine

gedunin

Figure 1. Continued.

Table L Botanical extracts used in this study

Species	Family	Active	Origin
Cedrela odorata	Meliaceae	gedunin	Costa Rica
Trichilia hirta	Meliaceae	3-hydroxy pregnane-2,16-dione, 2-hydroxy androsta-1,4-diene-3, 16-dione	Costa Rica
Trichilia glabra	Meliaceae	unkown	Costa Rica
Piper tuberculatum	Piperaceae	dihydropiper-longuminine	Costa Rica
Piper retrofractum	Piperaceae	piperine	Thailand
Piper guineense	Piperaceae	piperine	Guinea Conakry
Aglaia odorata	Meliaceae	rocaglamide	Thailand

TABLE II: Toxicity of 7 botanicals, at 5 different concentrations, on earwigs, after 24 hours. Means of extracts followed with the same letter are not signifcantly different (Tukey, P = 0.05).

TREATMENT	CONC.	WEIGHT GAIN (Tukey group)	RATING
Control	0	3.76 (a)	1.0
	125	3.24 (ab)	1.0
	250	2.22 (bc)	1.0
	500	2.53 (c)	1.0
	1000	2.08 (c)	1.0
Cedrela odorata	0	3.85 (a)	1.0
	125	1.01 (c)	2.0
	250	0.63 (c)	2.4
	500	0.10 (b)	2.5
	1000	0.00 (d)	4.0
Trichilia hirta	0	3.75 (a)	1.0
	125	0.60 (b)	1.4
	250	0.15 (bc)	1.8
	500	1.01 (c)	2.9
	1000	0.00 (d)	4.0
Trichilia glabra			
	0	3.74 (a)	1.0
	125	1.53 (b)	1.6
	250	1.01 (b)	1.6
	500	0.00 (b)	2.2
	1000	0.00 (c)	4.0
Piper guineense	0	3.71 (a)	1.0
	125	1.53 (b)	1.2
	250	1.01 (b)	1.9
	500	0.00 (c)	4.0
	1000	0.00 (c)	4.0
Piper tub-erculatum	0	3.75 (a)	1.0
	125	0.89 (b)	1.8
	250	0.60 (b)	2.4
	500	0.00 (c)	4.0
	1000	0.00 (c)	4.0
Piper retro-fractum	0	3.68 (a)	1.0
	125	0.70 (b)	1.8
	250	0.54 (b)	2.2
	500	0.00 (c)	4.0
	1000	0.00 (c)	4.0

Note: Weight gain was measured after a period of 24 hours. The rating value was assigned to the insect based on visual observations, as follows:
1) Insect active when disturbed. Insect appears unaffected by extract.
2) Insect movement slow and sporadic when disturbed. Insect appears partially paralyzed and hesitant to feed.
3) Insect appears fully paralyzed and unable to move, even when disturbed.
4) Insect dead.

Field trials with corn leaf aphid:

Corn leaf aphids, *Rophalosiphum maidis* (Fitch) (Homoptera: Aphidae) were raised under laboratory conditions from a colony obtained in the field in Ottawa. Aphid susceptible maize plants (bxbx lacking hydroxamic acids) were grown under greenhouse conditions, until the plants were developed to the mid-whorl stage. At this point, the plants were transferred outdoors to acclimatize for a week, then infested with a colony of five aphids. The plants were then covered with a nylon net, in order to isolate the aphid colony, and ensure that cross-contamination was not possible. The aphid colony was left undisturbed for a period of 7-10 days, in order to allow time for the colony to establish itself and increase.

After the aphid colony was established, the tassels of each plant was sprayed to runoff with the different botanical treatments and carrier controls. Piper extracts were formulated as in earwig trials, but Meliaceae extracts were formulated in MeOH. All treatments were diluted to a final concentration of 500 ppm or 125 ppm. Total number of aphids, number of dead and living aphids were counted after a period of 24 hours (5 replicates). Experiments were undertaken in August 1995.

In the field trials with maize aphids, the treatments showed concentration dependant effects on aphid mortality. All extracts tested at 500 ppm showed significant mortality in aphid populations but the carrying formulations produced no increase in mortality compared to controls (Table III). The Meliaceae extracts were significantly more active than the Piperaceae extracts and at 125 ppm, the *Piper guineense* treatment was not significantly different from the control. Overall the two *Trichilia* extracts appeared most effective in controlling aphids, but were not significantly different from each other or *C. odorata* extracts. The susceptibility of aphids to the Meliaceae extracts is similar to neem extracts that were also effective in controlling green peach aphid and the strawberry aphid at concentrations of 0.2-1.4% (10).

Taken together with previously published results (6-8) these findings suggest that the advanced Meliaceae and Piperaceae extracts have potential for control of aphids and earwigs as well Lepidoptera.

Synergism experiments:

An initial synergism experiment with dillapiol was conducted with the botanical phototoxin alpha-terthienyl with fourth instar mosquito larvae *Aedes atropalpus* Say (Diptera: Culicidae), since a well established lab protocol (12) allowed an accurate determination of LC_{50}'s and the resultant synergism ratio. Dillapiol alone had no significant effect on moratlity in the range tested. Alpha T had an LD_{50} of 39 and using α-T: D ratio of 1:5, the L_5C dropped to 11.3 (Table IV) with a synergism ratio of 3.45.

A second experiment (Table V) was conducted measuring the growth of second instar European corn borer larvae, *Ostrinia nubilalis* (Lepidoptera: pyralidae), given treated diets. Clear evidence was found of synergism between 10 ppm dillapiol and 100 ppm *C. odorata* extracts. There was no evidence of synergism between the *Piper* extract and dillapiol. Piperine already contains the PSMO inhibiting methylene dioxy functionality and may not be ammenable to dillapiol synergism for that reason.

Combinations of the botanical extracts with the monoterpene, linalol, produced additive effects with *C. odorata* and *P. guineense* extracts, suggesting that these materials can work together in a complementary, if not necessarily synergistic way.

TABLE III: Toxicity of botanicals, at two concentrations, on corn leaf aphid, after 24 hours under field conditions.   Means of treatments (back transformed from an arcsin square root transformation) with the same letter are not signifcantly different (Tukey, P=0.05).

TREATMENT	CONCENTRATION	PERCENT MORTALITY	TUKEY GROUPING
blank		5.47	A
water		8.45	AB
MeOH	125 ppm	6.02	A
	500 ppm	6.22	A
xylene	125 ppm	5.65	A
	500 ppm	11.5	ABC
T. hirta	125 ppm	40.81	B-E
	500 ppm	71.17	E
T. glabra	125 ppm	49.66	DE
	500 ppm	74.24	E
P. guineense	125 ppm	26.70	A-D
	500 ppm	46.26	C-E
P. tuberculatum	125 ppm	38.08	B-E
	500 ppm	57.14	DE
C. odorata	125 ppm	35.00	A-E
	500 ppm	65.88	DE
A. odorata	125 ppm	26.96	A-D
	500 ppm	50.66	DE

TABLE IIIb. Results of orthoganal contrasts following an ANOVA on arcsin square-root transformed corn leaf aphid mortality following spray applications of botanicals at 2 concentrations

CONTRAST	PROBABILITY
blank vs water	0.605
blank vs controls	0.683
controls vs treatment	0.0001
125 ppm vs 500 ppm	0.0001
Piperaceae vs Meliaceae	0.041
P. guineense vs *P. tuberculatum*	0.015
T. glabra vs *T. hirta*	0.436
Trichilia spp. vs *A. odorata*	0.003
Trichilia spp. vs *C. odorata*	0.197
A. odorata vs *C. odorata*	0.132

Table IV. Lethal concentration for 50% mortality (LC$_{50}$) of *Aedes atropalpus* larvae treated with alpha-T or alpha-T + dillapiol and UV light

	alpha-T	alpha-T + dillapiol
LC$_{50}$ (ppb)	39.6	11.2
95% confidence limits	25.4-55.2	8.6-15.6

TABLE V: Toxicity of 9 botanicals, at 3 different concentrations, to the European corn borer, after 24 hours. Means followed by the same letter are not signifcantly different (Tukey, P=0.05).

TREATMENT	CONC.	WEIGHT GAIN (mg)	TUKEY GROUPING
Control	10 ppm	3.82	A
	100 ppm	3.89	A
	1000 ppm	3.52	A
C. odorata	10 ppm	2.81	A
	100 ppm	3.15	A
	1000 ppm	1.06	B
C. odorata +Dillapiol	10 ppm	3.74	A
	100 ppm	0.61	B
	1000 ppm	0.45	B
C. odorata + Linalol	10 ppm	2.98	A
	100 ppm	1.84	AB
	1000 ppm	0.69	B
Dillapiol			
	10 ppm	3.03	A
	100 ppm	3.07	A
	1000 ppm	0.69	B
P.guineense			
	10 ppm	3.98	A
	100 ppm	2.75	B
	1000 ppm	1.59	C
Linalol			
	10 ppm	4.85	A
	100 ppm	4.00	A
P.guineense + Dillapiol	1000 ppm	2.60	B
	10 ppm	N/A	
	100 ppm	3.47	A
P.guineense + Linalol	1000 ppm	N/A	
	10 ppm	N/A	
	100 ppm	2.47	B
	1000 ppm	N/A	

Conclusion:

Piper insecticides were effective against highly active European earwig, because of their knockdown effect. The hydrophylic Meliaceae insecticides were more effective against phloem feeding aphids, possibly due to systemic action. The efficacy of the botanicals can be improved with the *Piper* synergist, dillapiol or repellents such as linalol.

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Chapter 5

Botanicals from the Piperaceae and Meliaceae of the American Neotropics: Phytochemistry

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Phytochemicals with insecticidal properties have been isolated and studied
from the neotropical Piperaceae, Meliaceae and related families. Investigation
of 16 neotropical *Piper* spp. led to the isolation of active amides, lignans and
prenylated benzoic acid derivatives. Recent investigations with the Meliaceae
and related families have led to isolation of humilinolides from *Swietenia*, of
C-D spiro-triterpenoids, a new of terpenoids from *Ruptiliocarpon* and new
steroids and limonoids from *Trichilia*.

A recent survey (1) indicates that natural products continue to be an important source of new
agrochemicals. While there are many families of plants of interest (2) for the development of
botanical insecticides, including the Annonaceae, Araceae, Asteraceae, Guttiferae, Meliaceae
and Piperaceae, we have concentrated on the latter two families as bioactive taxa which may
provide useful materials with low mammalian toxicity.

Insecticidal compounds from the Piperaceae

The Piperaceae (Pepper family) have long been used in traditional agriculture as
insecticides and are good candidates for safe botanicals because of their widespread use as
spices and medicinal plants as remedies for stomach, tooth and other ailments (3). The active
compounds include acutely acting amides and slower acting, growth reducing lignans which
may also act as insecticide synergists. Well studied botanicals from this family include extracts
of Guinea pepper, *Piper guineense* from Africa, as well as black pepper, *P. nigrum* and a
medicinally used wild pepper, *P. retrofractum* from Asia. *P. guineense* fruits contain piperine,
trichostachine and N-isobutyl-trans-2-4 eicosadienamide as active agents (4). *P. retrofractum*
fruits produce piperine (Figure 1), pipernonaline, piperoctadecalidine and pipereicosalidine
and a variey of other amides (5). Synergism between co-occurring pipericide and other
insecticidal amides of black pepper has been well established by Miyakado *et al.*(6)

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Less work has been done on new world *Piper spp.* with respect to their insecticidal activity. We recently completed a survey (7) of 16 neotropical *Piper* spp. for insecticidal activity and identified *P. tuberculatum* as the most active insecticidal extract in trials with European corn borer (ECB), *Ostrinia nubilalis* and mosquito larvae, *Aedes atropalpus*. Bioassay guided isolation led to the identification of dihydrolonguminine as the active principle (Figure 1). A second species, *P. aduncum* was found to have an active fraction containing large amounts of the phenylpropanoid dillapiol (Figure 1) which we had identified previously as a potent polysubstrate monooxygenase inhibitor. Similar compounds have been isolated by Nair from Jamaican *Piper* spp. (14). Subsequent study (8) of the active fraction of *P. decurrens* led to the identification of several co-occurring lignans including conocarpon, decurrenal and eupomatenoid-5 and -6 (Figure 2). *P. guanacastense* has recently afforded us several larvicidal prenylated benzoic acid derivatives. Methyl 3-(3-methyl-2-butenyl)-4-hydroxy benzoate (Figure 1) was isolated as the major principle which had noteworthy insecticidal activity to mosquito larvae. While these are not unique in the family (13), they are not common and have been shown to possess insecticidal activity now for the first time.

Tropical Meliaceae

Similarly, the Asian Meliaceae (Mahogany family) are noted for the production of useful antifeedant and growth reducing substances. These include the limonoids which have been commercialized: azadirachtin in the US and toosendanin from *Melia azedarach* in China (17). *Aglaia odorata* extracts from Thailand and neighboring S.E. Asian countries are also promising and contain an active benzofuran, rocaglamide, that is as active as azadirachtin (18). However, as yet no commercial Tropical American product has been developed, although considerable phytochemical work has been done on neotropical species.

Our examination of over 50 extracts of 35 species of neotropical Meliaceae was undertaken to identify a variety of promising extracts against the Lepidopteran pests, ECB and *Peridroma saucia* (9). The most active extract to ECB was *Ruptiliocarpon caracolito*, which was originally thought to be a member of the Meliaceae based on wood anatomy and other characteristics, but has recently been identified as the only American species of the Lepidobotryceae. The insecticidal fraction of this species has now yielded over a dozen distinct C-D spiro-triterpenoids some of which are illustrated in Figure 3. These represent an entirely novel biosynthetic class of terpenoid, possibly produced via a C-13, C-18 epoxyfriedelin derivative (10). The more highly oxygenated compounds of this family, especially spirocaracolitones E and F are very active against corn borer but supply of this rainforest endemic of the Osa peninsula of Costa Rica may limit any potential application. Their antifungal activity was modest as was their activity against malaria parasites (11).

Other active extracts included preparations of the genera *Cedrela*, *Trichilia* and *Swietenia*. The main active ingredient in the insecticidal fraction of wood of *C. odorata* was found to be the the the limonoid gedunin, which has moderate to good growth reducing activity to ECB and can be sourced in quantities from wood rather than scarce seed. An extraction method based on the use of toluene as the extraction solvent has been developed which gives rapid access to large amounts of gedunin. Sawdust obtained from some Central American sources yield up to 0.5% gedunin. In order to study the structural features of gedunin that were essential for activity, MacKinnon (11) synthesized and tested a series of derivatives for testing at 50 ppm in borer diets. Of these, the 1,2 epoxy-derivative and the 23 acetyl derivative were more active than gedunin (Figure 4). The 1,2 dihydro-3β-gedunol was inactive while the 1,2 dihydro-, hexahydro-, 21-acetyl- and 7-deactyl- gedunin derivatives
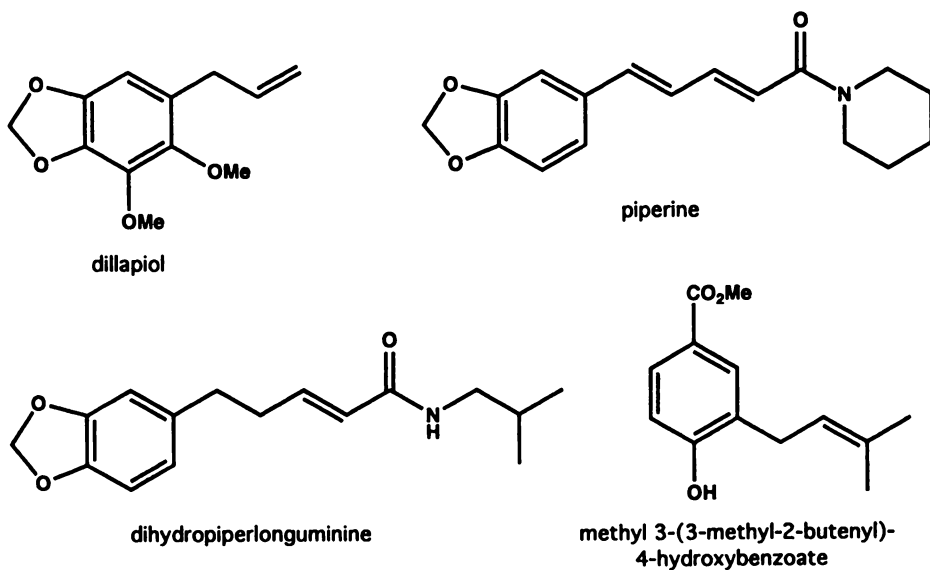
dillapiol

piperine

dihydropiperlonguminine

methyl 3-(3-methyl-2-butenyl)-
4-hydroxybenzoate

Figure 1. Compounds isolated from active neotropical *Piper* spp.
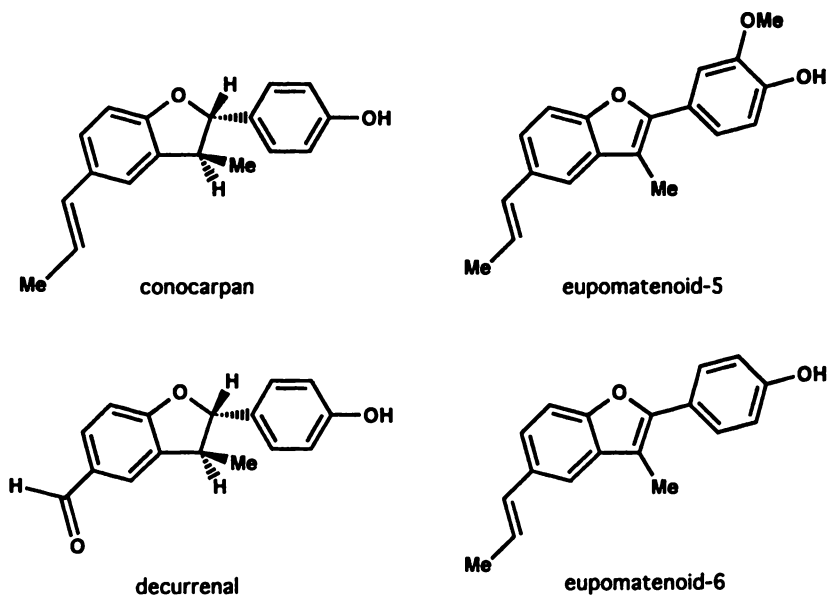
conocarpan

eupomatenoid-5

decurrenal

eupomatenoid-6

Figure 2. Neolignans form *Piper decurrens*

1, spirocaracolitone A
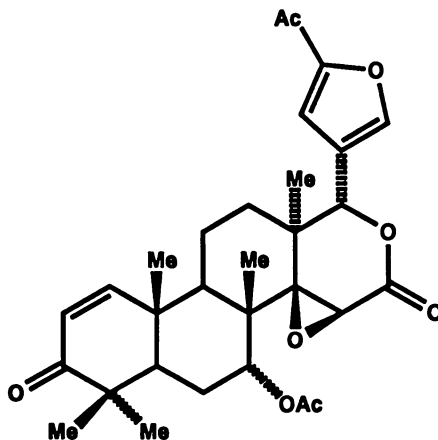

2, spirocaracolitone B: $R_1 = R_2 = R_3 = Ac$
3, spirocaracolitone C: $R_1 = H; R_2 = Ac; R_3 = COPh$
4, spirocaracolitone D: $R_1 = Ac; R_2 = H; R_3 = COPh$

5, spirocaracolitone E: $R = COC(CH_3)=CHCH_3$
6, spirocaracolitone F: $R = COPh$
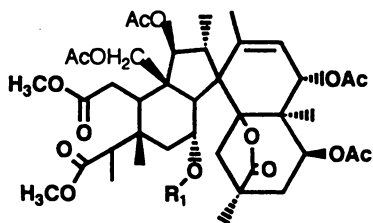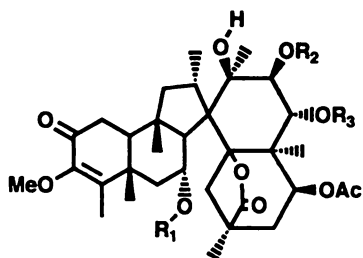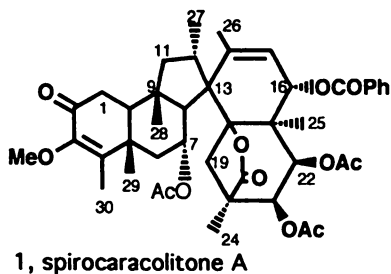
Figure 3   Spiro-caracolitones from *Ruptiliocarpon caraclito*

23-acetylgedunin

Figure 4. Gedunin derivative with greater activity than gedunin.

were less active than gedunin. Gedunin also has a potent antimalarial activity, and these derivatives are being studied in trials with the chloroquine resistant forms of *Plasmodium falciparum* in collabooration with Pezzuto and Angerhoffer at the University of Chicago.

Further study of seven species from the genus *Trichilia* identified *T. glabra* and *T. hirta* extracts as potent growth reducers for Lepidoptera (16). Two new steroidal compounds, 3-hydroxypregnane-2,16-dione and 2-hydroxyandrosta-1,4-diene-3, 16-dione (12) were identified from the insecticidal fractions obtained from *T. hirta* (Figure 5). *Trichilia martiana* fruits yielded two limonoids, methyl angolensate and a new limonoid as well as 2-(Z,Z)-8,11-heptadecadienylfuran (Figure 6). These compounds are currently undergoing evaluation for their bioactivity.

Study of *Swietenia humilis* by Mata's group at the Universidad Nacional Autonoma de Mexico (15) yielded several humilinolides. In evaluations with ECB, some of these compounds (Figure 7) were comparable to toosendanin in insecticidal activity. Extraction of *C. salvadorensis* bark by Mata's group has recently yielded the growth reducing limonoid, cedrelanolide (Figure 8), which is less active than the humilinolides, but could be produced in larger quantities from sawmill waste.

Conclusion:

With over 60 species of *Trichilia* in the Americas and over 100 *Piper* spp. in Costa Rica alone (19), the opportunities for developing useful natural products from the Meliaceae and Piperaceae are promising, since relatively·few of the species have been examined phytochemically. While the activity of the pure compounds has been or is being reported in separate research contributions in the companion paper by Assabgui *et al*, we evaluated the possibility of using standardized insecticidal extracts derived from these materials as practical insecticides.

3-hydroxypregnane-2,16-dione   2-hydroxyandrosta-1,4-diene-3,16-dione
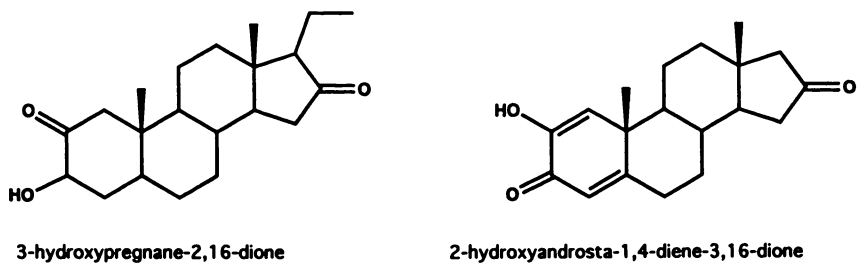
Figure 5. New compounds from *Trichilia hirta* wood.

Methyl angolensate   novel limonoid
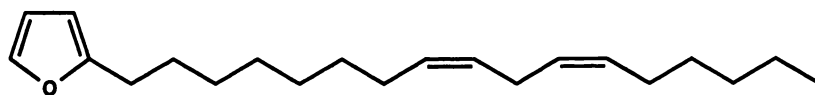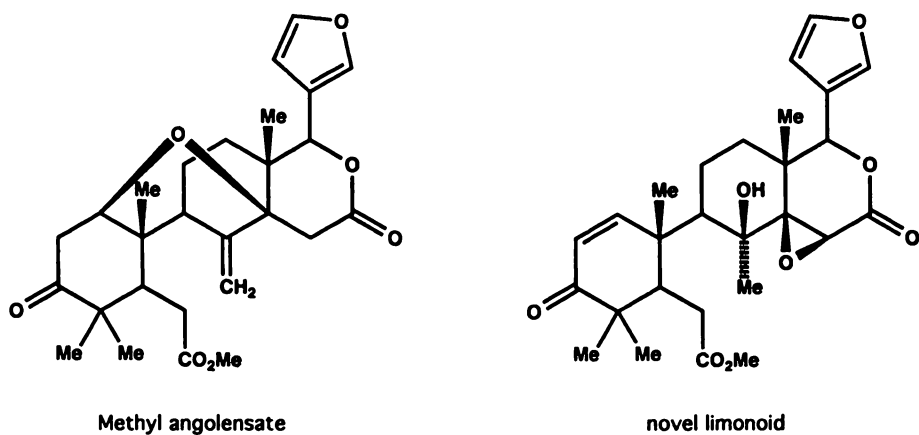
2-(Z,Z)-8,11-heptadecadienylfuran

Figure 6. Phytochemicals from *Trichilia martiana* fruits.
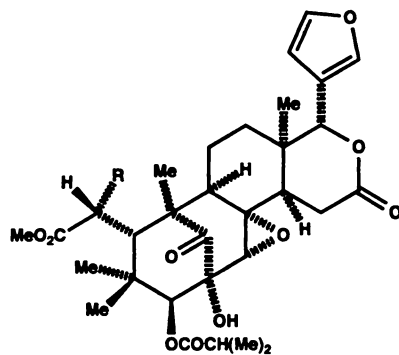
humilinolide A: R = OH
humilinolide B: R = OAc
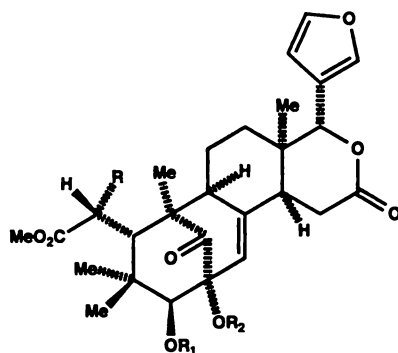
humilinolide C: R = H; R 1 = COC(Me)=CHMe; R 2 = Ac
humilinolide D: R = OAc; R 1 = Ac; R2 = H

Figure 7.Limonoids isolated from *Swietenia humilis.*
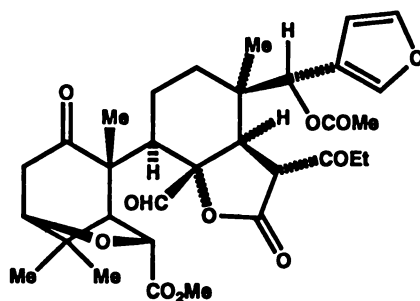
Cedrelanolide

Figure 8. A new rearranged limonoid from *Cedrela salvadorensis*

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Chapter 6

Phylogenetic Links in Plant Defense Systems: Lignans, Isoflavonoids, and Their Reductases

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Many lignans and isoflavonoids derived from (+)-pinoresinol/(+)-lariciresinol and isoflavone reductases, respectively, have important roles in plant defense against pests and pathogens. In addition to their somewhat comparable physiological functions, other similarities are noted between these apparently unrelated branches of phenylpropanoid metabolism which suggest a phylogenetic link. These include similar enzymatic mechanisms, gene sequence homology, and putatively conserved protein phosphorylation sites for the pivotal branch point reductases, as well as comparable roles in [inducible] defense systems. Taken together with a detailed chemotaxonomic analysis of metabolite occurrence, it is proposed that during plant evolution, (+)-pinoresinol/(+)-lariciresinol reductase served as progenitor of the isoflavone reductases.

Considerable emphasis continues to be placed upon deciphering, and ultimately biotechnologically exploiting, the natural defense mechanisms that plants employ in response to encroachment by herbivores, fungi, bacteria and viruses, e.g., as an attractive alternative to the application of pesticides, insecticides and other biocides. In this chapter, the intriguing parallels that have emerged between various lignans and isoflavonoids in plant chemical defenses are addressed. Both natural product classes appear to be derived from seemingly unrelated branches of phenylpropanoid and phenylpropanoid-acetate metabolism, which have originated in the early development of the modern seed plants [see (1) for a brief review of the phenylpropanoid pathway; and note that all references to angiosperm classification in this article are based on Dahlgren (2, 3)]. A number of similarities between the pathways has emerged, including striking homology of genes involved in presumed regulatory reductive steps, which leads to our reasoning that they are phylogenetically linked. Prior to elaboration of this hypothesis, a brief background on lignan and isoflavonoid nomenclature is necessary.

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Nomenclature.

Haworth (*4*) introduced the term "lignane" [later shortened to lignan] to define a class of dimeric compounds derived from cinnamyl units linked together via 8,8' [β,β] bonds, e.g., (±)-pinoresinols **1a/b**, Figure 1. A related term, neolignan, was subsequently proposed for all other coupling products [e.g., megaphone **2** (8,1' coupling), (±)-dehydrodiconiferyl alcohols **3a/b** (8,5' coupling) and *erythro/threo* guaiacylglycerol-8-*O*-4' coniferyl alcohol ethers **4a/b** (8-*O*-4' coupling)], but was later redefined to describe allylphenol coupling systems [e.g., magnolol **5** and isomagnolol **6**] (*5*). However, given that there appear to be only a relatively small number of distinct skeleta, the term *lignan* [or, in some instances, neolignan] can be used to encompass all skeleta provided that the linkage is stipulated. The most commonly observed are those linked via 8,8' bonds, although others are found, such as 8,1', 8,5', 8-*O*-4', 3,3' and 3-*O*-4' [Figure 1] (*1*, *5*). Note also that the 8,8'-linked lignan metabolites can be conveniently further divided into various subgroups, such as substituted furofurans, tetrahydrofurans, dibenzylbutanes, dibenzylbutyrolactones, aryltetrahydronaphthalenes and arylnaphthalenes [see Figure 2 for examples].

Isoflavonoids can be classified into isoflavone- and isoflavanone-derived subgroups and result from the merging of phenylpropanoid [giving rise to the B-ring] and acetate [producing the A-ring] metabolic pathways [Figure 3]. They differ from flavonoids by having a distinctive 3-phenylchroman skeleton, which arises via intramolecular aryl migration of a flavanone [2-phenylchroman] precursor. The isoflavone subgroup, with over 450 representatives reported thus far (*6*), includes the coumaronochromones, coumaronochromenes, substituted isoflavones, isoflavonequinones and rotenoids [Figure 3]. The isoflavanone-derived group [see Figure 4] has over 370 individual structures (*6*), including the isoflavanones, pterocarpanoids, isoflavans, isoflav-3-enes, arylcoumarins and coumestans. The major distinguishing feature between both broad subgroups is reduction of the 2,3 double bond of isoflavones to give rise to the isoflavanones [Figures 3 and 4], a conversion catalyzed by isoflavone reductase[s].

Biosynthesis.

Lignans. The biochemical pathways to the lignans are being delineated using *Forsythia* × *intermedia* Zabel and *F. suspensa* (Thunb.) Vahl [Oleales]. It is established that entry into the optically active 8,8'-linked lignans, bearing oxygenated functionalities at C_9/C_9', occurs through stereoselective coupling of two molecules of *E*-coniferyl alcohol **13** to afford (+)-pinoresinol **1a** [Figure 5]. The ~78 kDa protein responsible for engendering this *first* example of stereoselective bimolecular phenoxy radical coupling has been purified to apparent homogeneity [(*7*), Davin *et al.*, *Science*, 1996, in press]. Importantly, it lacks oxidizing capability by itself, and catalysis proper requires an auxiliary oxidase/oxidant. This is in stark contrast to the effect of all other known phenylpropanoid coupling enzymes, such as peroxidases, phenol oxidases and laccases, which only engender racemic coupling, e.g., with *E*-coniferyl alcohol **13** to give (±)-pinoresinols **1a/b**, (±)-dehydrodiconiferyl alcohols **3a/3b** and (±)-*erythro/threo*-guaiacylglycerol-8-*O*-4' coniferyl alcohol ethers **4a/b**. Consequently, the long-standing enigma of how stereoselective bimolecular phenoxy radical coupling occurs is now finally being resolved. Additionally, given the different [neo]lignan skeleta present in nature, it is our current working hypothesis that an entire class of such coupling proteins exists, albeit facilitating *different* coupling modes. We have coined the term "dirigent" protein [Latin = to align or guide] to describe the new class of proteins.
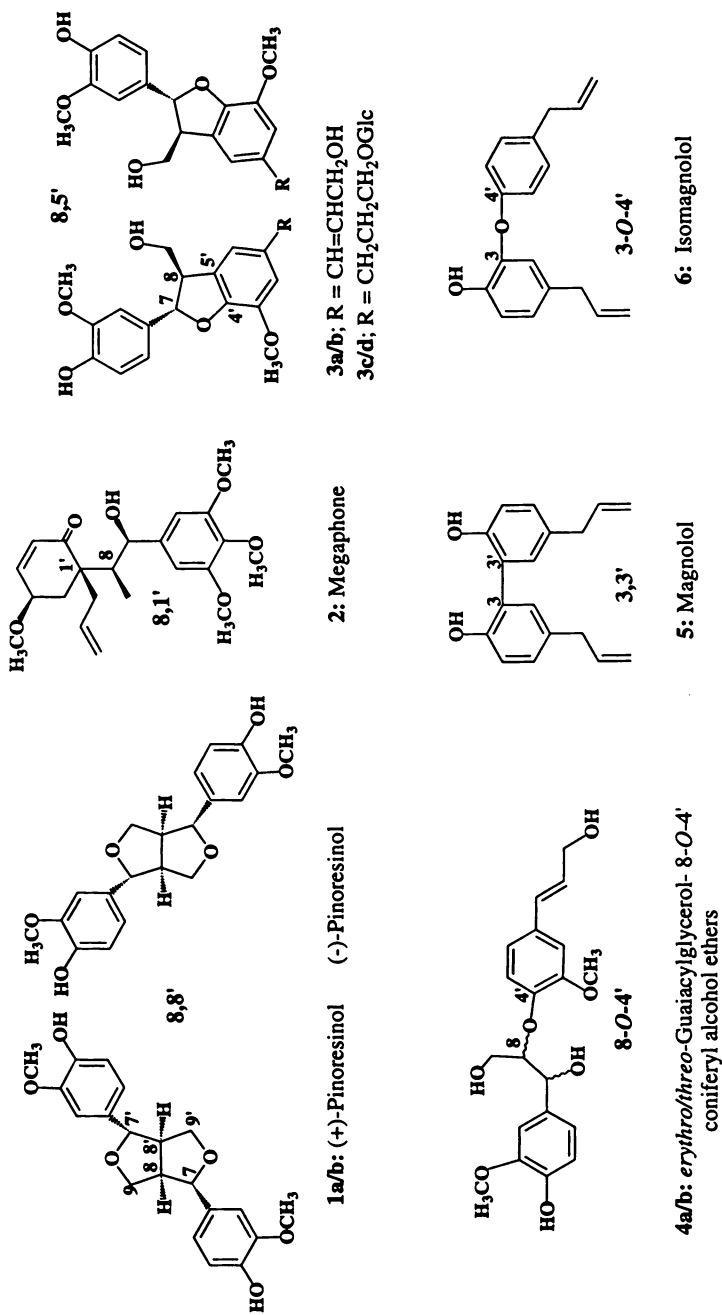
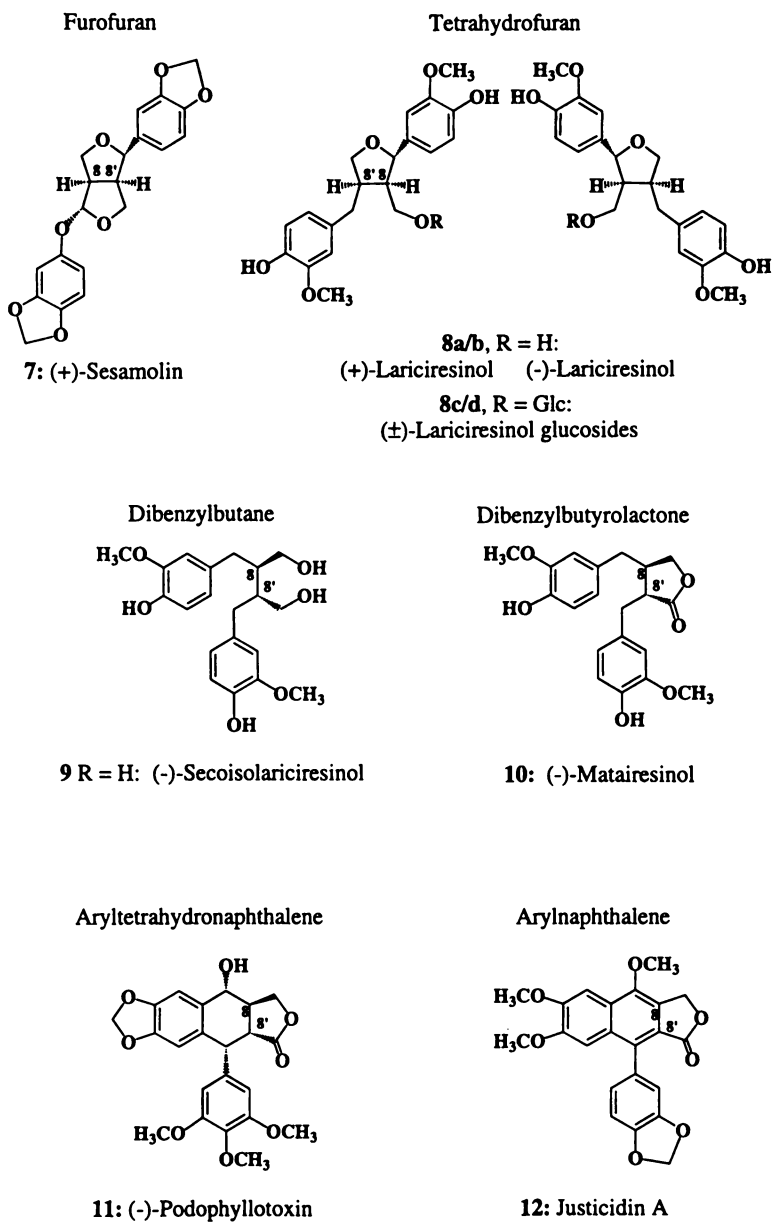Figure 1. Examples of various (neo)lignan linkages.

Furofuran

Tetrahydrofuran

7: (+)-Sesamolin

8a/b, R = H:
(+)-Lariciresinol   (-)-Lariciresinol
8c/d, R = Glc:
(±)-Lariciresinol glucosides

Dibenzylbutane

Dibenzylbutyrolactone

9 R = H: (-)-Secoisolariciresinol

10: (-)-Matairesinol

Aryltetrahydronaphthalene

Arylnaphthalene

11: (-)-Podophyllotoxin

12: Justicidin A

Figure 2.   Examples of various 8,8´-linked subgroups.
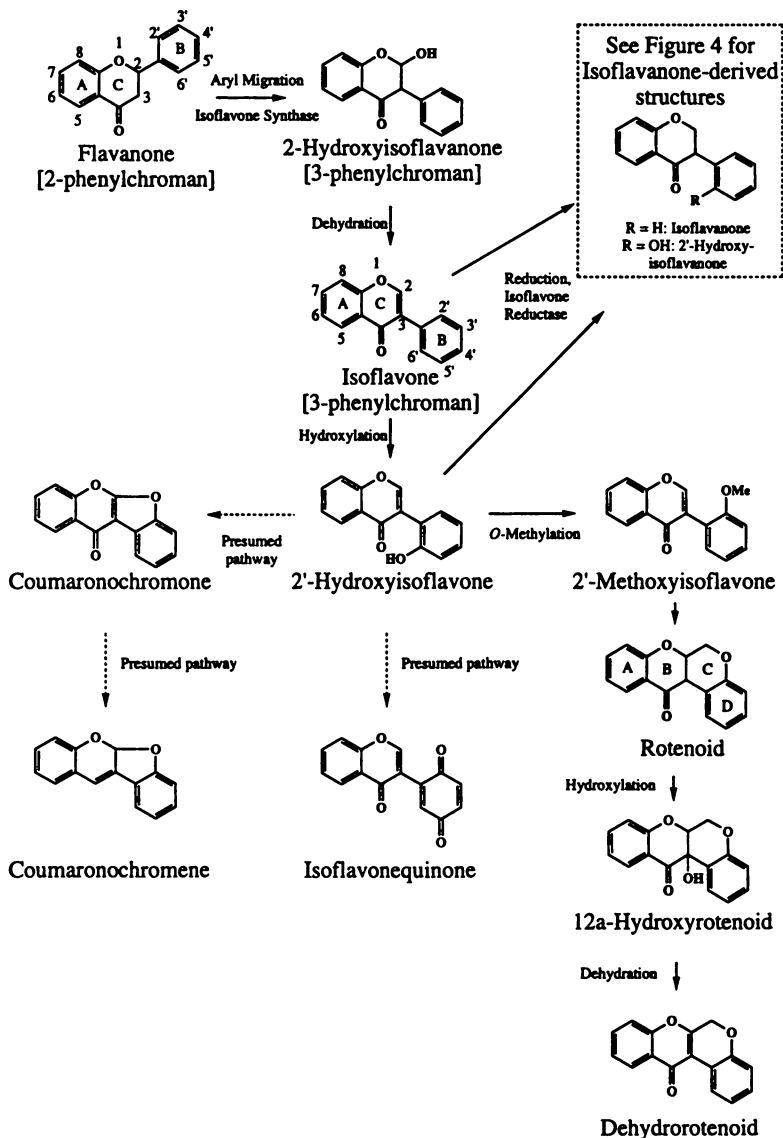
Figure 3. Simplified biosynthetic relationships between isoflavone-derived [3-phenylchroman] isoflavonoid skeletal types. Note: redrawn and modified from (6), with structures only revealing key structural features.
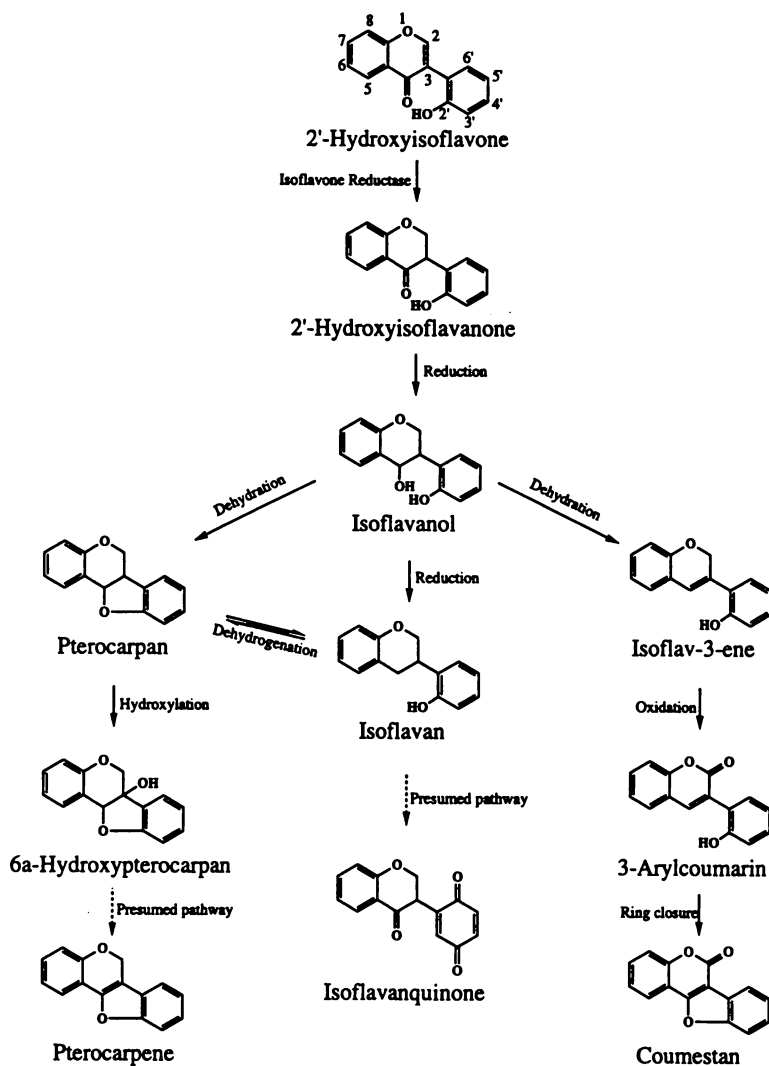
Figure 4. Simplified biosynthetic relationships between isoflavanone-derived isoflavonoid skeletal types. Note: redrawn and modified from *(6)*, with structures only revealing key structural features.
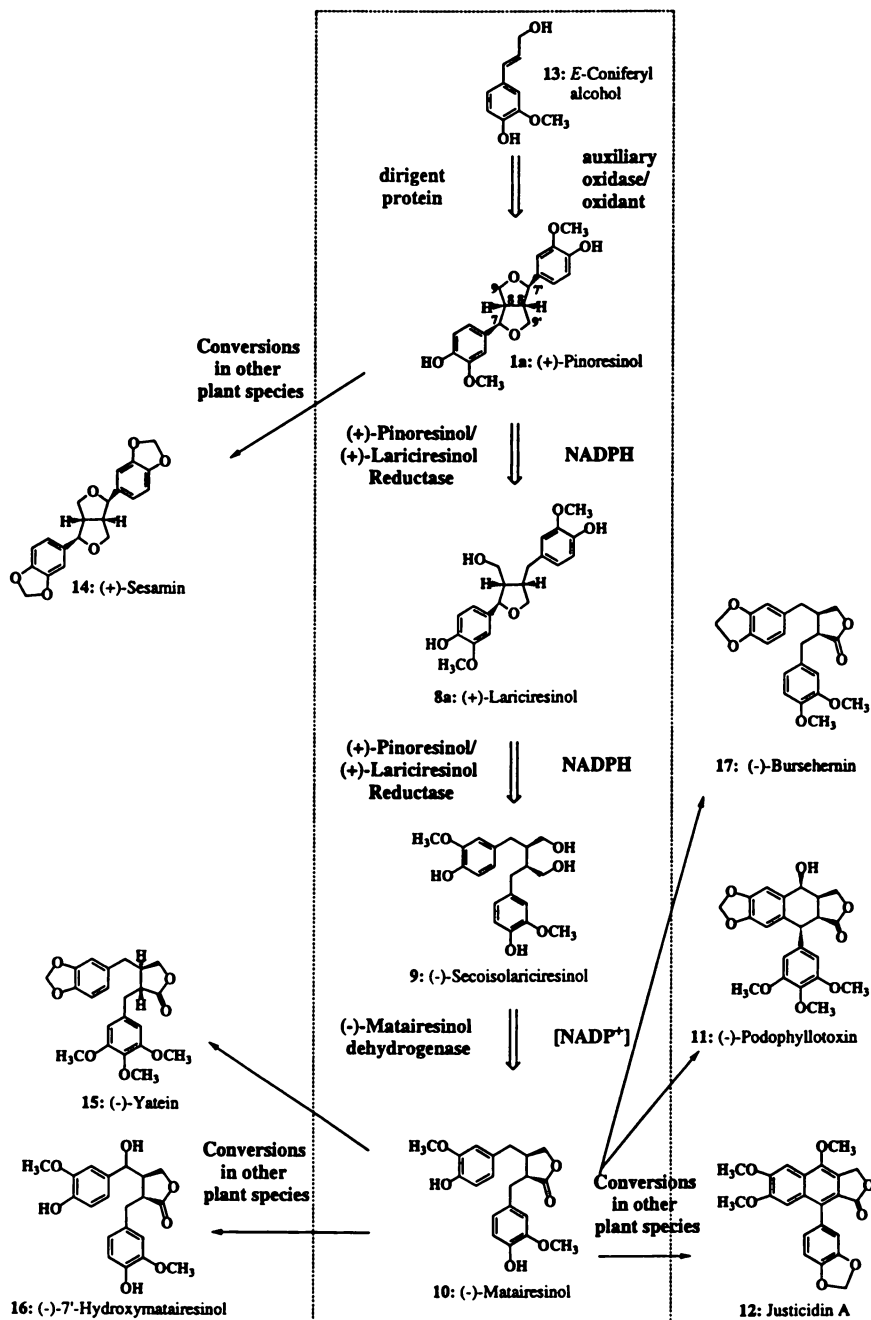
Figure 5. Proposed biosynthetic pathway to the main 8,8´-linked subclasses [compare to Figure 2]. - - - - = pathway shown in *Forsythia* sp.

Depending upon the species involved, the 8,8'-linked lignans can be found in a variety of distinct forms, as previously illustrated in Figure 2. The interrelationships between these different subgroups have been established using *Forsythia sp.* [Figure 5]. Thus, sequential enantiospecific reduction of (+)-pinoresinol **1a** occurs to give the furanolignan, (+)-lariciresinol **8a** and then the dibenzylbutane, (-)-secoisolariciresinol **9**. The responsible enzyme, (+)-pinoresinol/(+)-lariciresinol reductase, has been purified to homogeneity, and the cDNA encoding this ~35 kDa protein has been cloned (*8*). Enantiospecific dehydrogenation of (-)-secoisolariciresinol **9** next occurs to give the dibenzylbutyrolactone, (-)-matairesinol **10**, which is considered to be the precursor of other subgroups, such as aryltetrahydronaphthalene [e.g., (-)-podophyllotoxin **11**] and arylnaphthalene lignans [e.g., justicidin A **12**] as well as to other dibenzylbutyrolactones [e.g., **15-17**].

Thus, with the elucidation of the biosynthetic pathway leading to (+)-pinoresinol **1a**, (+)-lariciresinol **8a**, (-)-secoisolariciresinol **9** and (-)-matairesinol **10** in *Forsythia intermedia*, a general pathway to the various 8,8'-linked lignan subgroups has emerged [Figure 5].

Isoflavonoids. Over the past 20 years, our understanding of isoflavonoid biosynthesis has made considerable progress as summarized in general terms in Figures 3 and 4. Since excellent detailed accounts of isoflavonoid biosynthesis have been described elsewhere (*6, 9*), this section focuses essentially only on isoflavonoid synthase and the branch point enzyme[s], isoflavonoid reductase[s]. In this regard, a significant enzymological breakthrough came with the isolation of isoflavone synthase from elicitor-challenged soybean [*Glycine max* L.] cell suspension cultures (*10*). It catalyzes the intramolecular aryl migration [Figure 6A] of either (2*S*)-liquiritigenin **18** or (2*S*)-naringenin **19** into the isoflavones, daidzein **20** and genistein **21**, respectively. The next major advance came several years later with the purification and cloning of the branch point enzyme, isoflavone reductase, from various legumes, i.e., alfalfa [*Medicago sativa* L.] (*11*), chickpea [*Cicer arietinum* L.] (*12*) and pea [*Pisum sativum* L.] (*13*). Isoflavone reductases from alfalfa and pea catalyze the conversion of 2'-hydroxyformononetin **22** and 2'-hydroxypseudobaptigenin **23** into (3*R*)-vestitone **24** and (3*R*)-sophorol **25**, respectively. These are subsequently converted into the agronomically important pterocarpanoid phytoalexins, (-)-medicarpin **26** and (+)-pisatin **27**, as shown in Figure 6B.

Parallels Between (+)-Pinoresinol/(+)-Lariciresinol and Isoflavone Reductases: Amino Acid Sequence Homology and Enzymatic Mechanisms of Action.

Amino Acid Sequence Analysis. As summarized in Table I (*8, 11-13*)], (+)-pinoresinol/(+)lariciresinol reductase from *Forsythia* sp. has ~62% similarity and ~42% identity to isoflavone reductases from alfalfa [*M. sativa*], chickpea [*C. arietinum*] and pea [*P. sativum*], this representing the closest homology to any other reductase of known function. Based on this sequence similarity, it is proposed that lignan and isoflavone reductases are part of a closely related family. It is also interesting that there are additional members of this family of reductases, namely the so-called "isoflavone reductase homologs" from *Nicotiana tabacum* L. (*14*), and *Solanum tuberosum* L. [Solanales] (*15*), *Zea mays* L. [Poales] (*16*), *Arabidopsis thaliana* L. Heynh. [Capparales] (*17*), *Lupinus albus* L. [Fabales] (*18*) and *F. intermedia* [Oleales] [Gang and Lewis, unpublished results]. Yet, these apparently do not encode isoflavone reductases proper (*14, 16*) and thus their physiological roles are not defined. It is interesting, however, that they have very good homology to both lignan and isoflavone
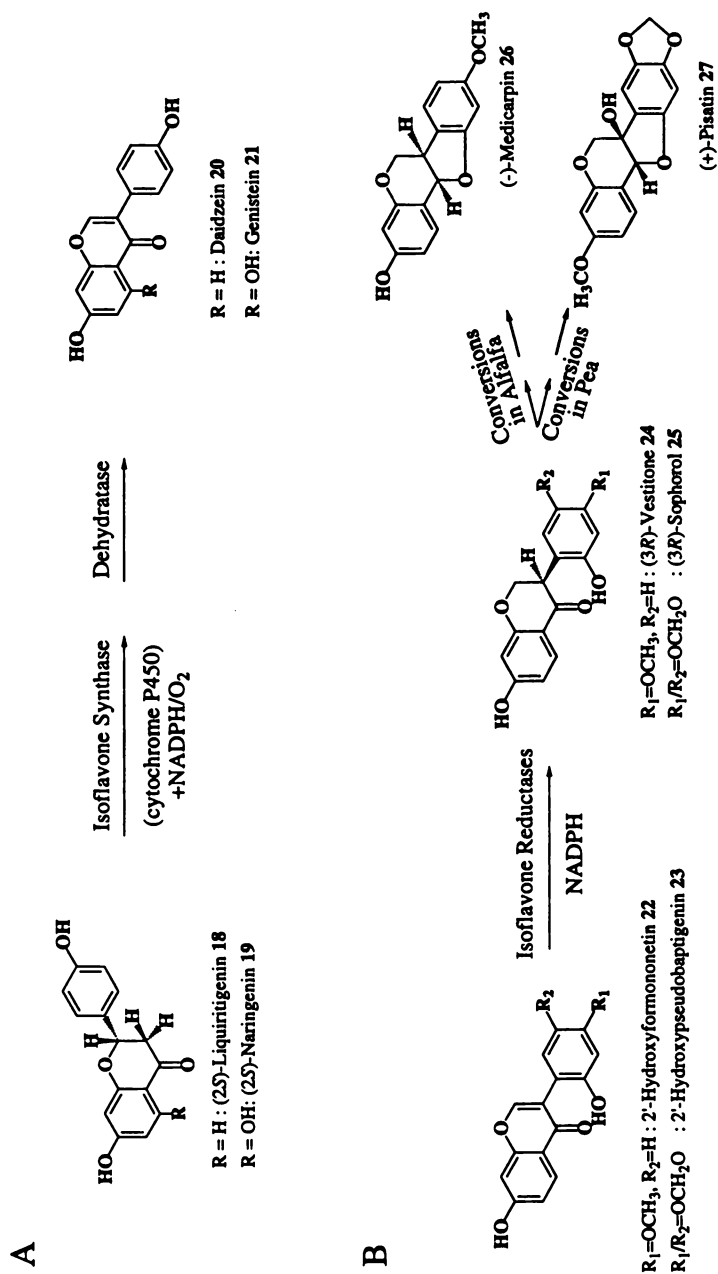
Figure 6. Biosynthetic steps in the formation of isoflavonoids catalyzed by isoflavone synthase [A] and isoflavone reductase [B].

Table I. Amino Acid Sequence Homology of (+)-Pinoresinol/(+)-Lariciresinol and Isoflavone Reductases and their "Homologs"

Enzyme	Plant Species	Abbreviation	Homology to *F. intermedia* (+)-Pinoresinol/ (+)-Lariciresinol Reductase		Homology to *M. sativa* [†] Isoflavone Reductases		Ref.
			% Similarity	% Identity	% Similarity	% Identity	
(+)-pinoresinol/ (+)-lariciresinol reductase	*Forsythia intermedia*	plr_fi	100	100	62.6	42.0	(8)
isoflavone reductase	*Medicago sativa*	ifr_ms	62.6	42.0	100	100	(11)
"	*Cicer arietinum*	ifr_ca	63.5	44.4	91.2	83.3	(12)
"	*Pisum sativum*	ifr_ps	61.6	41.3	95.6	91.5	(13)
"homolog"	*Nicotiana tabacum*	ifrh_nt	64.6	47.2	73.3	55.4	(14)
"	*Solanum tuberosum*	ifrh_st	65.5	47.7	72.6	58.6	(15)
"	*Zea mays*	ifrh_zm	62.0	45.2	71.0	51.5	(16)
"	*Arabidopsis thaliana*	ifrh_at	65.9	50.8	70.4	55.7	(17)
"	*Lupinus albus*	plrh_la	85.9	66.2	64.6	43.6	(18)
"	*Forsythia intermedia*	plrh_fi1	67.2	49.5	73.0	56.4	*
"	*Forsythia intermedia*	plrh_fi2	67.2	48.5	73.0	56.7	*

[†] Comparison to *M. sativa* isoflavone reductase was arbitrarily chosen for clarity.
* Unpublished results.

reductases. This homology is further illustrated in Figure 7, which pictorially displays the amino acid sequence alignment of (+)-pinoresinol/(+)-lariciresinol reductase, the isoflavone reductases and the "homologs" reported to date [see Table I for abbreviation descriptions].

As can also be seen [Table I], the *Lupinus albus* "homolog" has even higher homology to (+)-pinoresinol/(+)-lariciresinol reductase than to isoflavone reductases [~86% versus ~62% similarity; ~66% versus ~44% identity, respectively]. This may again be significant because this legume is not known to biosynthesize isoflavanone-derived isoflavonoids, and hence provides yet another reason for discontinuing the term "isoflavone reductase homologs". From an evolutionary standpoint, it will be instructive to determine whether these "homologs" preceded the appearance of the lignan and isoflavonoid reductases in the plant kingdom.

Additional analysis of the amino acid sequences of both (+)-pinoresinol/(+)-lariciresinol reductase and isoflavone reductases also revealed that they contain five conserved possible phosphorylation sites, including Thr-302 [casein kinase II-type protein phosphorylase site, which are conserved in the "homologs" as well, see reference (8)]. It can, therefore, be proposed that their enzymatic activities might be regulated by protein kinase cascades. This is highly likely, given the pivotal branch point positions the enzymes play in their respective biosynthetic pathways, their roles in the physiology of the whole organism [discussed later] and the ubiquity of protein kinase cascades in the regulation of biosynthetic pathways.

Proposed Enzymatic Mechanisms. In addition to this sequence homology, both (+)-pinoresinol/(+)-lariciresinol and isoflavone reductases are Class A dehydrogenases, as established by examining the stereospecificity of hydride transfer (*19-22*). For (+)-pinoresinol/(+)-lariciresinol reductase, the 4-pro-*R* hydride of NADPH is transferred sequentially to the C_7 and C_7 carbons of (+)-pinoresinol **1a** and (+)-lariciresinol **8a**, respectively (*19*). The incoming hydrides take up the pro-*R* positions in the product, resulting in an "inversion" of configuration at C_7/C_7. There are two possible mechanisms for ether bond breakage/hydride transfer. The first [Figure 8A] invokes a concerted SN2 mechanism, whereas the second, and preferred mechanism [Figure 8B], requires regeneration of the quinone methide [conjugated enone] intermediate followed by reduction. This latter mechanism is very similar to that for isoflavone reductase, where the 4-pro-*R* hydride of the NADPH cofactor is abstracted and added to the *si*-face of the isoflavone substrate. That is, direct hydride transfer occurs at C_2 of the α,β-unsaturated ketone [a conjugated enone] in a strictly stereospecific manner (*20*), thereby taking up the *pro-S* position in the product, while the corresponding proton is added *trans* across the double bond to C_3 [see Figure 8C] (*20-22*).

Occurrence of Lignans, Isoflavonoids, and Their Reductases in the Plant Kingdom

Lignans. Lignans are widely distributed throughout the plant kingdom, being found in various species within the hornworts [currently classified as bryophytes (*23*)], pteridophytes, gymnosperms and angiosperms (*1*). A recent detailed review describes our current understanding of their occurrence and biochemical pathway evolution in higher plants (*1*). They occur in various tissues from roots, stems [heartwood], and leaves, to flowers, fruits and seeds (*24*). Preliminary organelle fractionation using *Linum album* suspension cell cultures has suggested that they might accumulate in the vacuole (*25*).
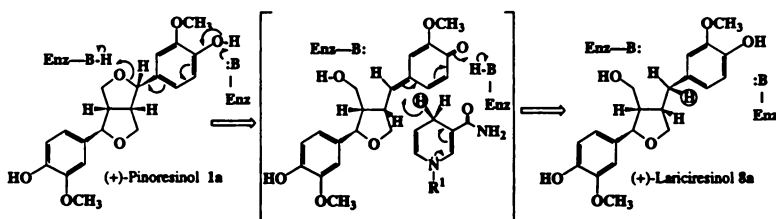
Figure 7. Amino acid sequence alignment of (+)-pinoresinol/(+)-lariciresinol and isoflavone reductases and their "homologs".
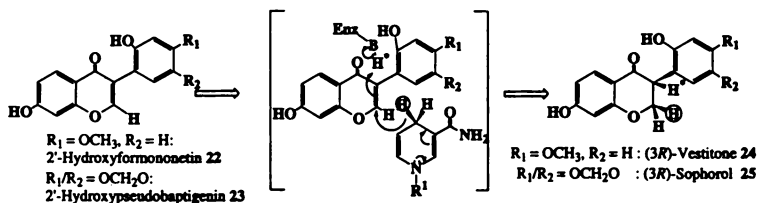
A: Concerted SN2 Mechanism
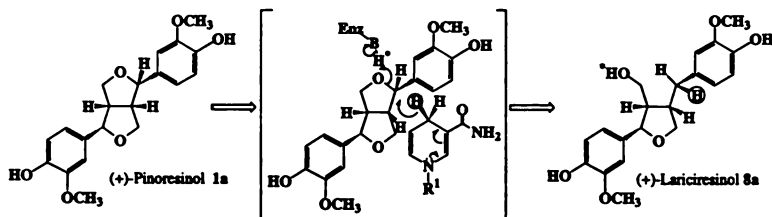
B: Conjugated Enone [Quinone Methide]

C: Conjugated Enone

R^1 = Ribose-diphosphate-ribose-adenine
[ ] = Putative Enzyme-bound Intermediate

Figure 8. Proposed catalytic mechanisms for (+)-pinoresinol/ (+)-lariciresinol reductase [A & B] and isoflavone reductase [C].

Based on current chemotaxonomic data, Table II attempts to correlate some of the most pertinent changes in lignan skeleta in terms of the evolutionary progression of plants, particularly with regard to mode of coupling and introduction of enantiospecific pinoresinol/lariciresinol reductase steps. However, note that only a few species have been comprehensively studied for their lignan constituents [see (*1*) and (*5*) for specific references], and it is anticipated that the trends described below will continue to be refined.

Lignans in Early Land Plants. The few "primitive" plant species that have had lignan constituents described include hornworts [e.g., *Megaceros flagellaris*] and ferns [e.g., *Blechnum orientale* (Table II and Figure 9)]. These accumulate the optically active lignans, (+)-megacerotonic **28** [7,8'] and (-)-blechnic **29** [8,2'] acids (*26-28*), respectively, which are *presumed* to result from stereoselective coupling of *p*-coumaric **30** and/or caffeic acids **31**. Neither lignan has been reported in the gymnosperms or angiosperms, and thus may be restricted to these early land plants. By contrast, the lignans reported present in the fern *Pteris vittata* are racemic, i.e., the glucosides of (±)-dihydrodehydrodiconiferyl alcohols **3c/d** [see Figure 1] and (±)-lariciresinols **8c/d** [see Figure 2] (*29*). Their formation can tentatively be considered to result from non-stereoselective 8,5' and 8,8' coupling of *E*-coniferyl alcohol **13** to first give (±)-dehydrodiconiferyl alcohols **3a/b** and (±)-pinoresinols **1a/b**. Formation of **3c/d** and **8c/d** could then occur by non-enantiospecific reduction and glucosylation. In any event, it can be proposed that a pinoresinol reductase step emerged at this point, albeit non-enantiospecific.

Lignans in Gymnosperms. The gymnosperms are replete with optically active 8,8'-linked tetrahydrofuran, dibenzylbutane, dibenzylbutyrolactone, aryltetra-hydronaphthalene and arylnaphthalene lignans [see Table II for examples] (*5, 30-33*). The vast majority can be considered to arise via stereoselective coupling of two *E*-coniferyl alcohol **13** molecules to give (+)-pinoresinol **1a**. They also differ from the metabolic products in the early land plants by undergoing presumed enantiospecific reductions via the action of (+)-pinoresinol/(+)-lariciresinol reductase [Figure 5], thereby providing entry to the different 8,8'-linked lignan subgroups. How this reductase differs from that putatively present in the earlier pteridophytes needs to be established.

Lignans in Angiosperms. The transition to the angiosperms was accompanied by large changes in both lignan structure and substitution patterns (*1*). The most widespread are again 8,8'-linked and Figure 10 illustrates their extensive distribution in the angiosperms. The presumed occurrence of (+)-pinoresinol/(+)-lariciresinol reductase is also included, based on the lignan structures known thus far. But other stereoselective coupling modes were also acquired in the angiosperms, mainly in the Magnoliiflorae, which contains 8,1', 8,5' and 8-*O*-4' coupled products in addition to 8,8'-linked lignans (*34-36*) [see Table II].

As the dicots evolved beyond the Magnoliiflorae, the diversity in lignan coupling modes appears more restricted with almost all known lignans belonging to the 8,8'-linked class, as illustrated by examples from the Asterales, Euphorbiales, Rosales and Scrophulariales [Figure 11]. Again, the structures observed clearly suggest the involvement of (+)-pinoresinol/(+)-lariciresinol reductase, e.g., heliobuphthalmin **37**/helioxanthin **38** from *Heliopsis buphthalmoides* [Asterales] (*37, 38*), diphyllin **34** from *Cleistanthus collinus* [Euphorbiales] (*39*), justicidin A **12** from *Justicia hayatai* [Rosales] (*40*) and (-)-olivil **39** from *Stereospermum kunthianum* [Scrophulariales] (*41*). By contrast, relatively few examples of lignans are known to date in the

Table II. Proposed Modifications of the Lignan Biosynthetic Pathway[s] During Plant Evolution

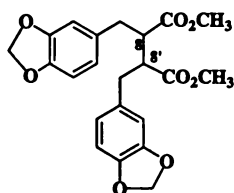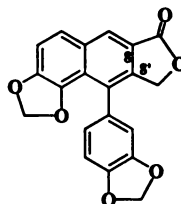| Presumed Coupling Mode	Earliest Known Occurrence	Resulting Lignan Class[es]/Subgroups	Specific Examples	Representative Species [Family]
Stereoselective 7,8'	Hornworts [Bryophytes]	7,8'-linked	(+)-megacerotonic acid **28**	*Megaceros flagellaris* [Anthocerotae]
Stereoselective 8,2'	Pteridophytes	8,2'-linked	(-)-blechnic acid **29**	*Blechnum orientale*, [Blechnaceae]
Racemic 8,5'	"	8,5'-linked	(±)-dihydrodehydro-diconiferyl alcohol glucosides **3c/d**	*Pteris vittata* L. [Adiantaceae]
Racemic 8,8'	"	8,8'-linked: tetrahydrofuran	(±)-lariciresinol-9-*O*-β-D-glucosides **8c/d**	"
Stereoselective 8,8'	Gymnosperms	8,8'-linked: furofuran	(+)-pinoresinol **1a**	*Larix leptolepis* [Pinaceae]
Stereoselective 8,8' Coupling with Enantiospecific Reduction	"	tetrahydrofuran	(+)-lariciresinol **8a**	"
		dibenzylbutane	(-)-secoisolariciresinol **9**	*Araucaria angustifolia* [Araucariaceae]
		dibenzylbutyrolactone	(-)-matairesinol **10**	*Podocarpus spicatus* [Podocarpaceae]
"	"	aryltetrahydro-naphthalene	(-)-plicatic acid **32**	*Thuja plicata* Donn. [Cupressaceae]
"	"	allylphenol-derived	dehydroguaiaretic acid **33**	*Taxus baccata* L. [Taxaceae]
"	"	arylnaphthalene	diphyllin **34**	*Taiwania cryptomerioides* Hayata [Taxodiaceae]
Stereoselective 8,1'	Angiosperms	8,1'-linked	(-)-megaphone **2**	*Aniba megaphylla* Mez [Lauraceae]
Stereoselective 8,5'	"	8,5'-linked	(-)-carinatonol **35**	*Virola carinata* Warb. [Myristicaceae]
Stereoselective 8-*O*-4'	"	8-*O*-4'-linked	(+)-surinamensin **36**	*Virola surinamensis* [Myristicaceae]
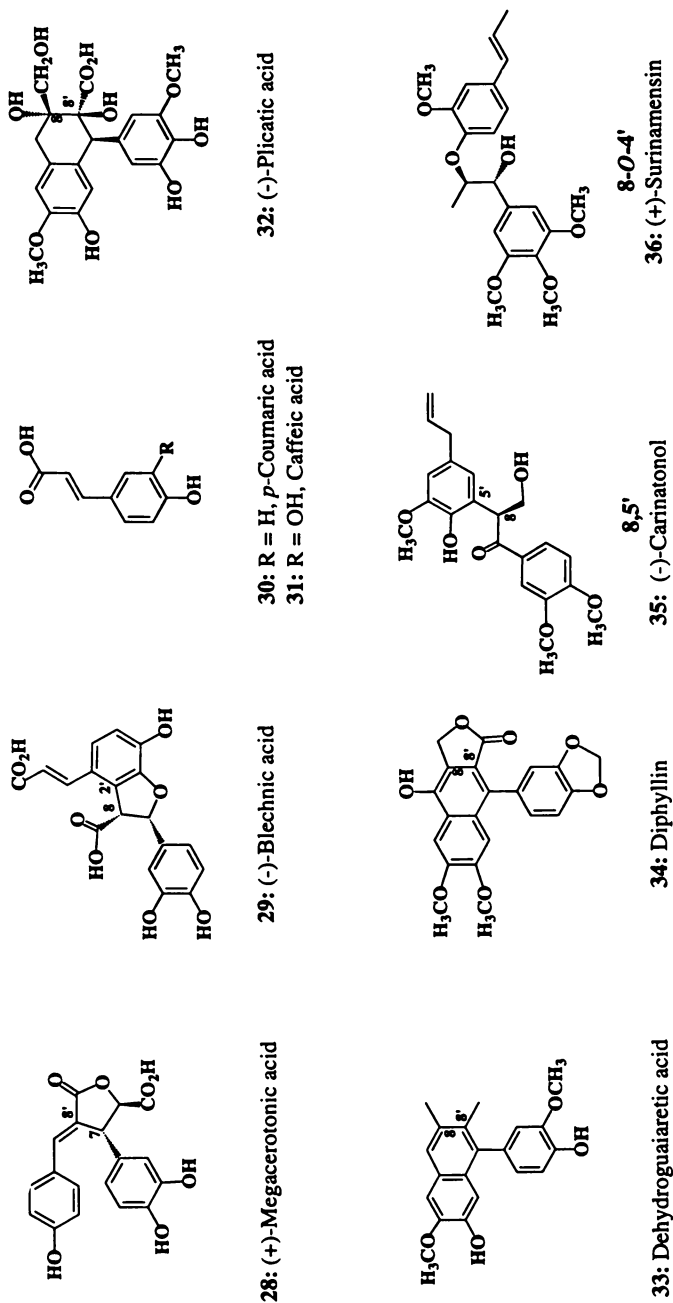
Figure 9. Selected lignans listed in Table II [and possible biosynthetic monomeric precursors mentioned in text].

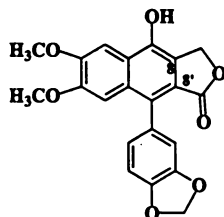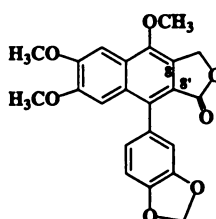Figure 10. Distribution of 8,8′-linked lignans and isoflavonoids in the angiosperms. Orders are indicated which are presumed to contain/lack lignans/isoflavonoids derived from (+)-pinoresinol/(+)-lariciresinol reductase [PLR] and isoflavone reductase [IFR].

ASTERALES

37: Heliobuphthalmin

38: Helioxanthin

EUPHORBIALES

34: Diphyllin

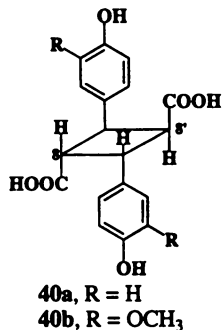## ROSALES

12: Justicidin A

SCROPHULARIALES
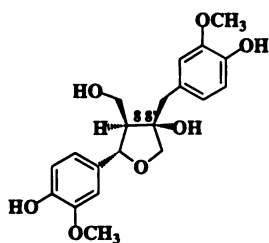
39: (-)-Olivil

POALES [Monocot]

40a, R = H
40b, R = OCH$_3$

Figure 11. Examples of lignans produced by members of the Asterales, Euphorbiales, Rosales, Scrophulariales and Poales.

monocots [see Lewis *et al.* (*1*)], these primarily consisting of cyclobutane dimers, such as the dihydroxytruxillic acids **40a/b** [Figure 11] from *Setaria anceps* cv. *Nandi* (*42, 43*). The latter are, however, believed to be formed by photochemical coupling of juxtaposed cell-wall bound cinnamoyl molecules, rather than by enzymatic coupling.

      Isoflavonoids. Like the lignans, isoflavonoids can occur in various plant tissues, from roots, stems and leaves to fruits and seeds (*44-57*). Similarly, they also appear to be stored in the vacuole (*58-60*). In contrast, based on available chemotaxonomic evidence, they do not appear to be as widely distributed throughout the plant kingdom as the lignans, with only a few examples of simple isoflavones and isoflavone derivatives reported in the bryophytes and gymnosperms [Table III]. However, none appear to be derived via isoflavone reductase catalyzed conversions, since their metabolites are unsaturated at carbons 2 and 3, e.g., orobol **41** from the moss *Bryum capillare* (*61*), genistein **21** and podospicatin **42** from *Podocarpus spicatus* (*9*) and a related isoflavone **43** from *Juniperus macropoda* (*6*). Thus, in contrast to the lignan biosynthetic pathways, there is no evidence for an isoflavone reductase prior to or during gymnosperm evolution.

      Both isoflavone and isoflavanone-derived metabolites are, however, found in the angiosperms as shown in Figure 10 [with representative examples given in Table III]. Interestingly, the only orders known thus far that contain metabolites derived from isoflavone reductases are those in the Asterales, Rosales, Annonales and Fabales. This is exemplified by the coumestans wedelolactone **44** and norwedelolactone **45** from *Wedelia calendulacea* and *Eclipta alba* [Asterales], padmakastein **46** from *Prunus puddum* [Rosales], and the pterocarpans, (-)-medicarpin **26** and (-)-maackiain **47**, from *Osteophloeum platyspermum* [Annonales] and *Medicago sativa* [Fabales] [see (*6, 9, 62, 63*) and Table III]. Significantly, these orders also contain plant species presumed to contain (+)-pinoresinol/(+)-lariciresinol reductases as previously indicated, i.e., the metabolites helioxanthin **38** [Asterales] (*5*), justicidin A **12** [Rosales] (*40*), and (-)-dihydrocubebin **48** [Annonales] (*64*) and secoisolariciresinol **9** [Fabales] (*65*). Consequently, given all of the similarities for both reductases, it can be tentatively concluded that the isoflavone reductases are derived from the corresponding lignan reductases.
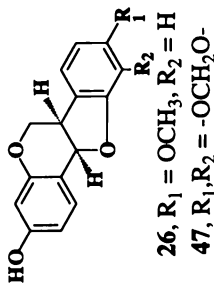
Comparable Physiological Functions?

With the above parallels in amino acid homology, putative regulatory processes [phosphorylation sites], metabolite occurrence and presumed vacuolar compartmentalization, it could be expected that the lignans and isoflavonoids have comparable physiological functions. This section therefore extends our discussions to comparing their known physiological properties.
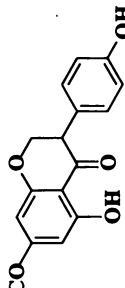
      It is worthwhile to first summarize our knowledge of the biological activities of the lignans and isoflavonoids that are *not* derived via (+)-pinoresinol/(+)-lariciresinol and isoflavone reductases [Tables IV and V, Figures 12 and 13]. Thus far, these are *primarily* insecticidal, growth inhibitory and piscicidal. Of the lignans, the 8,8', 8"-O-4'-linked (+)-haedoxan A **49** [Table IV and Figure 12] is perhaps the best studied (*66-69*). Administered orally in combination with piperonyl butoxide [a synergist], its potent insecticidal activity against several lepidopterous insect larvae and houseflies [*Musca domestica*] [LD$_{50}$ = 0.25 ng per fly] (*66*) is comparable to that of commercial synthetic pyrethroids. Its oral administration results in muscle relaxation, cessation of feeding, general paralysis and death, thereby causing similar effects to the insect neurotoxins, nereistoxin, ryanodine and reserpine (*66*). In a similar way, the 8,5'-linked lignan **50** from *Myristica fragrans* causes death in silkworm fourth instar larvae

Table III. Proposed Modifications to the Isoflavonoid Biosynthetic Pathway During Plant Evolution
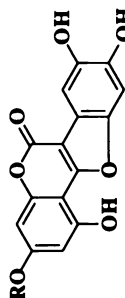
Distinguishing Feature	Earliest Known Occurrence	Resulting Class[es]	Specific Examples	Representative Species [Family]
1,2-Aryl migration	Bryophytes	isoflavone	orobol 41	*Bryum capillare* [Bryaceae]
	Gymnosperms	isoflavone	genistein 21	*Podocarpus spicatus* [Podocarpaceae]
			podospicatin 42	"
			5,7,3',5'-tetrahydroxy-4'-methoxy isoflavone 43	*Juniperus macropoda* Boiss. [Cupressaceae]
Reduction of 2,3 isoflavone bond	Angiosperms	coumestan	wedelolactone 44	*Wedelia calendulacea* [Asteraceae]
	"		norwedelolactone 45	*Eclipta alba* Hassk. [Asteraceae]
		isoflavanone	padmakastein 46	*Prunus puddum* Miq. [Rosaceae]
	"	pterocarpan	(-)-medicarpin 26	*Osteophloeum platyspermum* Warb. [Myristicaceae]
			(-)-maackiain 47	*Medicago sativa* L. [Fabaceae]

41, $R_1 = R_2 = R_5 = H$, $R_3 = R_4 = OH$
21, $R_1 = R_2 = R_3 = R_5 = H$, $R_4 = OH$
42, $R_1 = R_5 = OCH_3$, $R_2 = OH$, $R_3 = R_4 = H$
43, $R_1 = R_2 = H$, $R_3 = R_5 = OH$, $R_4 = OCH_3$

44, $R = CH_3$ 45, $R = H$

46: Padmakastein

26, $R_1 = OCH_3$, $R_2 = H$
47, $R_1, R_2 = -OCH_2O-$

Table IV. Known Insecticidal and Insect Anti-feedant Properties of Various Lignans

Lignan	Plant Species	Insect Species Known to be Affected	Biological Activity	Reference
(+)-haedoxan A **49**	*Phryma leptostachya* L.	housefly [*Musca domestica*], lepidopterous insect larvae	highly toxic when administered with synergist piperonyl butoxide	(66-69)
8,5'-linked **50**	*Myristica fragrans* Houtt.	silk worm [*Bombyx mori*]	inhibits fourth instar larval growth	(70, 71)
licarin A **51**	*Machilus japonica* Sieb & Zucc.	tobacco cutworm [*Spodoptera litura*]	reduces larval growth	(72)
(-)-machilusin **52**	*Machilus japonica*	"	"	(72)
magnolol **5**	*Magnolia virginiana* L.	mosquito [*Aedes aegypti*]	prevents larval growth	(73)
(+)-kobusin **53**	*Magnolia kobus* DC	silk worm [*Bombyx mori*]	inhibits fourth instar larval growth	(74)
(+)-sesamin **14**	*Magnolia kobus*, other species	"	"	(74)
(+)-epimagnolin A **54**	*Magnolia fargesii*	fruit fly [*Drosophila melanogaster*]	inhibits larval growth	(75)
(-)-piperenone **55**	*Piper futokadzura*	tobacco cutworm [*Spodoptera litura*]	deters larval feeding	(76)
(+)-sesamin **14**	*Sesamum indicum* L., other species	milkweed bug [*Oncopeltus fasciatus*]	synergist with natural juvenile hormone, prevents metamorphosis	(77)
(+)-sesamolin **7**	*Sesamum indicum*	"	"	(77)
(+)-sesamin **14**	*Sesamum indicum*, other species	several, synergistic with pyrethrins	antioxidant, prevents oxidative degradation of pyrethrins in insect midgut	(78, 79)
episesamin **56**	*Justicia simplex*, other species	"	"	(78, 79)

**Table V. Selected Isoflavonoids with Insecticidal/Insect Antifeedant Properties**

Compound	Plant Species	Affected Species	Activity	Reference
rotenone **57**	*Derris elliptica* [Wallich] Benth.	leaf-eating caterpillars, e.g., *Bombyx mori, Ceratomia catalpae, Oncopeltus fasciatus, Periplaneta americana, Popillia japonica* and *Tenebrio molitor*; fish	insecticidal, piscicidal	(81)
amorpholone **58**	*Tephrosia candida* DC	*Spodoptera litura* [armyworm]	"	(52)
deguelin **59**	*Derris elliptica*	same as for rotenone	"	(45, 51,81)
tephrosin **60**	*Tephrosia elata* Defl.	Insect larvae: *Spodoptera exempta* [African armyworm] and	insect feeding deterent [also a fish poison]	(46)
rotenone **57**	*Derris elliptica* root	Insect larvae: *Spodoptera exempta* [African armyworm] and *Eldana saccharina* [sugar-cane borer]	insect feeding deterent	(46)
hildecarpin **61**	*Tephrosia hildebrandtii* Vatke	Insect larvae: *Maruca testulalis* [legume pod-borer]	"	(53, 54)
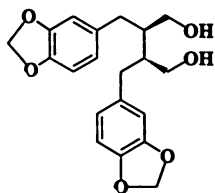
48: (-)-Dihydrocubebin
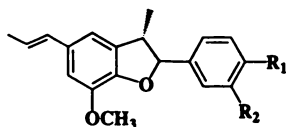
49: (+)-Haedoxan A

50, R₁,R₂ = -OCH₂O-:
8,5'-linked lignan

51, R₁ = OH, R₂ = OCH₃:
Licarin A
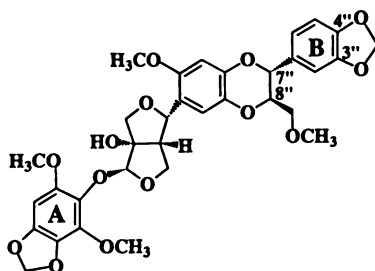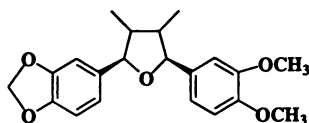
52: (-)-Machilusin
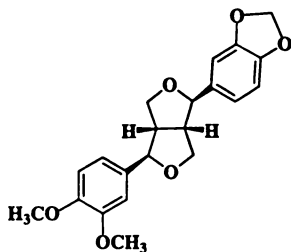
53: (+)-Kobusin
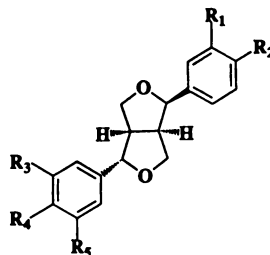
54, R₁ = R₂ = R₃ = R₄ = R₅ = OCH₃:
(+)-Epimagnolin A

56, R₁,R₂ = R₄,R₅ = -OCH₂O-, R₃ = H:
Episesamin
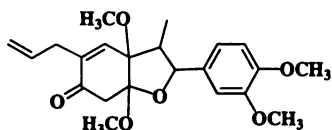
55: (-)-Piperenone

Figure 12. Examples of lignans listed in the text and Table IV.
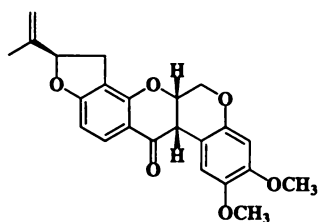
57: Rotenone
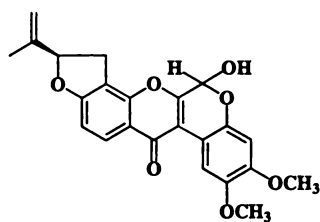
58: Amorpholone
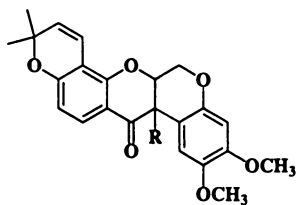
59, R = H: Deguelin

60, R = OH: Tephrosin
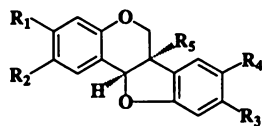
61, R$_1$ = R$_5$ = OH,
R$_2$ = OCH$_3$,
R$_3$,R$_4$ = -OCH$_2$O-:
(-)-Hildecarpin

62, R$_1$ = R$_3$ = OCH$_3$,
R$_2$ = R$_4$ = R$_5$ = H:
(-)-Homopterocarpin
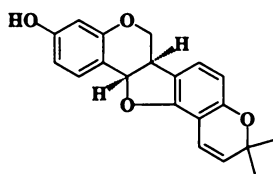
63: (-)-Phaseollin
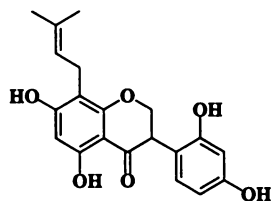
64: Kievitone

Figure 13. Examples of isoflavonoids listed in Table V and VI.

3 days after treatment (70, 71), but nothing is known about how it engenders this effect. Lignans such as licarin A 51 (72), (-)-machilusin 52 (72), magnolol 5 (73), (+)-kobusin 53 (74) and (+)-epimagnolin A 54 (75) [see Table IV], also have growth inhibitory effects, whereas some others, such as (-)-piperenone 55 (76), have antifeedant properties.

In a somewhat related manner, the 8,8'-linked (+)-sesamin 14 and (+)-sesamolin 7 from *Sesamum indicum* [Figures 2 and 5] act as synergists with natural juvenile hormone to prevent metamorphosis in the milkweed bug [*Oncopeltus fasciatus*] (77). (+)-Sesamin 14 and episesamin 56 function in a comparable way with pyrethrum insecticides, by inhibiting their oxidative degradation in the gut of the ingesting organism (78, 79).

Like the lignans, the isoflavonoids, rotenone 57 and related rotenoids, 58 and 59, primarily have potent insecticidal and piscicidal properties [see Table V and Figure 13] (80-84). They are believed to cause death by interference with normal respiration; for example, rotenone 57 has been shown to inhibit NADH-dehydrogenase [complex I] of the mitochondrial electron transport chain in isolated mitochondria, presumably by binding to the coenzyme Q binding site (80-84). Rotenoids also possess insect antifeedant properties as well. For example, tephrosin 60 from *Tephrosia elata* and rotenone 57 deter feeding of insect larvae [*Spodoptera exempta* and *Eldana saccharina*] without any obvious toxicity symptoms (46).

Some of the reductase-derived lignans [and isoflavonoids] display antifeedant and/or toxicity effects, e.g., (-)-yatein 15 (85), (+)-lariciresinol 8a (86) and hildecarpin 61 (53, 54). Lignans, such as (-)-matairesinol 10 and (-)-bursehernin 17, can also inhibit hatching of cyst forming nematodes [see Table VI] (87, 88), and justicidin A 12 from *Justicia hayatai* var. *decumbens* has been used for many centuries as a fish-poison (40).

The dominant biological effect of the reductase-derived lignans and isoflavonoids, however, appears to be that of antimicrobial action. For example, induced formation of the lignan, (-)-matairesinol 10 and related metabolites, e.g., 16, in *Picea abies* occurs after challenge by *Fomes annosus*, thereby limiting further growth of this pathogen (89) [see Table VI]. Indeed, this may help explain the massive, constitutive deposition [~20% by weight] of (+)-pinoresinol/(+)-lariciresinol reductase-derived lignans, such as plicatic acid 32 and its derivatives, in the heartwood of species such as western red cedar [*Thuja plicata* Donn ex D. Don] (33, 90, 91). In conjunction with the tropolones, these metabolites help confer protection to this plant species, thereby enabling it to reach life spans in excess of 3,000 years. Curiously, however, there has not yet been any systematic study conducted to date that comprehensively defines the physiological properties of the lignans, in spite of the emerging trends of their biological activities.

Interestingly, there are also a few examples of antimicrobial activities of lignans not derived from (+)-pinoresinol/(+)-lariciresinol reductase, e.g., the biphenyl lignans, such as magnolol 5 previously discussed, can inhibit growth of *Streptococcus mutans* (92, 93). They can also apparently function as phytoalexins, i.e., where they accumulate after *Fusarium solani* f. sp. *mori* challenge in the twig cortical tissue of *Cercidiphyllum japonicum* (94).

In a manner similar to several reductase-derived lignans, isoflavone reductase-derived isoflavonoids accumulate constitutively in the heartwood of leguminous trees, e.g., hildecarpin 61, in *Tephrosia hildebrandtii* Vatke, which has antifungal activity against *Cladosporium cucumerinum* (53, 54). As a result, these constitutively-formed isoflavone reductase-derived metabolites are believed to be of major importance for the longevity [survival] of such trees.

Table VI. Various Physiological Activities of Lignans

Compound	Example Plant Species	Target Organism	Physiological Activity	Reference
(-)-bursehernin **17**	*Bupleurum salicifolium*	*Globodera pallida* *G. rostochiensis*	nematocidal	(87, 88)
(-)-matairesinol **10**	"	"	"	(87, 88)
(-)-matairesinol **10**	*Picea abies* [L.] Karsten	*Fomes annosus*	"	(89)
7'-hydroxymatairesinol **16**	"	"	"	(89)
justicidin A **12**	*Justicia hayatai* var. *decumbens*	*Oryzias latipes*	piscicidal	(40)
magnolol **5** and other biphenyl lignans	*Myristica argentea*, *M. fragrans* *Magnolia obovata* Thunb., *Magnolia officinalis* Rehder & E. Wilson and *Magnolia virginiana* L.	*Streptococcus mutans*	bactericidal	(92, 93)
magnolol **5**	*Cercidiphyllum japonicum* Siebold & Zucc.	*Fusarium solani* f. sp. *mori*	antifungal phytoalexin	(94)

Table VII. Selected Isoflavonoids with Antifungal /Phytoalexin Properties

Compound	Plant Species [Source]	Affected Species	Activity	Reference
hildecarpin 61	*Tephrosia hildebrandtii*	fungus: *Cladosporium cucumerinum*	fungistatic	(53, 54)
(-)-medicarpin 26	*Medicago sativa* L.	fungi: *Helminthosporium carbonum, Stemphylium botryosum* and *S. sarcinaeforme*	phytoalexin, fungistatic	(97, 98)
(+)-pisatin 27	*Pisum sativum* L.	fungus: *Monilinia fructicola*	phytoalexin, fungistatic	(99)
(-)-maackiain 47	*Sophora tomentosa* L.	fungus: *Monilinia fructicola*	phytoalexin, fungistatic	(99)
(-)-homopterocarpin 62	*Pterocarpus* sp.	fungus: *Monilinia fructicola*	phytoalexin, fungistatic	(99)
(-)-phaseollin 63	*Phaseolus vulgaris* L.	fungi: *Monilinia fructicola, Rhizoctonia solani* and *Colletotrichum lindemuthianum*	phytoalexin, fungistatic	(99-102)
kievitone 64	*Phaseolus vulgaris* L. and *Vigna sinensis* Endl.	fungi: *Aphanomyces euteiches, Rhizoctonia solani* and *Fusarium solani* f. sp. *phaseoli*	phytoalexin, fungistatic	(103)

Pterocarpanoids and other isoflavone reductase-derived isoflavonoids are also extremely important inducible plant defense [phytoalexin] components (*95*).   For example, the pterocarpanoids, (-)-medicarpin **26**, (+)-pisatin **27**, (-)-maackiain **47**, (-)-homopterocarpin **62**, (-)-phaseollin **63**, and the isoflavanone, kievitone **64**, all possess potent fungistatic properties against a wide variety of fungi, such as *Monilinia fructicola* and *Rhizoctonia solani*; see Table VII (*96-103*).

Thus, in summary, growing evidence is demonstrating the importance of lignans and isoflavonoids in plant defense.   While those not derived via (+)-pinoresinol/(+)-lariciresinol and isoflavone reductases are primarily insecticidal, those resulting from the corresponding reductases appear to be mainly antimicrobial [and to a lesser extent, antifeedant].

Concluding Remarks

From our preceding discussions, notable similarities have emerged between two key reductases in the supposedly "unrelated" branches of lignan and isoflavonoid metabolism. These similarities include: comparable pivotal positions in their respective metabolic pathways; striking gene sequence homologies and conserved putative protein phosphorylation sites, with the latter suggesting a similar mechanism for regulation; closely related catalytic mechanisms; common physiological functions of their metabolites; and perhaps even similar subcellular location of their metabolites *in planta*. Taken together, these similarities suggest that (+)-pinoresinol/(+)-lariciresinol and isoflavonoid reductases are phylogenetically linked. Given that the lignan reductases apparently evolved prior to the isoflavonoid reductases, it can tentatively be proposed that evolutionary divergence of these pivotal branch point reductases has occurred. Further clarification to this hypothesis may occur by determining the physiological roles of the so-called "isoflavone reductase homologs", and establishing their evolutionary significance.

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Chapter 7

Potent Insecticidal Activity of *Ginkgo biloba* Derived Trilactone Terpenes Against *Nilaparvata lugens*

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Methanol extracts from 119 samples of 52 domestic plant species, 84 samples of 49 Indian plant species and 31 samples of 21 African plant species were tested for their insecticidal activities against the brown planthopper (BPH), *Nilaparvata lugens* Stål, using spray or topical application method. A foliar extract of *Ginkgo biloba* L. (Ginkgoaceae) revealed the most potent insecticidal activity against BPH. The active constituents were isolated by chromatographic techniques and characterized by spectral analysis as ginkgolides A, B and C, and bilobalide. Bilobalide not only revealed much more potent insecticidal activity against susceptible BPH than the commonly used carbamate insecticides carbofuran and fenobucarb, but was also highly effective against three strains of BPH resistant to diazinon, carbofuran, and fenobucarb, respectively. However, this compound was nontoxic to the tobacco cutworm, housefly, house mosquito, german cockroach, and two-spotted spider mite. At a dose of 0.01 μg/female, topically-applied bilobalide caused signs of toxicity such as tremors and paralysis, and the insects died within 30 min of treatment. This compound was relatively nontoxic to mice (LD_{50}, >1,000 mg/kg) and was not mutagenic, when tested against five strains of *Salmonella typhimurium*. Additionally, *G. biloba*-derived materials were not phytotoxic to rice plant seedlings at 2,000 ppm. As active ingredients of a naturally occurring insecticide, *G. biloba*-derived materials could be useful as a new control agent for BPH.

The brown planthopper (BPH), *Nilaparvata lugens* Stål, is one of the most important insect pests of the rice plant (*Oryza sativa* L.) in Asia. If not managed properly from the early growth stage of the crop, this insect species causes serious yield losses directly from adults and nymphs feeding excessively on the developing rice plants, and indirectly from transmission of stunt grassy disease. Losses due to this insect and the disease were estimated to be more than USD 300 million in 11 Asian countries alone (*1*).

Current control of BPH populations is primarily based on repeated applications of insecticides. Although they have effectively controlled this species, their continued use on rice paddy fields for several decades has disrupted biological control by natural enemies and led to resurgences in BPH populations (*2-4*), and the development of widespread resistance to various types of insecticides (*4-7*). Decreasing efficacy and increasing concern over adverse environmental effects of the earlier types of insecticides have brought about the need for the development of new types of selective control alternatives or of methods of crop protection without, or with reduced, use of synthetic insecticides.

Plants constitute a rich source of bioactive chemicals (*8*). Since these are often active against a limited number of species including specific target insects, are biodegradable to nontoxic products, and potentially suitable for use in integrated pest management programs, they could lead to the development of new classes of possibly safer insect control agents. Therefore, much effort has been focused on plant materials for potentially useful products as commercial insecticides or as lead compounds (*9-12*).

In the laboratory study described herein, we assessed insecticidal activity of compounds isolated from *Ginkgo biloba* leaves against six arthropod species and three strains of insecticide-resistant BPH.

Screening of Plant-Derived Materials against the BPH

We established a bioassay system suitable for rapid mass screening of synthetic organic compounds or plant-derived extracts for insecticidal activity with reproducible results, using only a minute quantity of compounds (*13-15*). The most important factor in the primary screening may be the starting concentration or dose. A concentration of 5,000 ppm or 1.0 μg of a plant extract in 0.25 μl of methanol (or acetone) did not cause any problem with solubility and allows detection of minor active compounds.

Methanol extracts from 119 domestic plant species in 52 families were tested for their insecticidal activities against susceptible BPH female adults. The plant species and tissue sampled are described elsewhere (*14-17*). A spray method was used at a concentration of 5,000 ppm, using a glass spray unit connected to a forced air supply (Pacific Chemical Co., Seoul, Korea). Twenty female adults were transferred into a test tube (3 x 20 cm) containing five 'Chuchung' rice seedlings wrapped with cotton and about 20 ml water. Test samples emulsified in distilled water with Triton X-100 added at the rate of 0.1 ml/L were used. Methanol extracts from 84 samples of 49 Indian plant species in 30 families and 31 samples of 21 African plant species in 13 families were also tested, using a topical application method. The plant species and tissue sampled are described elsewhere (*18, 19*). A dose of 1.0 μg of each sample in 0.25 μl of acetone was topically applied to the thoracic dorsa of 4- to 5-day-old female adults. All treated insects were held in a room at 25 $\pm$ 1°C, 50-60% relative humidity, and a photoperiod of 16 : 8 (light : dark). Mortalities were determined after 48 hr, and all treatments were replicated three times.

Insecticidal activity varied with both plant species and tissue sampled. In tests with domestic plants, a methanol extract of *G. biloba* (Ginkgoaceae) leaves had potent insecticidal activity (100% mortality). From Indian plant species, significant insecticidal activity (>90% mortality) was obtained with methanol extracts of *Adhota vasica* (Acanthaceae) leaves, *Annona squamosa* (Annonaceae) seeds, *Nerium indicum* (Apocynaceae) stems, *Pongamia pinnata* (Papilionaceae) seeds, *Prosopis chinensis* (Verbenaceae) stems, *Clerodendron inerme* (Verbenaceae) whole plants, and *Vitex negundo* (Verbenaceae) leaves. The economic importance of the majority of these Indian plants is described in detail elsewhere (*20*). Methanol extracts from whole plants of the African *Cassia occidentalis* and *C. tora* (Caesalpinaceae), and stems of *P. chinensis* were highly effective against BPH (>90% mortality). These domestic and tropical plant species might form a new source of BPH-management agents for the rice paddy ecosystem. Jacobson (*21*) pointed out that the most promising botanicals as sources of novel plant-based insecticides for use at the time (1989) and in the future are species of the families Meliaceae, Rutaceae, Asteraceae, Annonaceae, Labiatae, and Canellaceae.

Isolation and Identification. Fully developed leaves of *G. biloba* were collected during mid-July to August 1994-1995 at the College of Agriculture & Life Sciences, Seoul National University, Suwon, Korea.

These were dried in a oven at 60℃ for 3 days, finely powdered, extracted twice with methanol at room temperature for 48 hr and filtered (Toyo filter paper No. 2). The combined filtrate was concentrated *in vacuo* at 35℃ to give a yield of about 30% as a green tar. This extract (300 g) was partitioned between ethyl acetate (12.5 g) and water (287.5 g) for subsequent bioassay. For isolation, 0.05 μg of each *G. biloba*-derived fraction in 0.25 μl of acetone was topically applied to BPH. The organic phase alone was active.

The ethyl acetate portion (12.5 g) was further separated (Figure 1). This fraction was dissolved in H_2O-MeOH-acetic acid (70 : 30 : 1). Both the yellow precipitate and the dried filtrate showed potent insecticidal activity. The precipitate was further purified using various techniques such as silica gel column chromatography eluted with cyclohexane-EtOAc (6 : 4). Finally, three potent active principles were isolated. R_f values of the isolates I (white precipitate with purity >96%), II, and III in toluene-acetone (8 : 2) were 0.40, 0.38, and 0.19, respectively. The dried filtrate was chromatographed on a silica gel column (Merck 70-230 mesh, 500 g, 5.5 i.d. x 70 cm), and successively eluted with a stepwise gradient of chloroform-MeOH (**Figure 1**). The active fraction (5 %) was further fractionated on a Chromatotron (Harrison Research Model 7924 T, California), using a mobile phase of hexane-EtOAc (1 : 3 and 3 : 1, respectively). The fractions were analyzed by TLC (chloroform-MeOH, 10 : 3), and similar fractions combined. For further separation of the bioactive substances, a prep HPLC (Waters Delta Prep 4,000) was used. The column was 29 i.d. x 300 mm Bondapak C_{18} (Waters) using MeOH-H_2O (3 : 7) at a flow rate of 10 ml/min and detection at 280 nm. Finally, a potent insecticidal isolate (IV) was obtained.

Structural determination of the active isolates was based on spectral analysis. ^{1}H- and ^{13}C-NMR spectra were recorded with a Bruker AM-500 spectrometer and chemical shifts were given in δ(ppm). UV spectra were obtained on a Waters 490 spectrometer, IR spectra on a Biorad FT-80 spectrophotometer, and mass spectra on a JEOL JMS-DX 30 spectrometer. Unambiguous ^{1}H- and ^{13}C-NMR chemical shifts were obtained using various two-dimensional NMR techniques such as the [1]H-[1]H Cozy experiment, as well as [13]C-^{1}H correlation spectra and its long-range analog.

Bioassayed-guided fractionation and HPLC of the methanol extract of *G. biloba* leaves afforded active compounds which were characterized as the trilactone diterpenes ginkgolides A (I, 200 mg), B (II, 150 mg) and C (III, 200 mg), and the trilactone sesquiterpene bilobalide (IV, 96
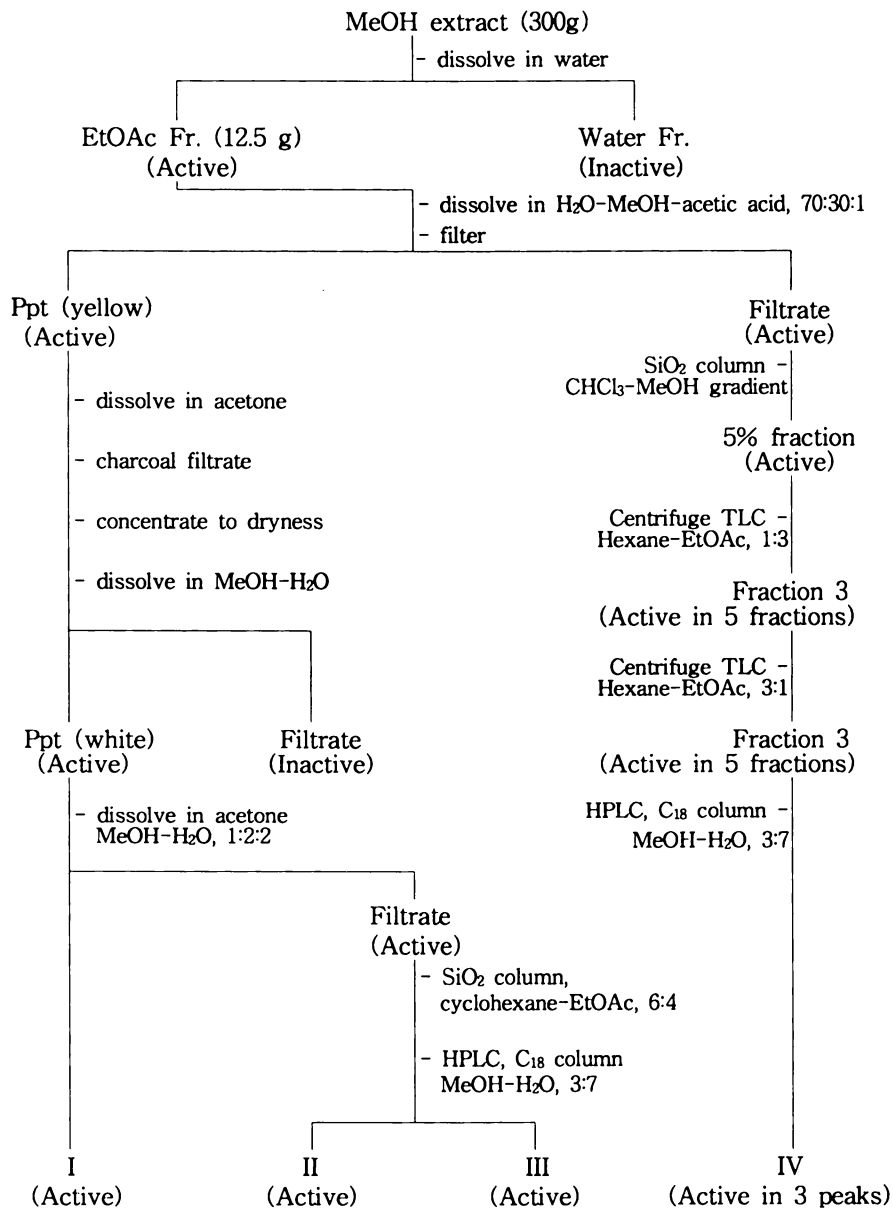
MeOH extract (300g)
- dissolve in water

EtOAc Fr. (12.5 g)   Water Fr.
(Active)   (Inactive)

- dissolve in H₂O-MeOH-acetic acid, 70:30:1
- filter

Ppt (yellow)   Filtrate
(Active)   (Active)
                                          SiO₂ column -
                                          CHCl₃-MeOH gradient

- dissolve in acetone   5% fraction
                                              (Active)
- charcoal filtrate
                                          Centrifuge TLC -
- concentrate to dryness   Hexane-EtOAc, 1:3

- dissolve in MeOH-H₂O   Fraction 3
                                          (Active in 5 fractions)

                                          Centrifuge TLC -
                                          Hexane-EtOAc, 3:1

Ppt (white)   Filtrate   Fraction 3
(Active)   (Inactive)   (Active in 5 fractions)

- dissolve in acetone   HPLC, C₁₈ column -
  MeOH-H₂O, 1:2:2   MeOH-H₂O, 3:7

              Filtrate
              (Active)
              - SiO₂ column,
                cyclohexane-EtOAc, 6:4

              - HPLC, C₁₈ column
                MeOH-H₂O, 3:7

I   II   III   IV
(Active)   (Active)   (Active)   (Active in 3 peaks)

Figure 1. Isolation procedure for insecticidal compounds from methanol extracts of dried *Ginkgo biloba* leaves. Insecticidal activity of the *G. biloba*-derived materials was determined at a dose of 0.05 µg/female by topical application.

mg) (Figure 2) by spectroscopic methods including MS and NMR, and comparision of the spectral data with literature values (*22-24*). For example, bilobalide was identified based on the following evidence: BP: >250°C; IR ν $^{KBr}_{max}$ cm^{-1}: 1809, 1790, 1775; UV λ^{MeOH}_{max} nm(ε): 221 (113); EI-MS (70 eV) m/z (% rel. int.): M$^+$ 326 (7), 270 (19), 224 (8), 196 (22), 129 (37), 15 (100); ^{1}H NMR (CD$_3$OD, 500 MHz): δ 1.21 (9H, s), 2.34 (1H, dd, J = 13.6, 7.0 Hz), 2.79 (1H, dd, J = 13.6, 7.0 Hz), 3.01 (1H, d, J = 18.1 Hz), 3.05 (1H, d, J = 18.1 Hz), 5.01 (1H, t, J = 7.0 Hz), 5.40 (1H, s), 6.37 (1H, s); ^{13}C NMR (CD$_3$OD, 100 MHz): δ 37.6 t (C1), 175.0 s (C2 and C11), 179.2 s (C4), 58.8 s (C5), 85.1 d (C6), 43.3 t (C7), 87.5 s (C8), 67.0 s (C9), 69.9 d (C10), 101.3 d (C12), 27.3 q [C(CH$_3$)$_3$], 38.7 s [C(CH$_3$)$_3$].

(I)

(II)
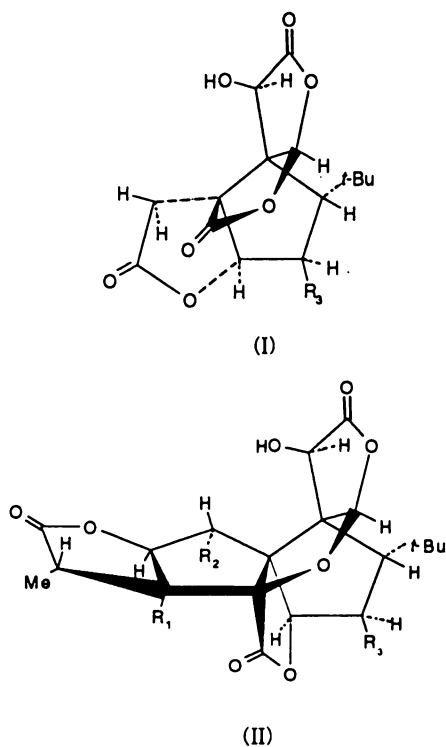
	R$_1$	R$_2$	R$_3$
Ginkgolide A	OH	H	H
Ginkgolide B	OH	OH	H
Ginkgolide C	OH	OH	OH

Figure 2. Structures of bilobalide (I), and ginkogolides A, B and C (II), potent insecticidal components isolated from *Ginkgo biloba* leaves.

Insecticidal Activity of *G. biloba*-Derived Trilactone Terpenes against the susceptible BPH. Insecticidal activity of the four active compounds (ginkgolides A, B, C, and bilobalide) against susceptible BPH female adults was determined following topical application. As shown in Table I, bilobalide (LD$_{50}$, 0.26 ng/♀) was much more toxic than diazinon (LD$_{50}$, 7.6 ng/♀), carbofuran (LD$_{50}$, 1.8 ng/♀), and fenobucarb (LD$_{50}$, 3.2 ng/♀). Efficacy of ginkgolide C was comparable to that of diazinon, whereas ginkgolides A and B were significantly less active than the insecticides. This is the first report on *G. biloba*-derived trilactone terpenes as naturally occurring insecticidal compounds against BPH, although Matsumoto and Sei (25) reported that these trilactone terpenes derived from *G. biloba* leaves possessed antifeedant activity against *Pieris rapae crucivora* larvae.

Observation of the poisoning symptoms of insecticides not only is of practical importance for insect control, but can also contribute to the elucidation of insecticide mode-of-action. Poisoning by bilobalide was compared with that of acetylcholinesterase (AChE) inhibitors via topical

Table I. Toxicity of Test Compounds to Susceptible *Nilaparvata lugens* Female Adults

Chemical	Slope ($\pm$SE)	LD$_{50}$ (ng/♀) (95% CL)	RT[a]
Bilobalide	2.71 ($\pm$0.39)	0.26 (0.19 – 0.35)	12.31
Ginkgolide A	2.27 ($\pm$0.22)	64 (55 – 73)	0.05
Ginkgolide B	1.81 ($\pm$0.16)	16 (14 – 19)	0.20
Ginkgolide C	2.29 ($\pm$0.18)	9.5 (8.2 – 10.0)	0.34
Diazinon	2.72 ($\pm$0.81)	7.6 (3.9 – 10.3)	0.42
Carbofuran	2.80 ($\pm$0.56)	1.8 (1.3 – 2.4)	1.78
Fenobucarb	2.75 ($\pm$0.68)	3.2 (2.3 – 4.6)	1.00

[a]Realative toxicity = LD$_{50}$ value of fenobucarb/LD$_{50}$ value of each chemical.

application (Table II). Bilobalide produced 100% mortality 24 hr after treatment even at doses as low as 0.01 μg/female, whereas lessermortality (30-67%) was obtained at equivalent doses of conventional insecticides. The lethal effect of bilobalide was observed within 30 min post-treatment at a dose of 0.01 μg/female, whereas the test insecticides produced less than 70% mortality at 24 hr post-treatment. Additionally, at 0.01 μg/female, bilobalide toxicity was characterized by tremors and paralysis. These results indicate that bilobalide is a fast contact poison comparable to AChE inhibitors, although its exact mode-of-action remains unknown.

Because of its potent activity in BPH, we determined the spectrum-of-action of bilobalide against several other pest species in laboratory bioassays as previously described (*13*). This compound was relatively nontoxic to the tobacco cutworm, housefly, house mosquito, german cockroach, and two-spotted spider mite (Table III).

Table II. Effects of Topical Application of Test Chemicals on the Mortality of *N. lugens* Female Adults at Different Times

Chemical	Dose, μg/♀	% Mortality (mean$\pm$SE)[a]				
		0.5	1	2	3	24 hr
Bilobalide	0.01	100a	100a	100a	100a	100a
	0.001	100a	100a	100a	100a	100a
Diazinon	0.01	13.3$\pm$1.7c	16.7$\pm$1.7c	20.0$\pm$2.9c	28.3$\pm$4.4c	56.7$\pm$6.0c
	0.001	8.3$\pm$1.7c	10.0$\pm$2.9c	13.3$\pm$1.7c	18.3$\pm$3.3c	33.3$\pm$3.3d
Carbofuran	0.01	28.3$\pm$1.7b	33.3$\pm$1.7b	38.3$\pm$3.3b	46.7$\pm$1.7b	71.7$\pm$3.3bc
	0.001	13.3$\pm$5.8c	18.3$\pm$1.7c	20.0$\pm$2.9c	23.3$\pm$1.7c	30.0$\pm$2.9d
Fenobucarb	0.01	38.3$\pm$3.3b	41.7$\pm$1.7b	50.0$\pm$2.9b	53.3$\pm$1.7b	76.7$\pm$1.7b
	0.001	30.0$\pm$2.9b	36.7$\pm$3.3b	38.3$\pm$4.4b	48.3$\pm$1.7b	66.7$\pm$1.7bc

[a]Means within a column followed by the same letter are not significantly different (p = 0.05, Scheffe's test). Mortalities were transformed to arcsine square root of mortalities before ANOVA. Means ($\pm$SE) of untransformed data are reported.

Various compounds including terpenoids, phenolics, and alkaloids exist in plants and jointly or independently contibute to insecticidal activities. About 18,000 secondary plant metabolites have been chemically identified (8). These are often active against a limited range of species including specific target insects. Based on our results, bilobalide has a narrow insecticidal spectrum. In contrast, *Azadirachta indica* (neem)-derived materials affect more than 200 species of insects including the hemipterous pests of rice (26, 27).

Effectiveness of Bilobalide against Insecticide-Resistant BPH. Control of BPH populations is primarily based on repeated or continued applications of insecticides. However, their extensive use for the decades has led to the development of widespread resistance (4-7). This species migrates every year to Korea from foreign breeding sources such as Mainland China (28-30). Independently developed resistance mechanism(s) with different genetic or biochemical bases might be involved in resistance (5). These facts suggest that careful

Table III. Insecticidal Activity of Bilobalide against Some Arthropod Pests

Test animal	Stage	Test method	LD_{50} or LC_{50}
Spodoptera litura (Lepidoptera: Noctuidae)	3rd instar	leaf dipping	>4,500 ppm
Musca domestica (Diptera: Muscidae)	female	topical	69 μg/♀
Culex pipiens pallens (Diptera: Culicidae)	female	water feeding	3,500 ppm
	3rd instar	immersion	>10 ppm
Blattella germanica (Orthoptera: Blattellidae)	male	diet feeding	>5,000 ppm
	male	topical	8.5 μg/♂
Tetranychus urticae (Acari: Tetranychidae)	female	spraying	>500 ppm

selection and rotation of insecticides should result in continued satisfactory control. Therefore, more emphasis has to be given to the need for selective BPH control materials for use in integrated pest management.

Toxicity of bilobalide and commercial insecticides against the resistant (R) strains of BPH was investigated. Strains resistant to diazinon, carbofuran, and fenobucarb were derived by selecting the S strain with diazinon 34% emulsifiable concentrate (EC), carbofuran 10% wettable powder, and fenobucarb 50% EC for 45 generations at a selection pressure of 30-50% mortality for successive generations. Hereafter, each strain is referred to as R_d-45, R_c-45, and R_f-45, respectively. As shown in Table IV, high levels of resistance to carbofuran and fenobucarb were observed in the R_c-45 (resistance ratio, 63-fold) and R_f-45 (RR, 107-fold) strains, respectively. However, the R_d-45 strain showed relatively less resistance to diazinon (RR, 34-fold).

Table IV. Toxicity of Test Compounds to Resistant (R) Strains of
N. lugens Female Adults

Chemical	Strain[a]	Slope ($\pm$SE)	LD_{50} (μg/♀) (95% CL)	RR[b]
Bilobalide	Rd-45	2.51 ($\pm$0.41)	0.00024 (0.00016 – 0.00036)	0.9
	Rc-45	3.12 ($\pm$0.56)	0.00017 (0.00011 – 0.00024)	0.7
	Rf-45	7.84 ($\pm$0.83)	0.00028 (0.00026 – 0.00030)	1.1
Diazinon	Rd-45	2.48 ($\pm$0.59)	0.257 (0.175 – 0.377)	33.8
Carbofuran	Rc-45	2.82 ($\pm$0.75)	0.114 (0.080 – 0.156)	63.3
Fenobucarb	Rf-45	2.53 ($\pm$0.76)	0.341 (0.188 – 0.466)	106.6

[a]R_d-45, R_c-45, and R_f-45 strains were derived by selecting the S strain with diazinon, carbofuran, and fenobucarb for 45 generations at a selection pressure of 30-50% mortality, respectively.

[b]Resistance ratio = LD_{50} value of resistant strain/LD_{50} value of susceptible strain.

Bilobalide was highly effective against the three resistant strains of BPH (Table IV), indicating that this compound could be useful as a new insecticidal product against field populations of BPH. We already reported that a decrease in AChE insensitivity and an increase in metabolic degradation by esterases play a great role in the carbofuran resistance in the BPH selected with carbofuran for 18 generations (5). These results indicate that the mode-of-action of bilobalide might be different from that of carbofuran. There is no cross resistance to α -terthienyl (T) derived from the plant family Asteraceae in malathion-resistant mosquito larvae (31), indicating that its mode-of-action is different from that of conventional insecticides. Arnason (31) reported that the ability of α-T to convert triplet oxygen to the singlet state accounts for its enhanced activity against various insect species in the presence of light. It has also been reported that neem derivatives including azadirachtin are highly effective against *Plutella xylostella* larvae resistant to chitin synthesis inhibitor acylureas (32) and to axonic nerve poison deltamethrin (33). This phenomena may be attributable to the differences in mode of action of these materials. Neem derivatives interfere with the neuroendocrine regulation of juvenile and molting hormone titers (27).

Safety of Bilobalide to Mammals.   Acute toxicity of bilobalide was studied in five-week-old male ICR mice (24 - 25 g) applied in corn oil with a gastric catheter. The compound had only modest acute toxicity to mice (LD_{50}, >1,000 mg/kg body weight). It is well acknowledged that plant-derived materials are biodegradable in the environment and many, but not all, are safe to mammals and humans. For example, incorporation of 20% neem cake in the diet increased the growth rate of sheep (34). Similar results were also reported with rats fed with a neem extract at a dose up to 600 mg/kg (35).

Mutagenic activity of bilobalide was investigated according to the method of Maron and Ames (36), using *Salmonella typhimurium* strains TA 1535, TA 1537, TA 1538, TA 98, and TA 100. Bilobalide was not mutagenic (Table V). Similarly, azadirachtin is not mutagenic in *S. typhimurium* (37) and normal human cells in culture are not affected by neem extracts (38). Lee (39) also reported that carvacrol derived from *Thujopsis dolabrata* var. *hondai* was not mutagenic against five strains of *S. typhimurium*.

Phytotoxicity.   *G. biloba*-derived materials (methanol extract, active EtOAc fraction, and bilobalide) were applied to 'Chucheong' rice plants (6 weeks after germination) grown in plastic pots (20 x 15 cm)

Table V. Mutagenicity of Bilobalide Using *Salmonella typhimurium*

Strain	Compound[a]	Dose (µg/plate)	−S-9 X±SE	+S-9 X±SE
TA-1535	Bilobalide	0	12±5	13±3
		312	17±3	10±4
		625	13±6	13±5
		1250	10±4	9±6
		2500	11±5	13±1
		5000	13±3	12±0
TA-1537	Bilobalide	0	10±3	12±4
		312	11±3	12±6
		625	12±1	14±4
		1250	8±6	11±3
		2500	8±2	9±3
		5000	9±6	12±7
TA-98	Bilobalide	0	26±3	41±2
		312	19±2	41±7
		625	19±4	35±4
		1250	25±3	30±4
		2500	25±9	31±10
		5000	20±3	34±2
TA-100	Bilobalide	0	103±6	105±5
		312	95±3	99±4
		625	97±5	100±3
		1250	97±7	96±7
		2500	104±4	96±4
		5000	90±10	93±1
TA-1535	SA	1	537±38	
TA-1537	9AA	50	177±2	
TA-98	2AF	1		609±45
TA-100	SA	1	505±25	

[a]Positive control; SA = sodium azide; 9AA = 9-amino-acridine; 2AF = 2-aminofluorene.

at a concentration of 2000 ppm. Test samples emulsified in distilled water with Triton X-100 added at the rate of 0.1 ml/L were used. No leaf burn or other symptoms of phytotoxicity were observed on treated plants. It has been observed that other plant derivatives cause no phytotoxicity. For example, neem treatments have no effects on seed germination and growth of rice plants (40), although their application in cabbage can cause a change of plant color and reduction of headsize (41).

Conclusion

Against the BPH, the toxicity of trilactone terpenes from *G. biloba* is comparable to that of the widely used insecticides diazinon, carbofuran, and fenobucarb. These terpenes were highly effective against three strains of BPH resistant to diazinon, carbofuran, and fenobucarb, respectively. At present, the toxicity of *G. biloba*-derived trilactone terpenes to natural enemies remains unknown, although other plant-derived materials and phytochemicals often have been shown to be selective towards natural enemies. Derivatives of neem are found to have a variety of biological activities against nearly 200 species of insects without adverse effects on most nontarget organisms (27, 42, 43). Natural products from *G. biloba* leaves might be useful as a new biorational management agent for controlling BPH populations on rice, based upon their modest toxicity to mammals and lack of mutagenic activity.

Acknowledgments

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Chapter 8

Mechanisms for the Initiation of Pathogenesis

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Infectious agents and pathogenic organisms represent a continually evolving threat to human health and agricultural production. Understanding the strategies that have allowed one organism to surmount the defenses of another will be critical to our successful control of such infections. In all specific interactions, the first stage of this process is host recognition, or xenognosis. This process represents the critical stage in the establishment of the host-pathogen interface and, as such, a reasonable target for intervention (*1*). In this context, the last several years has seen the discovery of several xenognostic agents controlling both pathogenesis and symbiosis across a diverse range of organisms.
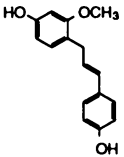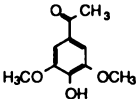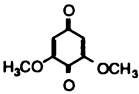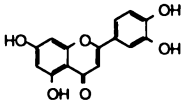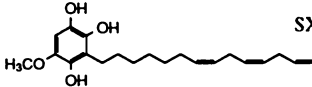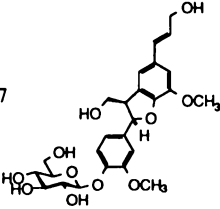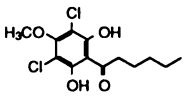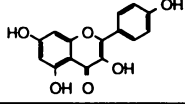
Table 1 summarizes the structures of some of these signal molecules. The aromatic ring and phenolic functional group are common features of these relatively simple structures. While phenols have been recognized for many years as important molecular signals, e.g. estrogen, thyroid hormone, the mitogenic neurotransmitter norepinephrine, they are most widely recognized for their more general role in allelopathy, as phytoalexins and as stress metabolites in plants (*2*). It is only more recently that these highly specific roles as xenognosins been generally accepted (*1, 3*).

The original members of the xenognosins, xenognosin A and B (*4, 5*), appear not to be perceived directly but are oxidatively converted by parasite enzymes to quinones (*6,7*) and it is these oxidation products, like DMBQ, that subsequently signal parasitic plants to switch from vegetative to parasitic growth (*6,8*). Acetosyringone, which also induces the transition to the pathogenic mode in the oncogenic *Agrobacterium tumefaciens*, appears be be recognized directly by receptor proteins (*9*). The signals that initiate the host-specific legume-Rhizobium interaction are flavaniods, such as luteolin (*10*). The Sorghum Xenognosin for Striga germination (*11*), SXSg, also appears to be very specific and, like the Rhizobium signals, directly perceived, although the signal transduction mechanism of these last two xenognosins are as yet insufficiently characterized (*12*).

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Table 1. Structures of the molecules

Year	Structure	Name	Function
1981		xenognosins A and B	Induce haustorial development in parasitic angiosperms
1985		acetosyringone AS	Induce *vir* gene expression in Agrobacterium
1986		DMBQ	Induce haustorial development in parasitic angiosperms
1986		luteolin	Induce *nod* gene expression in Rhizobium
1986		SXSg	Breaks developmental dormancy in parasitic angiosperms
1987		DCG	Induce cell division in tobacco
1987		DIF-1	Induces slime mold differentiation
1990		SA	Endogenous inducer of acquired resistance
1992		Kaempferol	Inducer of pollen germination and tube growth

The remaining entries in Table 1 are not xenognosins but rather endogenous signal molecules. The DCGs and their aglycones induce cell division in tobacco cells (*13,14*), DIF-1 induces slime mole differentiation (*15*), SA induces acquired resistance in tobacco (*16*), and kaempferol induces pollen germination and tube growth (*17*). The skeletal similarity between the endogenous signals, kaempferol and the DCG aglycone, and the xenognosins luteolin and xenognosin B, are striking. Structural similarities are also apparent with DIF-1 and SXSg as well as with AS, xenognosin A and even DMBQ. While these similarities may be evolutionally fortuitous with regard to their biological function, they appear to speak to a common mechanism for information reception and transfer. Clearly an understanding of their receptors and a comparison of the transfer mechanisms will open new strategies to manipulate the critical first step in xenognosis and cell-cell recognition.

Mechanism of Xenognosin Perception

Before discussing the mechanisms by which the xenognosins are perceived, it is important to consider both the biosynthetic origins of these materials as well as their general biological reactivity. Two principle biosynthetic pathways appear to contribute almost equally to the production of the known phenolic compounds (*2*). The polyketide pathway is illustrated in Figure 1 using the recently determined biosynthesis

A. Polyketide Pathway

B. Shikimate Pathway
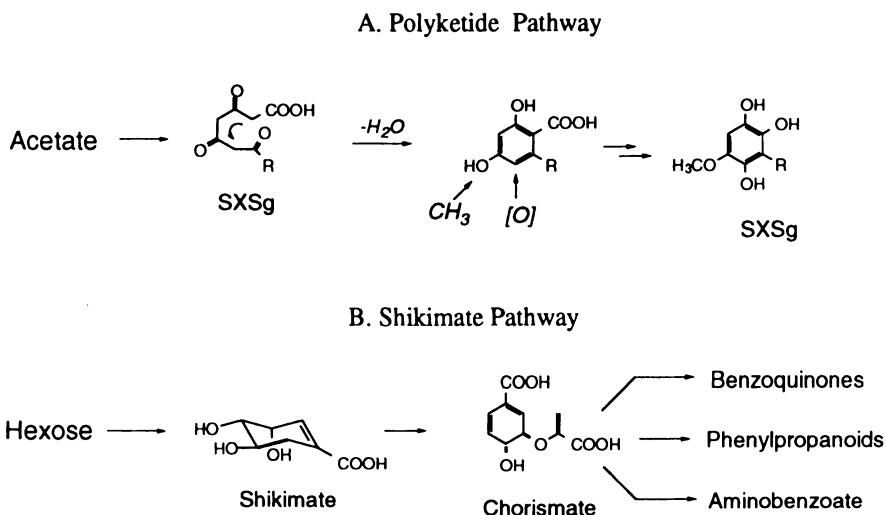
Figure 1. Biosynthetic pathways.

of SXSg (*18*). Monocyclic phenols or quinones are readily prepared through such dehydrating cyclizations. A somewhat different range of structural types are available through the shikimate pathway, including the benzoquinones and phenylpropanoids.

Importantly, the polyketide pathway starts with acetate as the precursor, whereas the shikimate pathway uses the hexose pool; these different origins will be critical to their regulation. The flavanoids, including both luteolin and kaempferol, and the DCGs are produced by a combination of both pathways (*2,19*).

There are three primary reaction manifolds open to phenols within a biological milieu (Figure 2). The resonance stabilization provided by the aromatic ring reduces the pKa of the phenolic O-H some seven orders of magnitude relative to aliphatic alcohols and places it in a range where it is readily ionized by biological bases. The pKa is further modulated, up to three or four orders of magnitude, with substituents on the aromatic ring and in environments of differing dielectric constant. This resonance stabilization also modulates a second reaction manifold, the homolytic cleavage of the O-H bond. An intermediate phenoxy radical is present in several enzyme reactions including ribonucleotide reductase, vitamin E mediated stabilization of autoxidation pathways and SXSg stabilization which is critical in defining the spatial distribution of parasitic commitment (*18*). The third reaction is oxidation-reduction, most notably oxidative phosphorylation and photosynthesis. Here the hydroquinone-quinone can serve as either a 1 or 2 electron carrier. In this paper we will discuss some of the evidence that implicates these reaction manifolds in xenognosin perception.
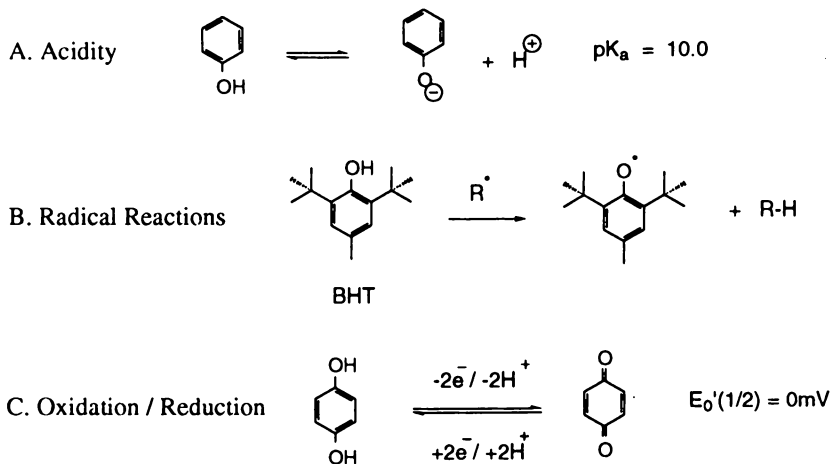
Figure 2. Reactions of phenols.

Agrobacterium Xenognosis. The remarkable success of *Agrobacterium tumefaciens*, the causative agent of crown gall tumors in plants, has been attributed to its ability to perceive a diverse range of structures (*3*). Tumorigenesis in crown gall disease involves the transfer and incorporation of DNA into susceptible plant cells (*20-23*); *A. tumefaciens* is still the only known natural vector for gene transfer into higher plants. The genes required for transformation, the *vir* genes, are encoded on

the Ti plasmid and their expression is controlled by the VirA/VirG two-component system (2). *virA* and *virG* share homology with a large number of environmental sensing regulatory systems which exist throughout the prokaryotes (*24-26*), and in at least several eukaryotes (*27-29*). Their name is derived from and reflects the apparent simple nature of signal transduction: a transmitter (sometimes referred to as a sensor), that is an autokinase; and a receiver, that after phosphorylation and/or dephosphorylation directly affects an output, in this case *vir* expression.
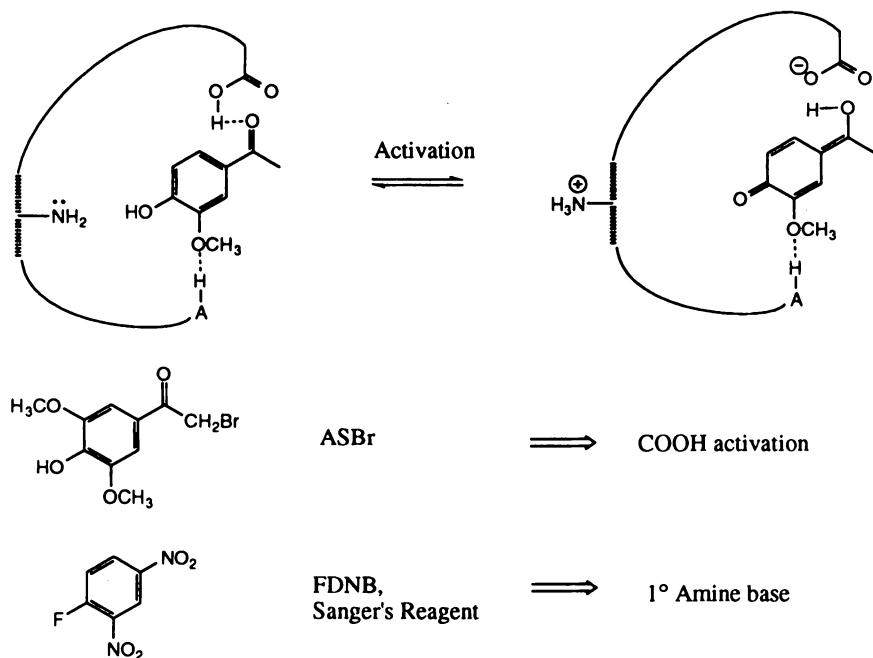
Figure 3. The proton transfer model for receptor activation shown with the two specific inhibitors ASBr and FDNB and the binding site residues identified.

    Signal recognition in two-component systems can be direct or indirect, and in most cases, the consequences of signal recognition are, at a structural and mechanistic level, unknown. An analysis of the active and structurally similar inactive xenognostic molecules in *vir* gene induction led to the development of the "proton-transfer model" of signal perception (*30*). In this model, deprotonation of the inducing phenol is critical and can be aided by a concomitant protonation of the para substituent. The resulting tautomerization, via the quinone methide (Figure 3), would effectively transfer a proton the length of the phenol, ~7 Å, to a basic residue in the binding site. Protonation within the hydrophobic, solvent-inaccessible site induces the conformational change in the receptor necessary for activation. Recognition is then

based on both the intrinsic binding energy and the modulated acidity of the xenognostic signal.

This reaction, the core of the proton transfer model (*30,31*), identifies two essential residues, one acidic and one basic, positioned on opposite sides of the binding site. To explore this proposal, α-bromoacetosyringone (ASBr), which positions an electrophilic carbon proximal to the conjugate base of the proposed acidic residue, was synthesized and found to be a specific and irreversible inhibitor of *vir* gene induction (*30*). Further studies with the amine specific Sanger's reagent also found it to be a specific and irreversible inhibitor (*31*). These competitive inhibitors provided support for the specific binding interaction of the xenognosin and identified two particularly reactive residues within the binding site. The existence of the reactive residues do not prove the proton transfer model, but further support the proposal that recognition was mediated as much by chemical reactivity of the xenognosin as by its affinity for a receptor binding site (*3*).

Striga Xenognosin. *Striga asiatica* (Schrophulariceae), is a holoparasitic plant with a host range restricted to monocots (*32*). The host attachment organ, known as the haustorium, is derived from the cells of the root apical meristem. The Sorghum Xenognosin for Striga haustoria, characterized as DMBQ, is not exuded by the host but rather oxidatively released from the host root surface by *Striga* enzymes (*6,7*). DMBQ is necessary and sufficient to induce the transition from vegetative growth to haustorial development (*1,8*).

An association between redox events and cellular development has been recognized for many years (*33*), yet neither the initiating events nor the mechanisms have been defined. In this case, a range of benzoquinones were found to be active inducers and structure-activity analyses suggested a redox dependence for the active compounds (*34*). A correlation with one electron reactions suggested the existence of a long-lived semiquinone. Attempts to legitimize the intermediacy of this radical anion led to the development of cyclopropyl-*p*-benzoquinone (CPBQ) as a specific inhibitor of haustorial development (*34,35*). The cyclopropane of CPBQ has now been shown to open following reduction (*35*) and, based on the chemistry of related radical anions (*36*), either radical recombination (*37,38*) or nucleophilic addition in the redox binding site provide a reasonable mechanism for the irreversible inactivation (Figure 4). These studies provide strong support for an oxidation-reduction reaction mediating the commitment to parasitism and has implicated this chemical reaction as a mechanism of signal perception.

Initiating Information Transfer

An understanding of how organisms acquire information about their environment, and how this information is processed to yield an "appropriate" response is a fundamental problem in the life sciences. In general, an environmental cue is perceived by a protein
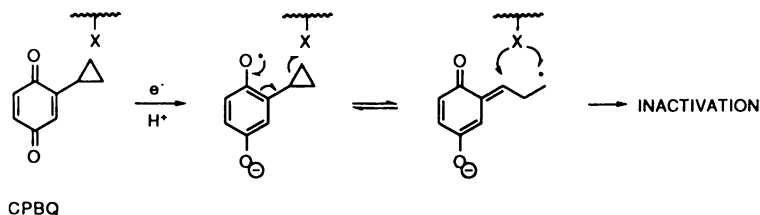
Figure 4.   Proposed mechanism of receptor inactivation following quinone reduction.

receptor which, in its "activated" state, initiates a series of biochemical processes leading to a new cellular behavior or activity. Often, the cell will respond by activating and/or inactivating the transcription of specific genes. This response can be the result of a relatively simple two-component system of the type seen in many bacterial responses (*39-41*) including Agrobacterium virulence, or may be highly complex involving a series of signals, membrane associated proteins, metabolites and transcription factors (*42*).

By this model, the energy of binding to the receptor must be sufficient to initiate the "conformational change" that constitutes activation. The xenognostic signals described above suggest an alternate mechanism for signal perception; the transfer of information resulting from the making and breaking of chemical bonds within the signal molecule itself. This mechanism carries the advantage of being high energy and mediated by a small highly diffusible molecule. An understanding of the breadth and generality of such recognition systems extends the possible mechanisms for signal perception and opens new strategies for their control.

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Chapter 9

Annonaceous Acetogenins as New Natural Pesticides: Recent Progress

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Since their discovery in 1982, the Annonaceous acetogenins have become one of the most rapidly growing classes of bioactive natural products. Chemically, they are C-32 or C-34 long chain fatty acids which have been combined with a 2-propanol unit at C-2 to form a γ-lactone. Biogenetically, double bonds along the fatty acid chain seem to epoxidize and cyclize to form one, two, or three tetrahydrofuran (THF) rings, often with flanking hydroxyls; other functional groups include hydroxyls, acetoxyls, carbonyls, and double bonds. Recently, we have found an acetogenin bearing a hydroxylated tetrahydropyran (THP) ring. Biologically, these compounds are among the most potent of the known inhibitors of complex I (NADH:ubiquinone oxidoreductase) in mitochondrial electron transport systems and of the plasma membrane NADH:oxidase that is characteristic of cancerous cells; these actions seem to induce apoptosis (programmed cell death), perhaps as a consequence of ATP deprivation. Applications as pesticides and antitumor agents hold excellent potential, especially in the thwarting of resistance mechanisms which require an ATP-dependent efflux. Two Annonaceous species, *Asimina triloba* Dunal ("paw paw") and *Annona muricata* L. ("sour sop", "guanabana"), are, respectively, abundant as fruit trees in temperate eastern North America and in the tropics worldwide. Both of these species have now yielded a variety of new acetogenins, and their crude extracts exhibit potent pesticidal effects. These crude extracts can be employed as safe, effective, economical, and environmentally friendly pesticides with an emphasis on the home garden, ornamental, greenhouse, and produce markets, pending regulatory approval.

An array of new natural and biopesticidal products are being developed and approved for control of insect pests, fungal and bacterial diseases, and weeds (1,2). New botanically-derived pesticides offer a more natural, environmentally friendly, approach to pest control, and a growing segment of our human population has grown wary of synthetics and is now geared toward "organic" methods of gardening and produce production (3). However, the regulatory problems associated with the approval of such products are extremely burdensome, and few

academic workers possess the financial resources to complete the required safety assessments (originally established for synthetics) concerning their discoveries (4). One can even convincingly argue that these regulations, when applied to natural products, now only function to disserve the environment and the public health by cementing in place the continued use of harmful and persistent synthetic pesticides. However, the recent approval of pesticidal products, containing azadirachtin and the related triterpenoid derivatives, from the seeds of the neem tree, *Azadirachta indica* A. Juss. (5), shows a hint of enlightenment on the part of the regulatory agencies.

Anticipating an expanding interest in natural pesticides, we, at Purdue University, began a search for new pesticides from higher plants in 1979. Our early progress and our development of brine shrimp lethality (BST), as a convenient pesticidal screen and monitor of bioassay directed fractionation (6,7), were reviewed in an earlier ACS symposium issue resulting from the Toronto Congress in 1988 (8). Our plant screening program has continued, and the most exciting leads from our investigation of over 3,000 plant species, to date, are with the extracts of the Annonaceae. This family is almost exclusively tropical and totals over 2,000 species in 120 genera (9).

Folkloric Leads

Numerous references to the folkloric uses of several Annonaceous species suggest that they contain potential pesticidal components and, in addition, might have the advantage of inducing emesis (vomiting) if ingested. For example, the powdered seeds of *Annona purpurea* Moc. and Sesse ("soncoya") and *A. squamosa* L. ("sugar apple") and the leaf juice of *A. reticulata* L. ("custard apple") are effective as pediculicides to kill head and body lice (10,11); the leaf extracts of *A. squamosa* are helpful in extracting Guinea worms, and the bark and/or roots of *Annona muricata* L. ("sour sop," "guanabana") and *A. chrysophylla* Boj. (syn. *A. senegalensis* Pers.) ("wild custard apple") are used as vermifuges (11). The seed extracts of *A. purpurea* destroy fleas, and the fruit of *A. muricata* relieves the bites of chiggers (11). Smoke from the burning twigs of *Goniothalamus macrophyllus* (Bl.). Hook. f. and Thomas is used to repel mosquitoes (12). The seeds of several species are powerfully emetic (11), and Eli Lilly even marketed an extract of the North American species, *Asimina triloba* Dunal ("paw paw"), at the turn of the century, for that purpose (13).

Discovery of the Annonaceous Acetogenins

Early chemical work on the Annonaceae revealed a storehouse of phytochemicals, including numerous benzylisoquinoline alkaloids, which are quite common in this plant family (9). These alkaloids were often erroneously implied as being responsible for the pesticidal and other biological effects exerted by Annonaceous plant extracts (11,14). Indeed, it is now well understood that the complexity of plant chemistry usually requires activity-directed fractionation of extracts if one wishes to identify the bioactive principles. Such activity-directed fractionations by Jolad et al. (15), using P388 (3PS) *in vivo* murine leukemia and the ethanol extracts of the roots of *Uvaria accuminata* Oliv. (Annonaceae), led to the isolation and structural elucidation of uvaricin (1), an unusual bioactive compound. Uvaricin (1) was the first example of a new class of extremely potent polyketide compounds that are now called the Annonaceous acetogenins.
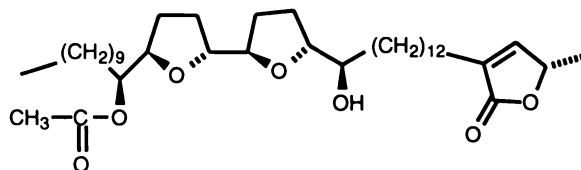
1, uvaricin

Chemically, the Annonaceous acetogenins are C-32 or C-34 linear fatty acids which have been combined with a 2-propanol unit at C-2 to form a γ-lactone. Biogenetically, double bonds along the long fatty acid chain seem to epoxidize and cyclize to form one, two, or three tetrahydrofuran (THF) rings often with flanking hydroxyls; other functional groups include hydroxyls (sometimes as vicinal diols), acetoxyls, carboxyls, and double bonds. We have recently found an acetogenin, mucocin (2) from *Rollinia mucosa* Baill. ("biriba"), which contains an hydroxylated tetrahydropyran ring (THP) ring (16). Our group has now isolated well over 100 acetogenins, and we have published three comprehensive reviews regarding the isolation, structural elucidation, stereochemistries, biogenesis, synthesis, and biological activities of over 160 of these compounds (17-19). A fourth review has been submitted for publication and will bring the total to over 220 distinct acetogenins that are currently known.
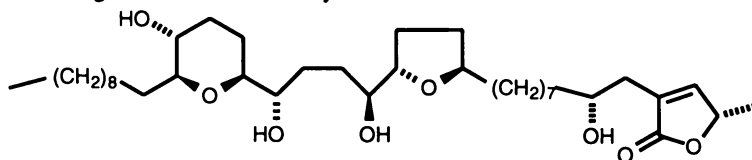
2, mucocin

Considerable progress with the Annonaceous acetogenins, resulting in over forty research papers, has also been made by Cave's group at Université Paris-Sud, and at least three reviews from his laboratory summarize this work (20-22). Similarily, Fujimotos' group at the Tokyo Institute of Technology has found nearly thirty acetogenins in the seeds of *A. squamosa* and *A. reticulata* and have summarized this work (in Japanese) (23). Other progress, especially with synthetic methods, has been recently reviewed by Yao and Wu (in Chinese) (24), Figadere (25), and Hoye (26).

Species of the Annonaceous genera, *Annona, Asimina, Goniothalamus, Rollinia, Uvaria,* and *Xylopia,* all yield extracts that contain complex mixtures of the acetogenins, e.g., from the bark of the Cuban species, *Annona bullata* Rich., our research group has isolated 45 different acetogenin compounds using activity-directed fractionations (17-19). However, the literature is becoming confused with redundancies and synonyms which result when researchers do not conduct thorough literature searches and do not identify the absolute stereochemistries of their isolated acetogenins, e.g., by the use of ^{1}H nmr analyses of Mosher esters (27). Our screens have detected biological activities in numerous additional Annonaceous genera, and these new genera, as well as those currently being explored, will undoubtedly yield novel acetogenins in the future. It is quite reasonable to predict that over 1,000 distinct acetogenins exist within all of the Annonaceous species, and many more of these compounds will be isolated and characterized within the next 20 years.

Asimina triloba Dunal. (the North American paw paw tree) and other species

Our work with the Annonaceous acetogenins began in the 1970's when the extracts of two species, *A. triloba* and *Goniothalamus giganteus* Hook. f. and Thomas, were screened under contracts from the National Cancer Institute (NCI) for *in vivo* murine P388 (3PS) antileukemic activity in mice. In both cases the mice succumbed at low doses of the extracts, and further contract fractionation work on these "toxic" species was abandoned at that time. However, the extracts of both species were extremely potent in the BST bioassay (6,7), and these extracts were submitted to Eli Lilly (Greenfield) for screening against a panel of seven indicator pests. The partitioned ethanolic extract of the barks (F020, F005) were, surprisingly, quite effective against Mexican bean beetles, melon aphids, mosquito larvae, blowfly larvae, and a nematode (*Caenorhabditis elegans*). Activity-directed fractionation was focused initially on the bark extracts of *A. triloba*, and, by monitoring with the BST bioassay and confirming the results with the Lilly pest panel, asimicin (3), a new 4-hydroxylated, pseudosymmetrical, adjacent *bis*-THF acetogenin was isolated as the major bioactive component of a complex, highly potent, acetogenin mixture (27,28). Subsequently, a U.S. patent was granted to Purdue University and the USDA (Peoria) where parallel work was conducted with the seed extracts (29); a divisional patent was then granted to us protecting the composition of matter of asimicin (30).
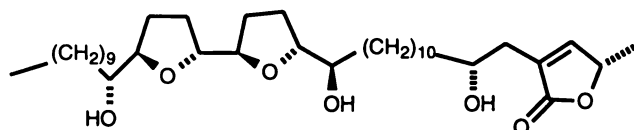
3, asimicin

Concurrently, patents were also being granted to the Bayer Company in Germany for the insecticidal use of an undefined material called "annonin" isolated from the seeds of *A. squamosa*; the Bayer patents, themselves, failed to identify "annonin" as an acetogenin; subsequently, however, some sixteen "annonins" from *A. squamosa* were published by the Bayer group, but several were identical to previously published acetogenin compounds (18); these Bayer patents have apparently been discontinued (31,32).

Activity-directed fractionation of the bark extracts of *G. giganteus* led us to two classes of bioactive components: a series of unprecedented styryllactones (7,33,34 *inter alia*) and several new types of Annonaceous acetogenins (17-19, 35 *inter alia*), including goniocin (4), the first adjacent *tris*-THF ring acetogenin (36). This species is native to Southeast Asia and is relatively inaccessible to us; thus, we have not pushed for its development as a pesticide source; although its extracts are quite active, even killing corn root worm, it apparently lacks the more potent, dihydroxylated, adjacent bis-THF ring acetogenins.
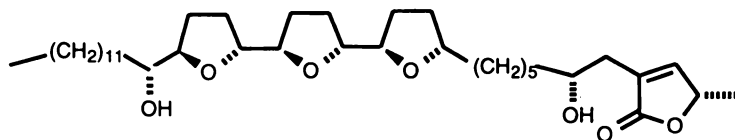
4, goniocin

Bioactivities of Paw Paw (Plant Parts, Monthly Variations, and Genotypes)

The North American paw paw tree, *A. triloba*, is the most abundant of the eight species in the genus, *Asimina* (9); it is native to the eastern United States (37) and is actually quite abundant in the wild, e.g., over 18 million trees are growing in Indiana (38). Our intial work was with bark and seeds. Various plant parts of the paw paw tree were extracted into standardized pesticidal extracts (F005), and the extracts were analyzed with the brine shrimp (BST) bioassay to determine their relative potencies (39). The small twigs (0-0.5 cm diameter) yielded the most potent extracts (LC_{50} values as low as 0.04 ppm); the stem wood (LC_{50} 4.9 ppm) and leaves (LC_{50} 53.7 ppm) yielded the poorest activities. The unripe fruits, seeds, root wood, root bark, and stem bark were notably potent (LC_{50} <0.2 ppm) and, generally, yielded >2% of their dry weight as F005. The smaller diameter stems were more potent than the larger stems. We concluded that, by pollarding or coppicing to collect the small branches and twigs, this biomass could be dried and processed to produce a potent acetogenin mixture. Such a biomass could initially be collected from the wild and eventually could be produced from plantations of trees grown as a new crop (40). Quantities of biomass needed for commercialization of the mixture of acetogenins as a new pesticidal product would be sustainable and renewable through regrowth from the parent trunk and larger branches. Thus, the wild stands of paw paw need not be decimated and, indeed, could be encouraged by elimination of competing species of trees.

In this work, we noticed that the bioactivity of extracts from paw paw biomass collected in November was apparently lower than that from biomass collected in July. This prompted a study of the monthly variation of bioactivity of twigs collected from the same trees. For this study (41), a more rapid extraction method was developed, and the extracts were, as usual, evaluated in the BST (6,7). The results were astonishing (Figure 1) and suggested a dynamic flux in acetogenin composition depending on the season. May, June, and July are optimum for biomass collection, and the winter months (when insects are less of a problem to the trees) are poorest for biomass collection. The potency differences were more than 25 times (February vs. May) in one of the trees. In a second tree at the same location (Amherst, NY), the bioactivity flux was not as dramatic, but the twig samples of this tree were generally more potent. This observation suggested that genetic differences are likely to exist between paw paw trees with some trees concentrating acetogenins more than others. A current project is underway to assay twig samples that were collected on the same day from 670 trees growing in a paw paw plantation in Maryland. Preliminary results show variations in bioactivities as high as 130 times from one tree to the next; however, most trees produce and maintain a useful level of potency (BST LC_{50} ca. 0.5 ppm). Growers of future plantations intended for biomass production of the paw paw pesticide would, thus, be advised to propagate only vegetative clones selected from the tree genotypes that give the highest potencies in their extracts.

Insecticidal Effects of the Extract (F005, F020) from Paw Paw

The ethanolic extracts of paw paw bark and seeds contain complex mixtures of well over thirty different Annonaceous acetogenins, each of which has its own unique features of biological activities. Most of our work on these compounds has been supported by the NCI and has been directed only toward those compounds that show selective cytotoxicities against human solid tumor cell lines. The complexities of these acetogenin mixtures, undoubtedly, serve as a benefit in the
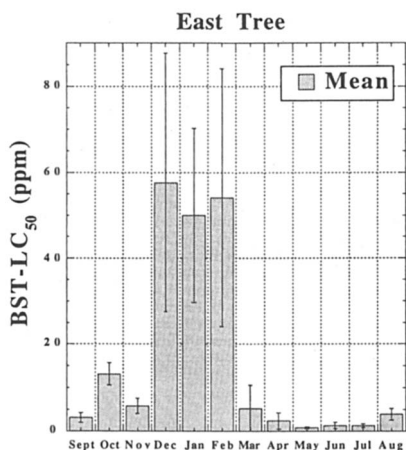
East Tree

Figure 1.   Monthly variation in the biological activity of twigs collected from a single tree of paw paw (*Asimina triloba*) growing in the Amherst, New York, area. (Reproduced with permission from reference 41. Copyright 1996 John Wiley.)
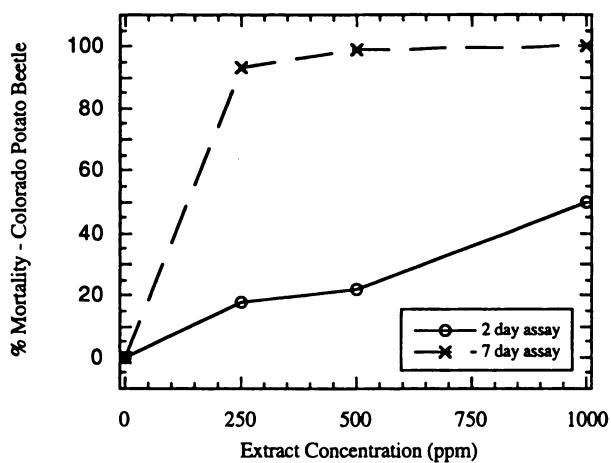

Figure 2.   Colorado Potato Bettle-4th Instar: 2 vs 7 Day Mortality with *Asimina triloba* Extract

protection of the trees against a host of microbes, pests, and herbivores. It is known that such a series of structurally related secondary plant products can even display a level of toxicity to pests that is lacking in the individual compounds (42). The recent work of Feng and Isman (43) showed that, after forty generations, green peach aphids, which were repeatedly treated with either pure azadirachtin or a refined neem seed extract (at equivalent concentrations), developed a nine-fold resistance to azadirachtin but developed no resistance to the neem extract. Such evidence helps to establish a good case for the use of crude, chemically unrefined, plant extracts, containing mixtures of the bioactive plant components, rather than the use of the pure individual components. Insect resistance is much less likely to develop, the environmental load of individual components is lessened, and the products of crude extracts are cheaper to prepare. Thus, we have concentrated our efforts on the pesticidal evaluation of the partitioned ethanolic extract (F005, F020) of paw paw bark (8,29,30).

Three experiments conducted at AgriDyne, Inc., are worthy of mention here and are best summarized in graphic form. Figure 2 shows 7-day vs. 2-day mortalities against Colorado potato beetles (4th instar) for a series of paw paw F005 concentrations applied in foliar sprays. The longer contact period led to excellent results with concentrations as low as 250 ppm being quite effective. This slow kill rate suggested that the acetogenins are more likely an ingestant rather than a contact poison.

One of our interests has been to market a mixed combination of natural pesticidal compounds. Two experiments illustrate that this proposal holds definite merit. Against white flies on cotton leaves the paw paw F005 extract and pyrocide (a natural pyrethrum extract) showed surprisingly effective synergism (Figure 3). The mixture of pyrocide at 250 ppm and F005 at 500 ppm gave more than the expected additive kill rate. Similarly, against Colorado potato beetles, the paw paw F005 extract at 300 ppm synergized very well with a standardized azadirachtin 720 neem extract at 10 ppm (Figure 4). Such experiments clearly demonstrate that the acetogenins need not be purified, beyond a crude level of concentration, to produce an effective pesticidal preparation. Furthermore, the application levels of both the acetogenin mixture and the synergistic product can be reduced in the synergistic mixture, saving money as well as reducing the environmental load of the individual pesticides. A combination of all three of these natural pesticides (acetogenins, pyrethrums, and neem) would quite likely be beneficial and could become a financial success.

Insecticidal Effects of F005 from Guanabana Seeds

Several of the edible, fruit-bearing, *Annona* species primarily from Latin America have been naturalized throughout the tropics (11). These include *A. cherimola* Mill. ("cherimoya"), *A. squamosa* L. ("sugar apple"), *A. squamosa* x *A. cherimola* ("atemoya"), *A. muricata L.* ("sour sop"), *A. reticulata* L ("custard apple"), *A. diversifolia* Safford ("ilama"), *A. purpurea* Moc. and Sesse ("soncoya"), and *A. chrysophylla* Boy. (syn. *A. senegalensis*) ("wild custard apple"). Of these, *A. muricata* appears to be the most abundant; it is called "sour sop" in English, "sir sak" in Dutch, "guanabana" in Spanish, and "corossol" in French. In tropical America, the name "guanabana" is most often used.

Guanabana is not only popular as a table fruit but it also forms the basis of a well-developed juice industry throughout both Latin America and the countries of tropical Asia. By weight, the seeds make up from 3-7% of the fresh fruit, and
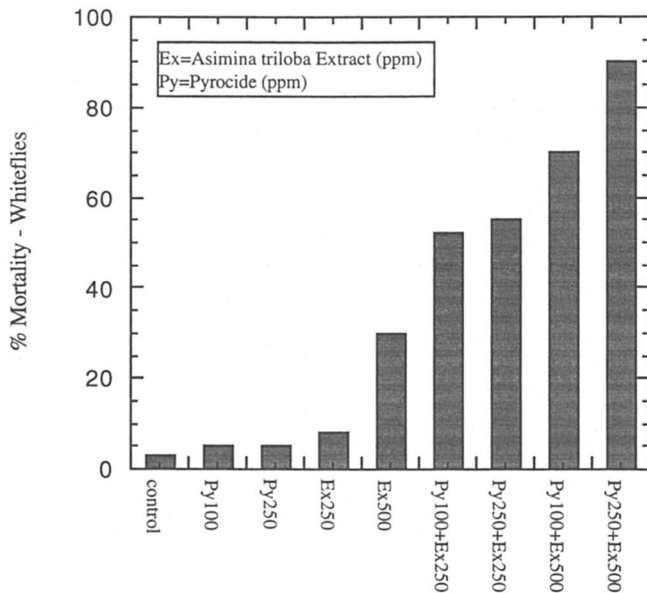
Figure 3.   Whitefly Bioassay on Cotton: *Asimina triloba* Extract Synergism
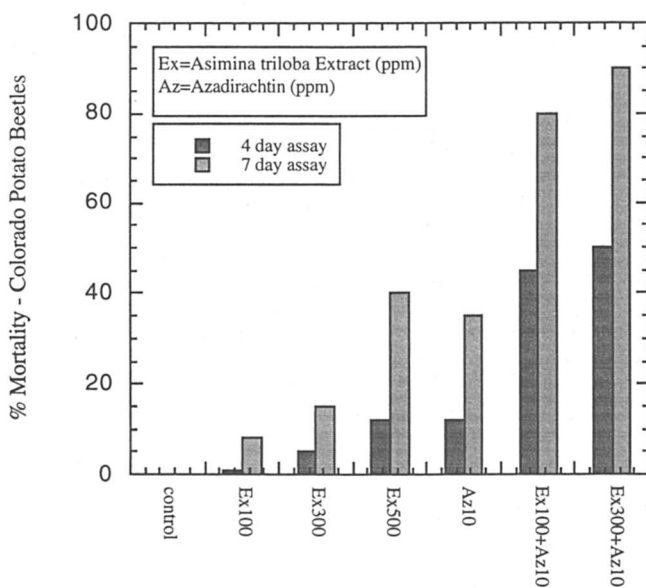with Pyrocide

Figure 4.   Greenhouse Cage Experiment on Colorado Potato Beetles: *Asimina
triloba* Extract Synergism with Azadirachtin 720 Extract

thousands of tons of the fresh fruit are processed each year. In the Philippines alone, about 500,000 trees of fruit-bearing age produce an estimated 8,500 tons of fruit annually (44). The large biomass of seeds that results is presently being discarded, and this material is a readily-available biomass source of acetogenins that might be suitable for pesticide production. Indeed, the F005 partitioned ethanolic extract of the defatted seeds is quite potent in the BST.

Again at AgriDyne, Inc., this F005 guanabana seed extract and the seed oil, from prior defatting of the pulverized seeds with hexane, were evaluated in several insecticidal experiments. Figure 5 shows their effects against Colorado potato beetles and shows that the F005 at 700 ppm, mixed with the seed oil at 1%, gives an excellent kill with a faster rate than the extract alone. In greenhouse cage experiments with sweet potato white flies on poinsettias, the F005 extract (at 750 ppm) and oil (at 0.5%) did an effective job at eliminating larvae and eggs (Figure 6). Similar complimentary effects were observed in reducing the number of green peach aphids on mums (Figure 7).

Acetogenins Isolated from F005 of Paw Paw Seeds and Bark

Since our discovery and patenting of asimicin (1) (28,30), we have isolated 35 bioactive compounds from the seeds and bark of *A. triloba* ("paw paw"). These include 18 adjacent bis-THF acetogenins which can be divided into three types (19): the asimicin type (asimicin, asimin, asiminacin, asiminecin, asiminocin, asimilobin, parviflorin, and *cis*- and *trans*-asimicinones) (28,45-50), the bullatacin type (bullatacin, bullatin, squamocin, motrilin, bullanin, and *cis*- and *trans*-bullatacinones) (48,49,51), and the trilobacin type (trilobacin, trilobin, and asitribin) (45,49,52). Also included are 13 mono-THF acetogenins (*cis*- and *trans*-annonacin A-ones, *cis*- and *trans*-gigantetrocinones, *cis*- and *trans*-isoannonacin, murisolin, 16,19-*cis*-murisolin, murisolin A, *cis*- and *trans*-murisolinones, and asiminenins A and B) (48,49,53,54). The absolute stereochemistries of many of these acetogenins have been determined by ^{1}H NMR analyses of Mosher esters (27), sometimes aided by the formation of formaldehyde acetal derivatives (55). Four bioactive nonacetogenin compounds were also isolated, but those are much less potent than the acetogenins (45).

The most potent Annonaceous acetogenins are the adjacent bis-THF compounds, e.g., asiminocin [(30S)-hydroxy-4-deoxyasimicin] gave an LC_{50} value of 4.9×10^{-3} ppm in the BST and cytotoxic ED_{50} values of 10^{-12} μg/ml against certain human solid tumor cell lines (47). The optimum chain length seems to be C-37 rather than C-35, e.g., parviflorin, from *A. parviflora* (Michx.) Dunal. ("dwarf paw paw"), is a less potent C-35 analogue of asimicin which is identical except that it is C-37 (56). *A. longifolia* Kral ("long-leafed dwarf paw paw") has yielded over a dozen acetogenins (57,58), including longimicins A-D; these four compounds represent the asimicin type compounds but the locations of the adjacent bis-THF ring systems are shifted toward the lactone ring to the detriment of their bioactivity (59).

Acetogenins Isolated from F005 of Guanabana Seeds and Leaves

Initial screening in the BST, of the defatted seeds of *A. muricata* ("guanabana"), showed the partitioned ethanol extract (F005) to be significantly bioactive (LC_{50} 1.6 ppm). Our bioactivity-directed fractionation of this plant material led to the isolation of seven known mono-THF ring acetogenins including the annonacin series (60-63); new compounds found were the muricatetrocins and
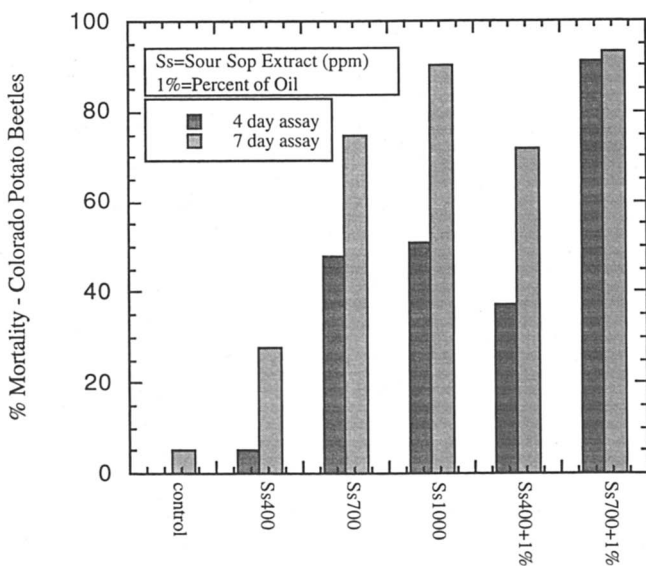
Figure 5.   Greenhouse Cage Experiment on Colorado Potato Beetles: *Annona muricata* (Sour Sop) Extract with / without Oil
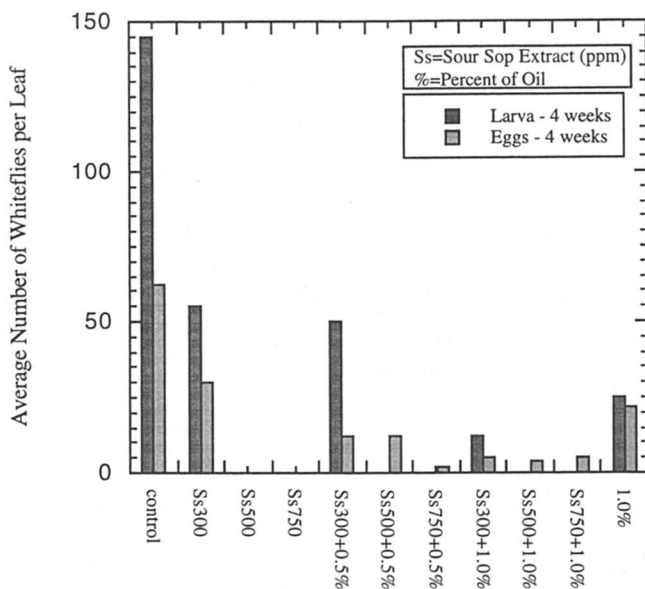

Figure 6.   Greenhouse Cage Experiment of Sweet Potato Whiteflies on Poinsettia Leaves: *Annona muricata* (Sour Sop) Extract with / without Oil
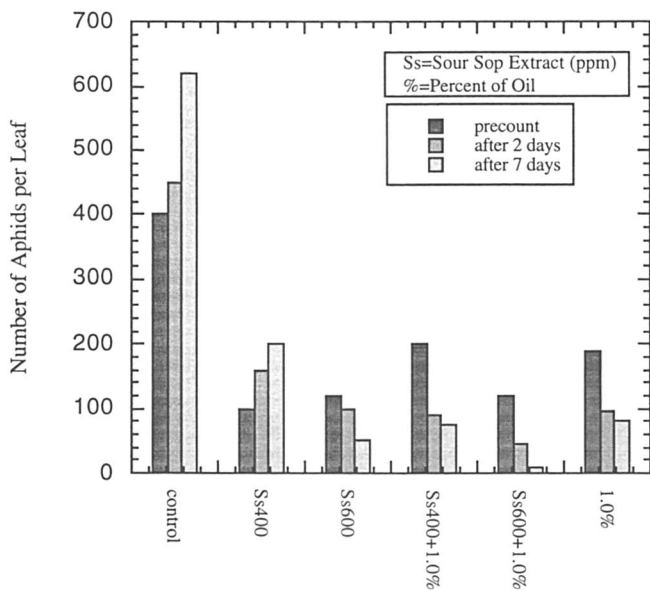
Figure 7. Greenhouse Cage Experiment of Green Peach Aphids on Mums: *Annona muricata* (Sour Sop) Extract with / without Oil

gigantetrocins (64) and muricatacin (a possible oxidation product which provided insights into the absolute stereochemistry of annonacin) (65). Most recently, five new diastereomeric mono-THF acetogenins, *cis*-annonacin and *cis*-annonacin-10-one (which are structurally related to the annonacin series), and *cis*-goniothalamicin, arianacin, and javoricin (which are structural isomers of goniothalamacin) have been found (66). Cavé and coworkers previously reported the antiparasitic effects (67) and the characterization of four new mono-THF acetogenins from the seeds: murisolin (68), corossolin, corossolone (69), and solanin (70). Gui et al. have recently isolated muricatalin, a new mono-THF compound with only one flanking hydroxyl (71), and Li et al. (72) have recently found muricatins A-C.

The leaves of guanabana, collected in Java, unlike the leaves of paw paw, showed considerable bioactivity (F005, BST LC_{50} 0.17 ppm). Encouraged by this level of potency, we conducted activity-directed fractionation and have now isolated 21 bioactive mono-THF acetogenins from the leaf extracts. Eight known compounds had been previously found in the seeds: gigantetrocin A, annonacin-10-one, muricatetrocins A and B, annonacin, goniothalamicin, (2,4-*trans*)-isoannonacin, and (2,4-*cis*)-isoannonacin; gigantetronenin and annonacin A, which are new to this species, were also found (73-75). New acetogenins from the leaves include annomuricins A and B (73); muricatocins A and B (74); annomuricin C and muricatatocin C (each containing five hydroxyl groups) (75); annomutacin, (2,4-*trans*)-10R-annonacin A-one, and (2,4-*cis*)-10R-annonacinA-one (76); and murihexocins A and B (77) and annohexocin (78) (each containing six hydroxyl groups, and the last including an unusual 1,3,5-triol). No bis-THF acetogenins, so far, have been reported from guanabana.

Mechanisms of Action of the Annonaceous Acetogenins

Londershausen et al. (79) initially observed that the toxicities caused by the Annonaceous acetogenins on insects resulted in lethargy and decreased mobility prior to death. Treated insects had substantially lower total levels of ATP, similar to the effect of antimycin A, a known inhibitor of the mitochondrial electron transport system (ETS). Mitochondrial enzymes were tested, and squamocin (annonin I) was 2.5-5 times as potent as rotenone in inhibiting NADH:ubiquinone oxidoreductase (79). Concurrently, Lewis et al. (80) observed a lower level of oxygen consumption in treated insects and experimentally located the site of action of asimicin (and F005) as mitochondrial complex I. Ahammadsahib et al. (81), working with bullatacin (the 24-epimer of asimicin) in SF9 insect cells as well as insect and mammalian mitochondria, at the same time, arrived at the same conclusion. Friedrich et al. (82) and Espositi et al. (83) found that the acetogenins bind competitively with respect to the ubiquinone site at complex I; whereas, rotenone binds non-competitively suggesting an alternative site. Hollingworth et al. (84) have concluded that bullatacin is the most potent of the several chemically diverse types of complex I ETS inhibitors examined. Landolt et al. (85) showed some structure-activity relationships among 20 different acetogenins in the inhibition of oxygen uptake by rat liver mitochondria, and some acetogenins appear to be more potent than bullatacin in this subcellular system. A brief summary of this mechanism has been published on our third review in the Annonaceous acetogenins (19).

A second mode of action helps to explain the selectivity of the acetogenins in inhibiting tumor vs. normal cells (86); an ubiquinone-linked NADH oxidase is activated in the plasma membranes of tumor cells, and this enzymatic activity is

potently inhibited by bullatacin (87). The net effect of this action and the action on the ETS results in intracellular ATP depletion, and apoptosis (programmed cell death) is a likely consequence (88). Resistance mechanisms which require an ATP-dependent transporter would be obvious targets for the useful application of these compounds as pesticides, antitumor agents, antimalarials, or any of the several systems in which such resistance is a significant factor.

Safety Studies

Using a modified guinea pig maximization test, F005 from paw paw was found to be only a weak skin sensitizer and asimicin was found to be only a weak skin irritant; neither produced the vesication or ulceration typical of urushiol (poison ivy) components (89). In our extensive work with the Annonaceae and the acetogenins over the past 15 years, our researchers have never experienced any form of dermatitis during plant collection, extraction, or isolation of the acetogenin compounds. One researcher experienced facial edema on two occasions, but this may have been caused by poison ivy. Another researcher rubbed one eye after his finger came in contact with a concentrated solution of pure acetogenins, and he experienced severe eye irritation; all pesticides (even soap) cause eye irritation.

Ames test results (Sitek Research Laboratories, unpublished results) on F005 from paw paw were negative in 9 out of 10 tests and only slightly positive (2.5% above background reversions) on one histidine mutant of *Salmonella typhimurium* after enzyme activation of the extract. This negative result demonstrates that the acetogenins are not likely to be mutagenic, and this result could have been expected because the acetogenins, unlike most other antitumor agents, do not exert their effects by poisoning DNA; they inhibit ATP production.

In unpublished feeding experiments, we found that mice tolerated F005 from paw paw mixed in their diet at 1% (a no choice diet). The mice ate this for four days without lethal effects. However, at 5% and above in their diets, they succumbed after three days, showing lethargy (as is typical from ATP deprivation), with their internal organs appearing normal. In other unpublished results (Asta Laboratories), bullatacin was emetic in pigs; this result demonstrated that the acetogenins very likely explain the former use of Eli Lilly's fluid extract of paw paw seeds as an emetic preparation (13). Thus, emesis is a definite safety factor should someone ingest these pesticidal materials either intentionally or unintentionally.

Conclusions

Activity-directed fractionations have identified a series of chemically diverse Annonaceous acetogenins as the bioactive principles of paw paw, guanabana, and over 20 other Annonaceous plant species. These compounds are powerful inhibitors of ubiquinone-linked oxidases in the mitochondria and in the plasma membranes of cancerous cells. Crude extracts containing these acetogenins are effective pesticidal agents whose animal toxicity is mediated by emesis. Their use in pesticidal products, including synergistic mixtures with other natural pesticides, offers an environmentaly friendly and safe alternative to synthetic pesticides, especially in the thwarting of resistance mechanisms, but awaits the approval of the regulatory agencies. The financial burden of this bureaucratic approval mechanism is the most serious detriment to the development of such products.

Acknowledgments

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Chapter 10

Novel Pesticidal Substances from the Entomopathogenic Nematode–Bacterium Complex

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Entomopathogenic nematodes in the genera *Steinernema* and *Heterorhabditis* and their associated bacteria *Xenorhabdus* and *Photorhabdus*, respectively, are commercially available for biological control of insect pests. Additionally, commercial products based on *Steinernema-Xenorhabdus* complex were developed against certain plant pathogenic nematodes. The mechanism of activity appears to be largely related to the biostatic or repellent substances derived from the bacterium. Recent discoveries have demonstrated that other substances derived from the bacteria have insecticidal, nematicidal and fungicidal activity. Advanced research is needed to fully understand the mechanism of activity and the commercial prospect of these substances.

Entomopathogenic nematodes in the families Steinernematidae and Heterorhabditidae have been known since 1929 and 1975, respectively (14), but they only became commercially available during the past decade (15). Since their discovery, tests indicated that these nematodes had the potential to control insect pests because they killed a wide range of insect species, especially under laboratory conditions. The laboratory host range is extensive because behavioral and ecological barriers are nonexistent, but in the field, the nematodes' host range is much more restrictive (22).

The biologies of *Steinernema* and *Heterorhabditis* have many similarities. Only the third-stage infective juvenile (500-1000 μ long depending on the species) can survive outside an insect host and move from one insect to another. The infective juvenile carries the symbiotic bacterium (*Xenorhabdus* for steinernematids or *Photorhabdus* for heterorhabditids) in its intestine. It releases the bacterial cells after entering the insect's hemocoel by way of natural openings (spiracles, mouth, anus) and, in the case of *Heterorhabditis*, directly through the

soft cuticle of certain insects. The bacteria cells proliferate, kill the insect host (usually in 24-72 hr) and render its interior favorable for nematode development. For *Heterorhabditis*, the entering infective juvenile develops into a hermaphroditic female, and hence only one nematode is needed for progeny production; for *Steinernema*, the infective juvenile develops into a male or female (amphimictic) and the mated female is required for progeny production. One or more generations develop until the host-derived nutrients are depleted. At this time, the nematodes synchronously become third-stage infective juveniles that leave the cadaver to forage for new hosts by detecting insect excretory products (22). At temperatures ranging from 18-28°C, the life cycle is completed in 6-18 d., depending on the host insect and nematode species.

The symbiotic bacteria are medium to long motile rods with peritrichous flagellae. They are Gram-negative facultative anaerobes that form spheroplasts (X = 2.6 μ diameter) in older cultures. They are non-spore formers, and thus do not have an environmentally resistant stage. They have been found nowhere naturally except inside the nematodes and infected insect hosts. Symbiotic bacteria produce two forms, designed as Phase I and II, when cultured *in vitro*. These phases can be distinguished by colony morphology, absorption of dyes from agar medium and antibiotic production (7). Both phases are pathogenic to insects, but phase one has a superior ability to provide nutrients for the nematodes in insects and artificial medium (3, 4) .

Breakthroughs in production and formulation made commercialization of nematode products possible. Major accomplishments were (a) *in vitro* production of nematodes at numbers sufficient for field applications at a cost generally competitive with chemical pesticides (15); (b) consistent production of high quality nematodes (i.e., viable, pathogenic); (c) development of nematode formulations that provided a shelf life sufficient for storage and transport to the site of use; and (d) formulations that made application rapid and simple. Nematode-based products that fulfill these requirements are presently available (15) and improved versions are being actively pursued.

Application strategies providing control levels comparable to those of standard chemical insecticides have been developed through extensive field research programs (22, 25). Selective control of pest species that spare their natural enemies can be achieved through an understanding of factors that limit the nematodes (e.g., desiccation, ultraviolet light, temperature extremes), knowing the ecology of the target insect (e.g., developmental stage, interaction with host plant) and application methodology (e.g., spot application, bait formulation, dispersion through drip irrigation systems).

This chapter addresses the recent discoveries of certain substances derived from the symbiotic bacteria shown to have activity against plant pathogenic bacteria, nematodes and fungi as well as insect pests (Table I).

Table I.   Activity of Substances from Symbiotic Bacteria *Xenorhabdus* and *Photorhabdus*

Substance/Type	Type of Effect	Reference
Unnamed antibiotics	Inhibit the growth of many bacterial and yeast species	1
Unnamed extracellular protein	Endotoxin against insect larvae	12
Lipopolysaccharide components	Endotoxin against insect larvae	11
Indole derivatives antibiotic	Bactericidal	27, 30
Hydroxystilbene derivative antibiotic	Antimicrobial	27, 28, 31
Xenorhabdins 2 antibiotic	Insecticidal and bactericidal	23
Xenocoumacins 1 and 2 antibiotics	Bactericidal	23, 25
Xenocoumacins 2 antibiotic	Fungicidal	25
Xenorxides antibiotic	Antimicrobial	31
Bacteriocins antibiotic	Bactericidal	8
Indole and stilbene derivative antibiotics	Inhibition of many plant-pathogenic fungi	9, 10, 31
Ammonia	Nematicidal	17, 31
Stilbene derivative antibiotic	Nematicidal	31

Insecticidal Activity of Symbiotic Bacteria

The usefulness of steinernematid and heterorhabditid nematodes for the control of insect pests depends entirely on their symbiotic associations with bacteria. The nematode functions as a vector transmitting its symbiotic bacterium into the host hemocoel and providing partial protection from the host's immune defense response. The bacterium multiplies, produces toxins that kill the host and provides and protects essential nutrients for the nematodes. Symbiotic bacteria phase one metabolites, but not phase two, exhibit a wide spectrum of antibiotic activity (2).

Like many other Gram-negative bacteria, *Xenorhabdus* spp. produce endotoxins. The *X. nematophilus* endotoxins are lipopolysaccharide components of the cell wall that are toxic for the hemocytes of *Galleria mellonella* (11). However, it is not clear that the endotoxin alone is sufficient to kill the host. The

damage to the hemocytes allows *Xenorhabdus* to multiply and produce exotoxins that are probably more immediately effective for killing the insect.

Exotoxin activity has been demonstrated in *P. luminescens*, *X. nematophilus* and *X. bovienii* by injecting culture supernatant into insects (2). Although these bacteria produce extracellular enzymes (7) that have been considered as toxins in other bacteria (proteases, phospholipases and lipases), these enzymes have not yet been proven to be the active agents in *Xenorhabdus* toxicity for insects. However, Ensign *et al.* (12) detected an extracellular protein in *P. luminescens* that was highly toxic when injected into fifth instar *Manduca sexta* larva (1 ng killing within 12-24 hours). The nature and mode of action of this toxin has not yet been determined but the toxin is apparently neither a protease nor a phospholipase (12).

McInerney *et al.* (24) reported the isolation, structure and biological activity of a series of five dithiolopyrrolone derivatives, xenorhabdins, which were isolated from *Xenorhabdus* spp. In larval feeding assays against *Heliothis punctigera* larvae, xenorhabdin 2 showed insecticidal activity with 100% mortality at 150 μg/cm^2. The LC$_{50}$ was 59.5 μg/cm^2. Xenorhabdin 2 also showed a considerable effect on the weight of surviving larvae. At a concentration of 37.5 μg/cm^2 there was only 18.8% mortality, but there was a reduction in weight of the survivors of 64.7% compared to controls. The effect on weight might suggest that xenorhabdin 2 is in some way interfering with feeding by the larvae. The other xenorhabdin compounds were not tested, but thiolution has been reported to have larvicidal activity against *Lucilia sericata* (24).

Antimicrobial Activity of Symbiotic Bacteria

Akhurst (1) reported that phase one of *Xenorhabdus* species and *Photorhabdus* produces antibiotics inhibit the growth of many bacterial and yeast species. Phase one of the symbiotic bacteria also inhibits the growth of blastospores of the insect pathogenic fungus *Beauveria bassiana* (5).

Several antibiotics and antimycotics from *Xenorhabdus* and *Photorhabdus* have been isolated and characterized. Among these compounds, xenocoumacin 1 isolated from *X. nematophilus* is active against Gram-positive bacteria, including animal and human pathogens (25).

Antimycotic substances produced by phase one of the symbiotic bacteria were tested for their activity against plant pathogenic fungi (Table II). Contrary to Barbercheck and Kaya (5), the insect pathogenic fungi *B. bassiana* and *Metarhizium anisopliae* have shown considerable resistance to the antimycotic(s).

That does not mean that all beneficial or nonpathogenic fungi are unharmed since Olthof *et al.* (26) found that when *S. feltiae* and *H. bacteriophora* were inundatively applied to control a sciarid fly infestation on mushrooms in glasshouses the mycelial development slowed.

Maxwell *et al.* (23) demonstrated that the use of nematode-bacterium complex against soil dwelling insect pests introduces xenocoumacins 1 and 2 into the soil via the dead insect fragments during the emergence phase of the nematodes. These antibiotics have a possible impact on soil bacteria.

Table II.   Spectrum of Antimycotic Activity of *Xenorhabdus* and *Photorhabdus* as Measured by Growth Inhibition of Fungal Species on Agar Plates[a]

Fungal Species	Major Plant Host
Alternaria sp.	Leaves, stems and fruits of ornamentals, fruits and field crops
Botrytis cinerea	All parts of vegetables, fruits, ornamentals and field crops
Ceratocystis dryocetidis	Canker on the bark of cacao, coffee, stone fruits and shade trees
Fusarium spp.	Roots of various plants, post harvest grains and legumes
Geotrichum candidum	Fruits and vegetables
Gloesporium perannans	Crownsard stems of cereals, grasses and vegetables
Monilinia fructicola	Stone fruits of peaches, plums and cacao
Pythium spp.	Roots of vegetable seedlings
Penicillium spp.	Post harvest fruits and vegetables
Rhizoctonia solani	Roots, tubes and stems of vegetables
Rhizopus stonifer	Post harvest fruits and vegetables
Venturia inaequalis	Leaves and fruits of apples
Verticillium spp.	Roots of various annuals and perennials

[a]: Modified from Chen *et al.* (10)

Nematicidal Activity of Nematode-bacterium Complex

Although most research with entomopathogenic nematodes has focused on their ability to infect insects, a few studies have reported interactions among entomopathogenic nematodes and plant-parasitic nematodes (6, 19). Ishibashi and Kondo (19) found that 10,000 *S. carpocapsae* per 100 cm^3 of sandy soil reduced population densities of tylenchid nematodes by 75-90% for 5 weeks after application. Using entomopathogenic nematodes as a preventive treatment, Bird and Bird (6) suppressed populations of *Meloidogyne javanica* associated with

tomato plants and reduced their reproductive capacity by adding 5×10^6 *S. glaseri* to each plant. A similar effect was observed by Ishibashi *et al.* (21) when an application of 10^5-10^6 *S. carpocapsae* per 200 cm^3 soil suppressed galling of tomato roots by *M. incognita*. In further work, Ishibashi and Choi (20) found that *S. carpocapsae* was positively attracted to and aggregated around the root tips of tomatoes. In irrigated turfgrass plots, population density of *Tylenchorhynchus* spp. was low in plots inoculated with a mixture of *H. bacteriophora* and *S. carpocapsae* (29). *S. riobravis* significantly affected the mean number of females per gram root of tomato plants, as well as the mean number of eggs per egg mass of the root-knot nematode *M. javanica* (Table III). Unpublished greenhouse and field trials have shown population reductions of *Radopholus similus*, *Criconemella* spp. and *Belonolaimus* spp. (Table IV). For example, in Florida, following commercial applications of *S. carpocapsae*, citrus growers reported a flush of new growth activity not associated with the mortality of the weevil target pest. Furthermore, in turfgrass applications of *S. riobravis* for control of mole crickets, golf course superintendents reported significant improvement in grass appearance, again not associated with the control of the target insect pest (Table V). Numerous successful trials have led to the introduction of products based on *S. riobravis* against nematodes infesting turfgrass. The long lasting effect of this nematode (Table V) is probably related to its ability to persist in various soil environments. (15, 16)

Table III. Percent Reduction[a] in the Population of Root-Knot Nematode *Meloidogyne javanica*-Infesting Tomato Due to the Application of Entomopathogenic Nematode *Steinernema* spp.[b]

Nematode	Root-knot Females/g Root	Root-Knot Eggs/Egg Mass
S. feltiae	46.6	50.0
S. carpocapsae	64.8	12.7
S. riobravis	61.8	27.0

[a]: Compared to untreated control. 2000 *M. javanica* per tomato seedling grown in 8 cm plastic pot
[b]: Modified from Gouge *et al.* (16)

There is no fully explained reason for the reduction of the population of the plant-parasitic nematodes in the presence of entomopathogenic nematodes. The first possibility is competition for space. Entomopathogenic nematodes do have an affinity with plant roots, partially due to their orientation towards their respective hosts along a CO_2 gradient (14). When applied at commercial field rates of between 2.5 and 7.5×10^9 nematodes per hectare, soil macropores will become crowded. Plant-parasitic nematodes may leave the root zone due to crowded conditions.

Table IV.   Plant-Parasitic   Nematodes   Successfully   Suppressed   with *Steinernema* spp. Entomopathogenic Nematode - *Xenorhabdus* spp. Bacterium Complex[a]

Nematode	Method (Reference)[a]
Root-knot nematodes	
Meloidogyne incognita	Cucumber seedlings in pots (19)
M. javanica	Tomato seedlings in pots (16)
Meloidogyne spp.	Turfgrass, field trials
Sting nematodes	
Belonolaimus longicaudatus	Turfgrass, field trials
Ring nematodes	
Criconemella spp.	Turfgrass, field trials
Lesion nematodes	
Pratylenchus penetrans	Turfgrass, field trials
P. coffeae	Cucumber seedlings in pots
Tylenchorhynchus spp.	Turfgrass, field trials (29)
Burrowing nematodes	
Radopholus similus	Wheat seedlings in pots
	Banana plantation, field trials
Cyst nematodes	
Heterodera spp.	Potato, field trials

[a]: biosys, unpublished data unless otherwise specified

Table V.   Population   Reduction   of   Sting   Nematodes,   *Belonolaimus longicaudatus*, and Root-Knot Nematodes, *Meloidogyne* spp. Infesting Turfgrass, Due to the Application of Devour® (based on *Steinernema riobravis*) and Nemacur® (Fenamiphos)

Treatment	Number of Nematodes/100 cc soil[a]		
	Pretreatment	28 DAT	63 DAT
Root-knot Nematodes			
Devour	62	9	6
Nemacur	67	0	5
Check	58	138	263
Sting Nematodes			
Devour	18	4	5
Nemacur	20	4	2
Check	11	22	139

[a]: Application rate was 5×10^9 nematodes/ha for Devour and 25 lb. ai/ha for Nemacur. biosys, unpublished data.

A second possibility is the release into the soil of the symbiont bacteria of the entomopathogenic nematodes after the infective juvenile's death. These symbionts produce natural biostatic substances (17). According to Hu *et al.* (17), second-stage juveniles of *M. incognita* and a mixed population of *Bursaphelenchus xylophilus* were killed when exposed to the cell-free culture filtrate of *X. bovienii*, a bacterial symbiont of several *Steinernema* spp. The percentage mortality of J2 *M. incognita* was negligible when exposed to 25 or 50 % concentrations of 12-hour and 24-hour culture filtrate, but was 100% when exposed to 48-hour to 120-hour culture filtrate at concentrations of 50% or more. The percentage mortality of *B. xylophilus* was only about half that of *M. incognita* at high concentrations (50% or more) of the culture filtrate. Neither water nor uninoculated tryptic soy broth culture medium, corrected to the same pH as the cell-free culture filtrate, resulted in a mortality greater than 2%. The major nematicidal compound of the culture filtrate, as detected by evaporation, neutralization and infrared spectroscopy of the corresponding salt, was ammonia. Further investigations demonstrated that *P. luminescens* produces stilbene derivative(s) that caused 94% mortality of *B. xylophilus* at 50 ppm but had no effect on *M. incognita* (31).

A third mechanism may be a typical predator-prey response. There are many predatory organisms that feed on free-living nematodes. Commercial application of entomopathogenic nematodes will increase the availability of prey and predator populations should increase accordingly. Soil predators are usually generalist feeders and would probably consume both insect parasitic and plant-parasitic nematodes. Nematode predators typically have short generation times from 5-7 days with high reproductive capacity. Evidence for this possibility comes from various studies conducted in greenhouses and fields (19, 21). These studies showed a decline in *S. carpocapsae* population and an increase in predatory nematode populations.

Conclusion

Except for the success of using nematode-bacterium complex for the control of certain plant-parasitic nematodes, there is significant basic research that needs to be conducted to determine the commercial feasibility of substances derived from the symbiotic bacterium. Some important areas of investigation are: identification and characterization; mode of action; stability and persistence in the environment; safety to mammals and natural enemies; and the ability to synthesize the molecules or produce sufficient quantities of the active ingredient in fermenters. Basic research on the cost effectiveness of production and the required dosage for effective control is critical.

Generally, antibiotic production in the soil is limited by nutrient restriction (32). However, in view of the production of antibiotics in the nutrient-rich insect cadaver (23) and in dead nematodes (13), it is possible that they may have substantial impact on plant pathogens.

Currently there are relatively few commercially available products derived from microbials. Based on preliminary data, substances derived from *Xenorhabdus* and *Photorhabdus* appear to hold promise as chemical control agents of insects and plant pathogenic nematodes, bacteria and fungi. Most present research concentrates on identifying novel, useful molecules. New substances, including those from *Xenorhabdus* and *Photorhabdus*, are being discovered at the rate of several hundred per year. Continued advances in biotechnology, particularly in genetic manipulation, as well as improved culturing systems and advanced formulations, will enable *Xenorhabdus* and *Photorhabdus* bacteria to be increasingly exploited as sources of useful chemicals in many sectors, including use as control agents of agricultural pests.

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Chapter 11

Physical and Biological Properties of the Spinosyns: Novel Macrolide Pest-Control Agents from Fermentation

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The spinosyns have commercial levels of activity against lepidopteran pests. The spinosyns also exhibit activity in screening assays on leafhoppers/planthoppers, mites, and cockroaches. The spinosyns are generally not active against aphids or nematodes. Acid addition salts are much more water soluble than the free bases but have a similar insecticidal spectrum and activity. Small changes in the chemical structure of the spinosyns lead to large differences in physical and biological properties. For example, the presence of a methyl group at 6-position (spinosyn D) reduces water solubility from 235 to 0.329 ppm at pH 7 and increases the melting point from 118 °C to 169 °C relative to spinosyn A. Similarly, the absence of a methyl group at the 3'-position (spinosyn J) decreases tobacco budworm activity by more than 200-fold relative to spinosyn A.

The spinosyns are a new group of macrolide pest control agents isolated from the actinomycete, *Saccharopolyspora spinosa* (*1*). A total of 23 naturally occurring spinosyns A-Y (Figure 1) have been isolated; these molecules vary in the substitution of one or more methyl groups on the tetracyclic core, the forosamine sugar, or the rhamnose sugar (*2,3*). Spinosad, a mixture of spinosyns A and D, has achieved development status as a natural agent for the control of insect pests. Spinosad is currently being developed as the first member of the Naturalyte[1] class of products for the control of lepidopterous pests on cotton and vegetables (*4,5*).

Since the inception of this project in 1988, most of the natural factors have not been available in the quantities needed for broad insecticidal testing. Most of the factors are produced in very small amounts from the fermentation of the wild type strain of *S. spinosa*, which produces primarily spinosyns A and D. However, the recent discovery and large scale fermentation of *O*-transmethylase blocked mutants of *S. spinosa* (*2,6*) has now made available gram quantities of many minor spinosyns,

[1]Please see note under Acknowledgments, page 153.
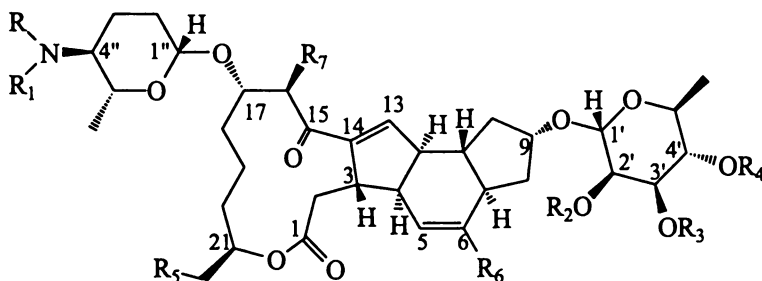
Item	Spinosyn	R	R1	R2	R3	R4	R5	R6	R7
1	A	Me	Me	Me	Me	Me	Me	H	Me
2	B	H	Me	Me	Me	Me	Me	H	Me
3	C	H	H	Me	Me	Me	Me	H	Me
4	D	Me	Me	Me	Me	Me	Me	Me	Me
5	E	Me	Me	Me	Me	Me	H	H	Me
6	F	Me	Me	Me	Me	Me	Me	H	H
7	H	Me	Me	H	Me	Me	Me	H	Me
8	J	Me	Me	Me	H	Me	Me	H	Me
9	K	Me	Me	Me	Me	H	Me	H	Me
10	L	Me	Me	Me	H	Me	Me	Me	Me
11	M	H	Me	Me	H	Me	Me	H	Me
12	N	H	Me	Me	H	Me	Me	Me	Me
13	O	Me	Me	Me	Me	H	Me	Me	Me
14	P	Me	Me	Me	H	H	Me	H	Me
15	Q	Me	Me	H	Me	Me	Me	Me	Me
16	R	H	Me	H	Me	Me	Me	H	Me
17	S	Me	Me	H	Me	Me	H	H	Me
18	T	Me	Me	H	H	Me	Me	H	Me
19	U	Me	Me	H	Me	H	Me	H	Me
20	V	Me	Me	H	Me	H	Me	Me	Me
21	W	Me	Me	Me	H	H	Me	Me	Me
22	Y	Me	Me	Me	Me	H	H	H	Me
23	A tartrate	Me	Me	Me	Me	Me	Me	H	Me
24	D tartrate	Me	Me	Me	Me	Me	Me	Me	Me

Figure 1. Chemical Structures of the Spinosyns.

permitting measurement of physical properties and spectrum of activity against other key pests.

Previous reports on the spinosyns have focused either on the physical and biological properties of the major factor spinosyn A (3,4) or on the activity of the spinosyns on tobacco budworm, (*Heliothis virescens*), neonate larvae (6,7). This study expands on these previous works and reports on the physical and biological properties of 22 spinosyns and acid addition salts of spinosyns A and D. The physical properties examined include physical state, melting point, and solubility in water and organic solvents. Biological activity on lepidoptera, homoptera, orthoptera, mites, and nematodes is reported.

Chemical Structure of the Spinosyns

The chemical structures of spinosyn A-Y are shown in Figure 1. The structure of the spinosyns is composed of a tetracyclic core (containing a 12-membered macrolide ring) substituted at both ends with hydroxyl groups containing the neutral sugar tri-*O*-methyl rhamnose and the amino sugar forosamine. The sugars may be removed sequentially through acid hydrolysis; the forosamine is lost first and then the rhamnose sugar. The carbon atoms of the molecules are numbered beginning with the lactone carbonyl and then proceeding along the carbon chain of the tetracyclic framework. The rhamnose sugar carbon atoms are numbered using a single prime (') while the forosamine sugar is numbered with a double prime ("). As shown in Figure 1, the spinosyns are very closely related in structure, varying only by the replacement of hydrogen atoms for methyl groups on the tetracyclic core or on the two sugars. However, these small substitution changes lead to dramatic differences in physical and biological properties of the spinosyns.

Physical Properties of the Spinosyns

For these studies, the spinosyns were purified by preparative reversed phased HPLC (10 μm, C18, Kromasil, Eka Nobel) using a mobile phase of 43:43:14, methanol/acetonitrile/0.25% ammonium acetate. Fractions containing the spinosyns in greater than 95% purity were combined, concentrated on a rotary evaporator, extracted into dichloromethane, and evaporated to dryness. Materials were used without further manipulation.

Table I shows some of the physical properties for spinosyn A, spinosyn D and tartrate salts of spinosyns A and D. The spinosyns exists as odorless, colorless solids with melting points that range from 79-185 °C. The melting points of 13 spinosyns are presented in Table II. These melting points were obtained on a capillary melting point apparatus and were corrected with the melting point reference standard benzoic acid (mp = 122.4 °C)

Spinosyn Q has the highest melting point and spinosyn F has the lowest melting point of the spinosyns examined. The introduction of a methyl group at the 6-position dramatically increases the melting point of the spinosyns as observed by comparing spinosyn A to D and spinosyn J to L. Spinosyn O is an exception to this trend and has a much lower melting point than do the other 6-position substituted spinosyns D, L, and Q, Table II. Removal of the 2'-O methyl group also increases

Table I. Physical Properties of Spinosyn A, Spinosyn D, and Tartrate Salts of Spinosyns A and D

Property	spinosyn A	spinosyn D	A tartrate	D tartrate
Color	white	white	white	white
Physical State	crystalline solid	crystalline solid	crystalline solid	crystalline solid
Odor	none	none	none	none
MP	118 °C	169 °C	139 °C	142 °C
Vapor Pressure	2.4×10^{-10} mmHg	2.0×10^{-10} mmHg	--	--
pKa	8.1	7.8	--	--
UV λmax in methanol	243 nm	243 nm	--	--
Molar absorptivity, ε in methanol	11,000	11,000	--	--
Specific rotation, $[\alpha]_{436}$ at 27 °C in methanol	-262.7 °	-297.5 °	-213.3 °	-236.9 °
Solubility				
Water (pH 5)	290 ppm	28 ppm	--	--
(pH 7)	235 ppm	0.329 ppm	--	--
(pH 9)	16 ppm	0.04 ppm	--	--
Water distilled	20 ppm	1.3 ppm	>33 w/v%	>26 w/v%
Methanol	19 w/v%	0.25 w/v%	23 w/v%	>50 w/v%
Acetone	17 w/v%	1.0 w/v%	0.8 w/v%	47 w/v%
Dichloromethane	>50 w/v%	45 w/v%	>50 w/v%	>50 w/v%
Hexane	0.45 w/v%	0.07 w/v%	0.1 w/v%	0.3 w/v%

Table II.   Melting Point and Water Solubility of the Spinosyns

Item	Spinosyn	Melting point, °C	Distilled water solubility, ppm
1	A*	118-124	20
2	B	89-95	100
3	C	95-103	140
4	D	169-174	1.3
5	E	118-120	9.4
6	F	79-91	40
7	H	175-177	26
8	J	93-105	83
9	K	101-111	220
10	L	160-161	4.1
11	M	83-96	--
12	O*	118-123	36
13	Q	185-187	0.2

* recrystallized from methanol/water.

the melting point as observed by comparing spinosyn A to H. The higher melting spinosyn D has reduced solubility in water and most organic solvents relative to spinosyn A, Table I.

The spinosyns are weak bases and their solubility in water is very pH dependent. The pKs for spinosyns A, B, C, and D are 7.87, 8.77, 8.50, and 8.1, respectively. The pKs were measured for these compounds by the method of capillary electrophores (8). The spinosyns have very low water solubility at neutral and basic pH and the solubility increases as the pH decreases due to protonation of the amino sugar, Table I. Below pH 4 the spinosyns are freely soluble in water. However, hydrolysis of the forosamine sugar occurs at pH 2 and below so the spinosyns should not be stored for prolonged periods under highly acidic conditions.

The solubility of 12 spinosyns in distilled water is shown in Table II. Water solubility was measured by stirring a suspension of the spinosyn in distilled water for three days at ambient temperature and analyzing the concentration in solution by HPLC.   In this test, spinosyn K is the most water soluble while spinosyn Q is the least soluble and spinosyn A is intermediate in solubility. The addition of a methyl group at the 6-position significantly reduces water solubility relative to spinosyn A (A vs. D). This effect is magnified in buffered water where the solubility of spinosyn D is over 700 times less than spinosyn A at pH 7, Table I.   The loss of a methyl group from position 16 increases solubility 2-fold (A vs. F) while the loss of a methyl group from position 22 decreases solubility 2-fold relative to spinosyn A (A vs. E) . Also, demethylation of the rhamnose sugar or the forosamine sugar increases water solubility 5 to 10-fold relative to spinosyn A (A vs. B, C, H, J, or K). The solubility of the spinosyns in distilled water is both pH and time dependent and one can get different values depending on the conditions used in the measurement.   The spinosyns also adhere to glass surfaces and may form micelles in aqueous solution further complicating measurements of water solubility. Therefore, the values in Table II are best treated as relative numbers rather than absolute numbers.

Acid addition salts of the spinosyns are formed with carboxylic acids and may be stored in the solid state for prolonged periods. The salts of spinosyn A are generally amorphous solids with the exception of the L-tartrate salt (1:1, spinosyn A to L-tartaric acid) which readily crystallizes from acetone or ethyl acetate. L-tartaric acid also forms crystalline salts with most of the spinosyns. The formation of salts leads to greater water solubility relative to the free-bases as exemplified by the tartrate salts in Table I. The water solubility of spinosyn A salts generally vary between 5% to 50% in distilled water and the pH of these solutions are between 4 and 5. In addition to improved water solubility, these salts also maintain good solubility in many common organic solvents such as methanol, acetone, tetrahydrofuran, and ether. Indeed, spinosyn D tartrate salt is more soluble in organic solvents than is spinosyn A.

Activity Spectrum of the Spinosyns

Screening results for 22 spinosyns are presented in Table III. In these bioassays, 400 ppm solutions were applied to host plants or insect diet, depending on the species tested. The spinosyns are generally active against larval lepidoptera. Twenty-one spinosyns are active against tobacco budworm (TBW, *Heliothis virescens*). Only spinosyn R was found to be inactive. Eighteen spinosyns are active against beet armyworm (BAW, *Spodoptera exigua*). Spinosyns P, T, and R were found to be inactive against this species. Most spinosyns are also active against leafhoppers and planthoppers. Eighteen spinosyns are active against either the corn planthopper (CPH, *Peregrinus maidis*) or the aster leafhopper (ALH, *Macrosteles fascifrons*); only spinosyns P, R, and W were inactive.

The spinosyns vary in acaricidal activity, 9 spinosyns are active against two-spotted spider mite (TSM, *Tetranychus urticae*). Spinosyn R, which is not active against any of the other species screened, is active against two-spotted spider mites. The spinosyns also vary in toxicity to orthoptera.   Seven spinosyns have demonstrated activity against German cockroach (GCR, *Blattella germanica*).

The spinosyns generally have little aphid activity. None of the spinosyns tested were active against cotton aphid (CA, *Aphis gossypii*) in this assay. The spinosyns also have little nematicidal activity.   Only spinosyns D and O demonstrated weak activity against root-knot nematode (RKN, *Meloidogyne incognita*).

Lepidoptera Activity of Spinosyns

The activity of 19 spinosyns to tobacco budworm is presented in Table IV. The neonate drench assay is mainly a measure of topical activity since newly hatched larvae are drenched in aqueous solutions of the spinosyns. Activity to second instars feeding on treated cotton leaves and to eggs/neonates on insect diet is also presented.

In the neonate drench and cotton leaf assays, spinosyn A is the most active molecule against tobacco budworm in the series. In comparison to cypermethrin, a pyrethroid insecticide widely used to control this insect pest in cotton, spinosyn A is slightly more active in the neonate drench assay and equivalent in the leaf dip assay. Loss of a methyl group from the forosamine nitrogen has little effect on activity in

Table III. Screening Activity of the Spinosyns Against Several Insect, Mite, and Nematode Species

		Percent mortality at 400 ppm							
Item	Spinosyn	TBW	BAW	CPH	ALH	TSM	GCR	CA	RKN
1	A	100	100	100	--	100	100	0	0
2	B	100	100	100	--	85	90	0	0
3	C	100	100	100	--	100	100	0	0
4	D	100	100	100	--	100	100	0	50
5	E	100	100	100	--	0	100	0	0
6	F	100	100	100	--	0	0	0	0
7	H	100	100	100	--	0	0	0	0
8	J	100	98	100	--	0	0	0	0
9	K	100	100	100	--	100	70	0	0
10	L	100	100	100	--	0	0	0	0
11	M	100	100	100	--	0	0	0	0
12	N	100	100	100	--	0	0	0	0
13	O	100	100	100	--	95	80	0	50
14	P	100	0	--	0	0	0	0	0
15	Q	100	100	100	--	0	0	0	0
16	R	0	0	--	0	90	0	0	--
17	S	100	100	--	100	0	20	0	--
18	T	100	0	--	100	0	0	0	--
19	U	100	100	--	100	0	0	0	--
20	V	100	--	--	--	--	--	--	--
21	W	100	60	--	0	80	0	0	--
22	Y	100	100	--	100	100	--	0	--
23	A tartrate	100	100	--	100	0	80	0	0
24	D tartrate	100	100	--	100	100	60	0	--

TBW = tobacco budworm, BAW = beet armyworm, CPH = corn planthopper,
ALH = aster leafhopper, TSM = two-spotted spider mite, GCR = German
cockroach, CA= cotton aphid, RKN = root-knot nematode, (--) = not tested.

Table IV. Activity of the Spinosyns Against Tobacco Budworm

Item	Spinosyn	Neonate drench[a] LC50, ppm	Cotton leaf dip[b] LC50, ppm	Percent mortality on diet[c] 25 ppm
1	A	0.31	1.1	100
2	B	0.36	6.0	100
3	C	0.82	18	94
4	D	0.80	3.1	100
5	E	4.6	6.6	100
6	F	4.5	18	90
7	H	3.2	>25	84
8	J	>64	>25	62
9	K	1.0	>25	84
10	L	26	~25	76
11	M	>64	>25	96
12	N	13	--	--
13	O	1.4	13	100
14	P	>64	--	--
15	Q	0.39	31	76
16	R	15	--	--
17	S	53	--	--
18	T	>64	--	--
19	U	22	--	--
20	A tartrate	0.27	--	--
21	D tartrate	1.3	--	--
22	cypermethrin	0.61	1.3	94

[a]LC50s determined 24 hr after treatment. [b]LC50s determined 4-6 days after treatment on 2nd instar larva. [c]LC50s determined 4-6 days after treatment on eggs/neonates. (--) = not tested.

neonate drench activity relative to spinosyn A, a much greater decrease (~5-fold) in activity occurred in the cotton leaf assay. Loss of the other methyl group from this nitrogen reduces activity 2.5-fold in the neonate drench assay and 16-fold in the cotton leaf assay relative to spinosyn A. Addition of a methyl at 6-position reduces activity slightly relative to spinosyn A in both assays. However, a methyl group at this position tends to increase activity if methyl groups are missing from other parts of the molecule (spinosyns L vs. J, N vs. M, O vs. K, Q vs. H). Absence of methyl groups at positions 16 or 22 decreases tobacco budworm activity 15-fold in the neonate drench assay relative to spinosyn A and 6 to 16-fold in the cotton leaf assay. The observed differences in neonate drench versus cotton leaf activities may indicate that differing physical properties affect the interaction of spinosyns with a leaf surface or it may reflect differences in the abilities of neonate versus second instar to detoxify the materials.

Methylation of the rhamnose sugar is very important for tobacco budworm activity, the loss of any methyl from this sugar decreases activity relative to spinosyn A, particularly on cotton leaves. The neonate drench results indicate the relative effects of methylation on this ring. Absence of a methyl at the 3'-position has a dramatic effect on activity, spinosyns without a methyl at 3' are 40 to >200-fold less active than spinosyn A. Demethylation at positions 2' and 4' has much less effect on activity. Absence of a methyl at the 4'-position causes a 3-fold decrease in activity; absence of a methyl at the 2'-position causes a 10-fold decrease in activity. However, absence of methyl groups at both the 2' and 4'-positions causes a much greater (70-fold) decrease in activity.

Demethylation in more than one region of the molecule tends to cause a greater decrease in activity than the absence of individual methyl groups. Spinosyn R (the N-demethyl, 2'-O demethyl analog of spinosyn A) is 50-fold less active than spinosyn A and spinosyn S (the 22-demethyl, 2'-O-demethyl analog of spinosyn A) is 170-fold less active than spinosyn A.

Acid-addition salts such as the tartrate salts of spinosyn A and spinosyn D are equal to the free-bases in tobacco budworm activity.

The activity of spinosyn A against several economically important lepidoptera larvae is presented in Table V. Spinosyn A is highly active against tobacco budworm,

Table V. Activity of the Spinosyns Against Several Species of Lepidoptera Larvae

Species	Common name	Family	Neonate drench LC50, ppm
Heliothis virescens	Tobacco budworm	Noctuidae	0.31
Spodoptera exigua	Beet armyworm	Noctuidae	0.28
Agrotis ipsilon	Black cutworm	Noctuidae	9.4
Cydia pomonella	Codling moth	Tortricidae	6.0
Ostrinia nubilalis	European cornborer	Pyralidae	0.42
Pectinophora gossypiella	Pink bollworm	Gelechiidae	0.05

beet armyworm, European cornborer, and pink bollworm. There are significant differences among species, however, as indicated by the much lower activity against black cutworm and codling moth.

Conclusions

The 22 spinosyns examined here vary in the presence or absence of one or more methyl groups at various positions on the macrolide, rhamnose, and forosamine parts of the molecule. These minor differences in structure cause large differences in the physical properties of the spinosyns. For example, the presence of a methyl group at 6-position reduces water solubility from 235 to 0.329 ppm in pH 7 buffer and increases the melting point from 118 °C to 169 °C relative to spinosyn A. Similarly, the absence of a methyl group at position 2' increases the melting point from 118 °C to 175 °C. These minor structural differences also have a large effect on insecticidal activity.   The absence of a methyl group at the 3'-position decreases tobacco budworm activity by more than 200-fold relative to spinosyn A.

The spinosyns have commercial levels of activity against lepidopteran pests. The spinosyns also exhibit screening levels of activity on leafhoppers/planthoppers, mites, and cockroaches. The spinosyns are generally not active against aphids or nematodes. Acid addition salts are much more water soluble than the free-bases but have a similar insecticidal spectrum and activity.

Acknowledgments

[1] Naturalyte is a registered trademark of DowElanco.

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Chapter 12

Phytoalexins from Brassicas: Overcoming Plants' Defenses

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In response to pathogen attack, plants synthesize phytoalexins, which are part of the plant's defense mechanism. Phytoalexins from brassicas have an indole ring and at least one sulfur atom as a common structural feature. Despite their close biogenetic relationship, these phytoalexins possess significantly different structures. Such structural differences suggest that brassica phytoalexins have different biological activity.
We have determined that the virulence of the blackleg fungus, a fungal pathogen of brassicas, correlated with its ability to rapidly metabolize and detoxify the phytoalexin brassinin. This process appears to be a mechanism by which the pathogen overcomes the plant's chemical defenses. We have now compared the toxicity of three additional phytoalexins of brassicas, brassicanal A, brassilexin, and camalexin, and investigated their biotransformation by the blackleg fungus. Most interestingly, the biotransformation of brassicanal A was different from that of brassinin, and occurred at a much slower rate. Additionally, unlike brassinin, these phytoalexins did not significantly affect the synthesis of blackleg phytotoxins. Overall, these results suggest that the blackleg fungus does not have specific enzymes to carry out those biotransformations.

Brassicas are cultivated worldwide and constitute an extremely valuable group of plants. Many brassicas are consumed as vegetables, as for example broccoli, cauliflower, cabbage, and turnip. Among the most economically valuable brassica crops are the oilseeds canola, rapeseed, and mustard. Brassica oilseeds are the third most important world source of edible vegetable oils, after soybean and palm. Specialty brassica oils are also produced for specific industrial use. Additionally, rapeseed ranks third in the production of seed meal, which is utilized as a protein source in animal nutrition. These commercial applications of brassicas, the amenable genetic manipulation of their oil biosynthetic pathway, and their adaptation to diverse agroclimatic conditions justify the ever increasing value in international trade (1).

Herein we review work on the phytoalexins of different *Brassica* species and other crucifers, as well as literature data concerning the phytoalexins elicited by rapeseed pathogens, and the possible ecological significance. Finally, we present new results concerning the biotransformation of some of these phytoalexins by the blackleg fungus of canola and rapeseed, and discuss implications of these results.

Chemical Structures and Elicitation.

Canola (*Brassica napus*, *B. rapa*), rapeseed (*B. napus*, *B. rapa*), and other brassicas (part of the *Cruciferae* family, syn. *Brassicaceae*) have defense mechanisms that can be associated with phytoalexin biosynthesis (2, 3). Phytoalexins are secondary metabolites synthesized *de novo* by plants in response to diverse forms of stress, which include pathogen attack (4). The phytoalexins from brassicas have an indole or indole or indole related ring system and at least one sulfur atom as common structural features. Most interestingly, crucifers appear to be the only plant family producing these sulfur metabolites. Several of these phytoalexins are produced by more than one *Brassica* spp., and can be elicited by diverse pathogens and/or abiotic factors. Brassinin (**5**), methoxybrassinin (**14**), and cyclobrassinin (**9**) were the first brassica phytoalexins to be reported (Table I, reference 10). These novel indole alkaloids were elicited by either *Pseudomonas cichorii*, or with ultraviolet irradiation. Subsequently, several other new phytoalexins have been isolated from cruciferous plants, including diverse *Brassica* spp.; the most representative examples are shown in Table I.

We isolated spirobrassinin (**23**) from canola cotyledons, leaves, and stems infected with a virulent isolate of the blackleg fungus [*Leptosphaeria maculans* (Desm.) Ces. et de Not., asexual stage *Phoma lingam* (Tode ex Fr.) Desm.]. Furthermore, we observed that spirobrassinin was also elicited by sirodesmin PL, one of the non-specific blackleg phytotoxins (2). Several other reports have shown accumulation of different phytoalexins in canola tissues infected with different pathogens (9, 17). Additionally, a recent report indicates that brassilexin and cyclobrassinin sulfoxide accumulated rapidly (within hours) with abiotic elicitation; however, with elicitation by a virulent isolate of the blackleg fungus, spirobrassinin was the main phytoalexin detected (22). Not surprisingly, these results demonstrate that abiotic elicitation of phytoalexins can evoke a plant reaction different from that evoked by microorganisms. This is not only because phytopathogenic microorganisms may elicit a more selective response than abiotic factors, but also because some of these pathogens can metabolize phytoalexins. We have published the first reports of the metabolism and detoxification of a brassica phytoalexin by a fungal pathogen of canola and rapeseed (23-25); however, the metabolism of several phytoalexins from different plant families by fungal plant pathogens was previously demonstrated (for a recent review see reference 26).

Biogenesis and Mode of Action.

L-Tryptophan is the biogenetic precursor of some of the brassica phytoalexins (Table I), and brassinin (**5**) appears to be an advanced precursor of spirobrassinin (**23**), cyclobrassinin (**9**), and possibly other related metabolites (27). An obvious structural similarity between the indole glucosinolates **25** - **27**, and brassinin (**5**), methoxybrassinin (**14**), and 4-methoxybrassinin (**15**), respectively, suggests that their biogenesis is interrelated. Recent work (27) proved these earlier speculations, although no glucosinolates have yet been reported as precursors of any of the brassinins **5**, **14**, and **15**. Interestingly however, a direct correlation between the accumulation of some phytoalexins and the presence of indole glucosinolates in turnip tissue was recently reported (14). It would be most important to ascertain

TABLE I. Phytoalexins from Brassicas and Other Crucifers, and their Elicitors

Phytoalexin (#)	Plant Species (elicitor)	Ref. #
Brassicanal A (**1**)	*B. rapa (P. cichorii), R. sativus (P. cichorii)*	5, 6, 7
Brassicanal B (**2**)	*B. rapa (P. cichorii)*	5
Brassicanal C (**3**)	*B. oleracea (P. cichorii)*	7
Brassilexin (**4**)	*B. juncea (L. maculans, A. brassicae), B. nigra, B. carinata, B. napus, B. oleracea, B. rapa (L. maculans, abiotic)*	8, 9,
Brassinin (**5**)	*B. rapa (P. cichorii, abiotic), B. oleracea (P. cichorii, L. maculans) R. sativus (P. cichorii), B. napus (L. maculans)*	6, 9, 10, 11, 12, 13, 14
Brassitin (**6**)	*B. rapa (P. cichorii), R. sativus (P. cichorii)*	6
Camalexin (**7**)	*C. sativa (A. brassicae), A. thaliana (P. syringae)*	15, 16
(-)-3-Cyanomethyl-3-hydroxyoxindole (**8**)	*B. oleracea (P. cichorii)*	7
Cyclobrassinin (**9**)	*B. rapa (P. cichorii, abiotic), B.oleracea (P. cichorii, L. maculans), B. napus, B. rapa, B. nigra, B. carinata, B. juncea (L. maculans)*	9, 10, 11, 12, 13, 14, 17
Cyclobrassinin sulphoxide (**10**)	*B. juncea, B. napus, B. rapa, B. nigra, B. carinata, B. oleracea (L. maculans)*	9, 18
Cyclobrassinone (**11**)	*B. oleracea (abiotic)*	19
Dehydro-4-methoxy cyclobrassinin (**12**)	*B. rapa (P. cichorii)*	14
Dioxibrassinin(**13**)	*B. oleracea (P. cichorii)*	7
Methoxybrassinin (**14**)	*B. rapa (P. cichorii, abiotic), B. oleracea (P. cichorii, abiotic), B. oleracea, B. napus, B. rapa, B. carinata (L. maculans), R. sativus (P. cichorii)*	6, 9, 10, 11, 12, 13, 14, 17, 19
4-Methoxybrassinin (**15**)	*B. oleracea (P. cichorii), B. rapa (P. cichorii)*	12, 14
Methoxybrassenin A (**16**)	*B. oleracea (P. cichorii)*	20
Methoxybrassenin B (**17**)	*B. oleracea (P. cichorii)*	20
Methoxybrassitin (**18**)	*B. rapa (P. cichorii), B. oleracea (P. cichorii, abiotic), R. sativus (P. cichorii)*	6, 11, 12, 19
6-Methoxycamalexin (**19**)	*C. sativa (A. brassicae)*	15
1-Methoxyspirobrassinin (**20**)	*B. oleracea (abiotic)*	19
N-Methoxyspirobrassinol (**21**)	*R. sativus (P. cichorii)*	6
N-Methoxyspirobrassinol methylether (**22**)	*R. sativus (P. cichorii)*	6
Spirobrassinin (**23**)	*R. sativus (P. cichorii), B. oleracea (P. cichorii, abiotic), B. rapa (P. cichorii, abiotic), B. napus (L. maculans)*	2, 5, 6, 12, 13, 14, 19, 21, 22

B.= Brassica; R.= Raphanus; C.= Camelina; A.= Arabidopsis; (P.= Pseudomonas); (L.= Leptosphaeria); (A.= Alternaria)

1

2

3

4

5 R=H; R'=S; R"=H
6 R=H; R'=O; R"=H
14 R=OCH₃; R'=S; R"=H
15 R=H; R'=S; R"=OCH₃
18 R=OCH₃; R'=O; R"=H

7 R=H
19 R=OCH₃

8

9 R=SCH₃
10 R=S(O)CH₃

11

12

13

16 R=H$_2$
17 R=O

20 R=OCH$_3$
23 R=H

24

21 R=H
22 R=CH$_3$

25 R=H; R'=H
26 R=OCH$_3$; R'=H
27 R =H; R'=OCH$_3$
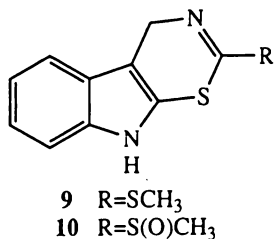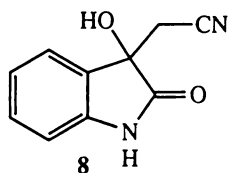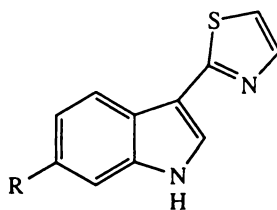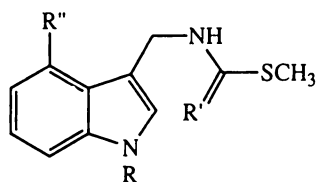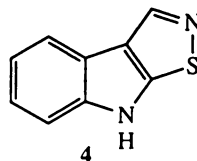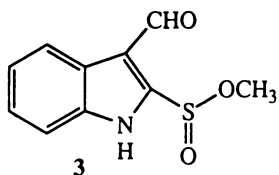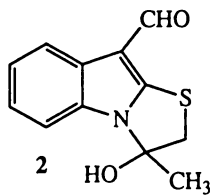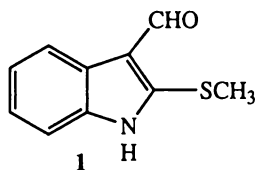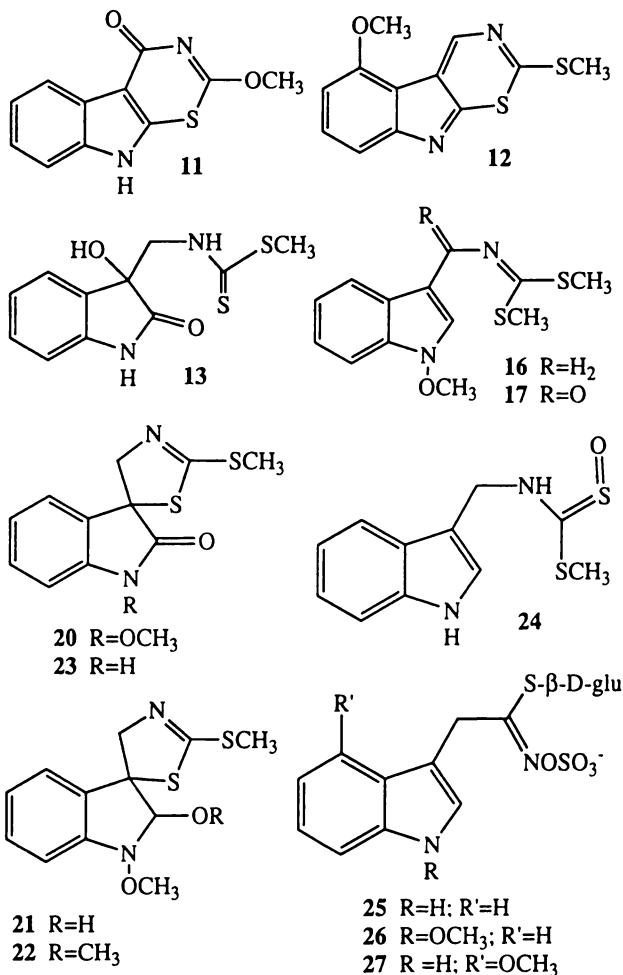
whether this direct correlation is general, because one of the goals of diverse rapeseed breeding programs has been to obtain plants with zero content glucosinolates. If that correlation exists, the presence of glucosinolates in plant tissues will be highly desirable and should be maintained.

Despite their close biogenetic relationship, brassica phytoalexins possess significantly different structures with various ring systems. These structural differences suggest that these phytoalexins have different biological activity; however, little is known about their antifungal or any other antimicrobial activity. Recently, we have investigated the biotransformation of brassinin by the blackleg fungus, the causative agent of the blackleg disease of brassicas (23-25). This fungal pathogen has caused significant canola losses in recent years in Canada and is considered a serious agricultural problem worldwide (28). We have determined that the virulence of the blackleg fungus correlated with its ability to rapidly metabolize and detoxify the phytoalexin brassinin (25). Because brassinin is a biogenetic precursor of several other phytoalexins, the rapid metabolism of brassinin by virulent isolates of the blackleg fungus may deprive the plant of other important phytoalexins. This process appears to be a mechanism by which the pathogen overcomes the plant's chemical defenses. The overall effect is a plant more susceptible to further fungal colonization.

It is also worthy to note the effect of brassinin on the biosynthesis of the blackleg phytotoxins (25). Virulent isolates of *P. lingam* start synthesizing the phytotoxins sirodesmins several hours after spores are incubated in liquid culture (25, 29). However, incubation in the presence of brassinin (5) inhibited the synthesis of those phytotoxins. By contrast, incubation with an oxidation product of brassinin, the S-oxide 24 or related intermediates did not affect sirodesmin production (25). These unprecedented results suggest a possible mode of action for brassinin and perhaps other phytoalexins, that is the inhibition of the pathogen's non-specific phytotoxins.

Applications

A possible strategy for deterring the blackleg fungus would be the inhibition of the enzymes involved in the detoxification of brassinin. However, before such inhibitors can be rationally designed, it is important to determine whether the blackleg fungus metabolizes and detoxifies other brassica phytoalexins. Ultimately, a correlation between the bioactivity of the phytoalexins and of their catabolites will allow the understanding of detoxification mechanisms utilized by the blackleg fungus to overcome the plant's defenses. It should then be possible to rationally design antifungal agents selective against the blackleg fungus.

Additionally, because other fungal species cause significant crop losses in brassicas, it is of great importance to determine the antifungal activity of these phytoalexins on those fungi. From this information a "phytoalexin blend" which could make brassica's defense mechanisms more effective against diverse fungal pathogens may be established. The targeted phytoalexin blend could be introduced either through genetic manipulation of biosynthetic pathways, or by screening brassica lines generated in diverse breeding programs..

Integrated in a research program aimed at understanding and controlling fungal diseases of canola and other economically important brassicas, we investigated the metabolism of brassinin (23-25). We determined that the virulence of the blackleg fungus correlated with its ability to rapidly metabolize and detoxify the phytoalexin brassinin (5). We have now examined the metabolism of three additional brassica phytoalexins, brassicanal A (1), brassilexin (4), and camalexin (7), by virulent isolates of the blackleg fungus. Herein we report these results and compare the toxicity of these phytoalexins towards *P. lingam*.

Synthesis of Phytoalexins

Camalexin (**7**) was synthesized according to a literature procedure (30); brassicanal A (**1**) (31, 32) and brassilexin (**4**) (33) were synthesized by modification of literature procedures, as briefly described.

Brassicanal A (**1**): phosphorus pentasulfide (1.33 g) was added to a mixture of oxindole (0.47 g), sea sand and benzene . The reaction mixture was heated under reflux with stirring for 80 min, then allowed to cool, and the benzene layer was decanted. The insoluble residue was extracted with benzene and ethyl acetate. The benzene and ethyl acetate extracts were combined and evaporated to give crude 2-indolinethione. A mixture of 2-indolinethione (1.1 g), iodomethane (2.8 g), Na_2CO_3 (1.2 g), and acetone (20 ml) was stirred at room temperature for 4 hours and filtered. The filtrate was evaporated to leave a brown oily residue which was extracted with benzene. The benzene extract was purified (silica gel column chromatography, hexane-acetone, 9:1) to give 2-(methylthio)-indole (0.8 g).

Phosphorus oxychloride (1.66 g) was added with stirring under argon atmosphere to DMF (3 ml). A solution of 2-(methylthio)-indole (0.17 g) in DMF (1 ml) was added dropwise with continuous stirring. The reaction mixture was kept at room temperature for 45 min and then poured onto crushed ice. The clear solution was treated with NaOH solution (3.8 g in 20 ml, very slow addition, exothermic reaction). The crystals of brassicanal A were filtered off, washed with water and dried (0.11g, 70% yield). The NMR spectroscopic data of the product was in complete agreement with those reported for the natural product (5).

Brassilexin (**4**): indole-3-carboxaldehyde (290 mg, 2 mmole) was dissolved in acetic acid (16 ml) with stirring at 60 °C and sulfur monochloride (1.6 ml, 20 mmol) was added dropwise. The reaction mixture was stirred at 60 °C for 1 hour, followed by evaporation of the acetic acid under reduced pressure. A solution of ammonia in methanol (60 ml, saturated) was added to the reaction mixture with stirring at 0 °C for 1 hour, and then allowed to stand overnight at room temperature. The reaction mixture was evaporated to dryness under reduced pressure and the residue was extracted with ethyl acetate . The extracts were evaporated to yield crude brassilexin (0.176 g).

The crude material was subjected to column chromatography over silica gel, elution with CH_2Cl_2 - MeOH (98:2); crude brassilexin (70 mg) was further purified by prep. TLC (CH_2Cl_2 - MeOH, 96:4) to yield brassilexin (22 mg). The NMR spectroscopic data of the product was in complete agreement with those reported for the natural product (8).

Biotransformation of Phytoalexins.

Feeding Experiments. *P. lingam* virulent isolates Laird 1, ENG-53 and BJ-125 were obtained from G. Séguin-Swartz and R. K. Gugel, Agriculture and Agri-Food Canada Research Station, Saskatoon, SK; *P. lingam* virulent isolates PHW 1276 and PHW 1317 were obtained from P. H. Williams, University of Wiscosin-Madison, Madison, WI.

The fungal isolates were grown on V8 agar as described previously (25). To initiate liquid cultures, fungal spores (final conc. 2×10^8 spores per ml) were inoculated in minimal media supplemented with thiamin (25). The cultures were incubated at 25 ± 2 °C on a shaker at 200 rpm.

Metabolism of Brassicanal A (1). Liquid shake cultures (100 ml media in 250 ml Erlenmeyer flasks) inoculated with fungal spores (isolates ENG-53, BJ-125) and incubated for 35 hours at 27 ± 2 °C were used for the metabolism studies. Control cultures of each isolate were grown separately. Solutions of brassicanal A (final concentration 5×10^{-4} M and 2×10^{-4} M) in DMSO [final concentration 0.5% (v/v)] were administered to 35-hour-old liquid cultures and to uninoculated media. Samples (10 ml) were withdrawn at 8-24 hours intervals up to six days and were either immediately frozen or filtered and extracted with ethyl ether, as previously described (25). The extracts were analyzed by HPLC, as described below. Brassicanal A was stable in uninoculated medium up to six days.

Metabolism of Brassilexin (4). Liquid shake cultures (100 ml media in 250 ml Erlenmeyer flasks) inoculated with fungal spores of the isolate PHW 1276 (4 x 100 ml) and incubated for 48 hours were used in these metabolism studies. Solutions of brassilexin (final concentration 2×10^{-4} M) in DMSO [final concentration 0.5% (v/v)] were administered to 48-h-old liquid cultures and to uninoculated medium and incubated at 25 ± 2 °C. Samples (10 ml) were withdrawn and worked up as described above, up to six days. The extracts were analyzed by HPLC, as described below. Brassilexin was stable in uninoculated medium up to six days.

Metabolism of Camalexin (7). Liquid shake cultures (100 ml media in 250 ml Erlenmeyer flasks) inoculated with fungal spores of three different isolates (PHW 1276, PHW 1317, and Laird 1) and incubated for 48 hours were used in these metabolic studies. Solutions of camalexin (final concentration 5×10^{-4} M) in DMSO [final concentration 0.5% (v/v)] were administered to 48-h-old liquid cultures and to uninoculated media and incubated at 25 ± 2 °C. Samples (10 ml) were withdrawn and worked up as described above, up to 10 days. The extracts were analyzed by HPLC, as described below. Camalexin was stable in uninoculated medium up to 10 days.

Analysis and Isolation of Metabolites. The analyses of the metabolic products were performed with a high performance Hewlett Packard liquid chromatograph equipped with quaternary pump, automatic injector, and diode array detector (wavelength range 190 - 600 nm), and a Hypersil ODS column (5 mm particle size silica, 4.6 i.d. x 200 mm), equipped with a guard column filled with the same stationary phase (HPLC grade solvents were degassed during operation through continuous bubbling of a stream of He). The retention times obtained employing a mobile phase 75% water - 25% acetonitrile to 100% acetonitrile, 35 min., flow rate 1.0 ml / min, are shown in Table II.

The structures of the products and/or intermediates were determined from analyses of the spectroscopic data (NMR, MS, FTIR, UV) of the purified metabolites, and confirmed by synthesis where indicated.

The isolation of metabolites resulting from the fungal metabolism of brassicanal A was carried out by flash column chromatography (cc), followed by preparative TLC. The organic extract was subjected to cc over silica gel (CH_2Cl_2 - MeOH, 98:2), followed by prep. TLC (CH_2Cl_2 - MeOH, 96:4), to obtain metabolites **28-30**. The structure of sulfoxide **28** was further confirmed by oxidation of brassicanal A with MCPBA; the structure of alcohol **29** was further confirmed by reduction of brassicanal A sulfoxide with $NaBH_4$.

TABLE II. Retention Times of Phytoalexins from Brassicas

Phytoalexins	Retention Time (min)
Brassicanal A (1)	10.2
Brassilexin (4)	11.7
Brassinin (5)	18.7
Camalexin (7)	18.0 (broad)
Brassicanal A sulfoxide (28)	6.0
3-(Hydroxymethyl)indole-2-methylsulfoxide (29)	3.4 (broad)
3-Methylindole-2-methylsulfoxide (30)	8.7
Indole-3-carboxaldehyde	6.3
Indole-3-carboxylic acid	5.6
Sirodesmin PL (phytotoxin)	14. 5
Deacetylsirodesmin PL (phytotoxin)	8.0
Phomamide (fungal metabolite)	7.7

Results and Conclusions.

The toxicity of the phytoalexins, brassicanal A (1), brassilexin (4), brassinin (5), and camalexin (7), to virulent isolates of the blackleg fungus employing a mycelial radial growth assay (24) was compared. Brassilexin completely inhibited the mycelial growth of the blackleg fungus and brassinin significantly decreased the mycelial growth rate, while brassicanal A and camalexin did not have any significant effect.

Most interestingly, the biotransformation of brassicanal A was different from that of brassinin (23-25), and occurred much slower (Scheme I). The aldehyde group of brassicanal A was initially reduced to the corresponding alcohol 29, and then further to the corresponding 3-methylindole 30 (Pedras, M.S.C., University of Saskatchewan, unpublished data). However, similarly to brassinin, the 2-methylsulfide group was oxidized to the corresponding sulfoxide 28; this product was detected in the cultures two hours after incubation with brassicanal A. The final product of the biotransformation of brassicanal A, metabolite 30 was detected in the cultures 48 hours after incubation and remained in the cultures for at least six days. On the other hand, camalexin (7) was stable in the culture medium; none of the blackleg isolates tested metabolized camalexin or appeared to be affected by a concentration of 5 x 10^{-4} M (Pedras, M.S.C., University of Saskatchewan, unpublished data).

After incubation of isolate PHW 1276 with brassilexin (4) for six days, the major components of ethereal and acidic extracts were isolated by prep. TLC. Analysis of each fraction by HPLC and ^{1}H NMR indicated that the major components of the extract were sirodesmin PL, deacetylsirodesmin PL, and phomamide, whose structures were confirmed by comparison with authentic samples (34). Traces of brassilexin were present in mycelial extracts, but not in extracts of the culture broth. No metabolites resulting from brassilexin metabolism could be detected, even at earlier incubation stages (24 to 72 hours). Possible metabolic products of brassilexin might be very polar and more soluble in the aqueous medium

than in organic solvents (Et_2O, or EtOAc), thus precluding detection and isolation. Attempted oxidation of brassilexin with MCPBA resulted in an intractable mixture, insoluble in organic solvents (Pedras, M.S.C., University of Saskatchewan, unpublished data). HPLC analysis of this reaction mixture did not reveal the presence of any UV active components. We are currently investigating brassilexin metabolism employing radiolabeled material. A previous study indicates that brassilexin is metabolized, but no biotransformation products or fungal metabolites were reported (3).

Scheme I
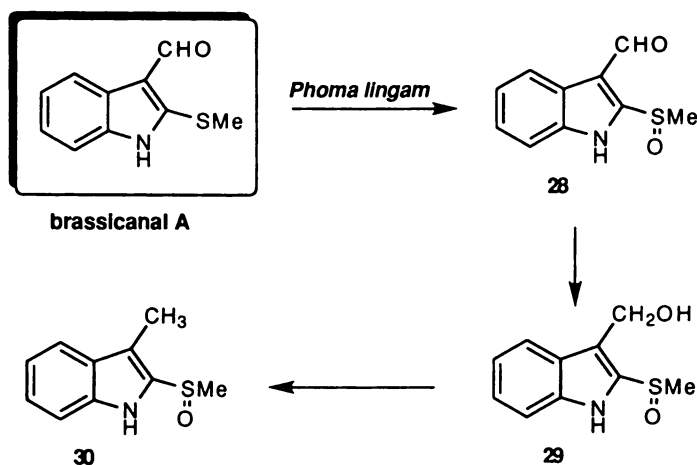
brassicanal A

The biotransformation of brassicanal A (**1**) and brassilexin (**4**) by virulent isolates of the blackleg fungus was slower than the biotransformation of brassinin (**5**) (which occurred within 48 h). No traces of 3-indolecarboxaldehyde or of 3-indolecarboxylic acid, the final metabolites of brassinin (**25**), were detected at any time. Thus, there is a significant difference in the metabolism of brassinin and that of the phytoalexins examined in this study. While the biotransformation of brassinin by virulent blackleg isolates rapidly yielded the corresponding carboxylic acid, the biotransformation of brassicanal A and brassilexin was slower, and that of camalexin did not occur at all. Furthermore, in contrast with the effect of brassinin, none of these phytoalexins appeared to inhibit the biosynthesis of the non-specific blackleg phytotoxins.

Pathogens may use phytoalexin metabolism as a detoxification mechanism to overcome plant chemical defenses (26). However, contrary to studies of the metabolism of brassinin (5), the results of this study do not indicate that the blackleg fungus can metabolize either camalexin (**7**), brassicanal A (**1**), or brassilexin (**4**) effectively. Considering that brassilexin has a much stronger antifungal activity than any of the other three phytoalexins reported here, brassilexin could be a very useful phytoalexin for engineering blackleg disease resistance in brassicas. Towards this end, we are currently investigating the metabolism of radiolabeled brassilexin by the blackleg fungus and other brassica pathogens. These results will indicate whether the presence of brassilexin in brassicas would be beneficial. Additionally, the mechanism of brassilexin biotransformation will give important clues for designing selective antifungal agents.

Overall, our results suggest that the blackleg fungus may utilize different enzymes to transform diverse phytoalexins. These could be non-specific enzymes used in general detoxification processes. However, much more work must be carried out, which should include additional fungal species and phytoalexins, before inhibitors of those enzymes that allow brassica pathogens to overcome the plant's defenses can be rationally produced.

Acknowledgments.

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Chapter 13

Natural Products as Leads in Structural Modification Studies Yielding New Agrochemicals

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Chemical investigations of interactions between organisms has led to the isolation and identification of biologically active principles. Some of the biologically active natural products have served as leads to the discovery and development of commercialized agrochemicals. Several examples of the chemical modification of such natural products yielding new agrochemicals are reviewed.

1. INTRODUCTION.

The struggle between man and pest took on serious proportions at the beginning of the farming age. Expansion of the farming area, as a result of reclamation of deep forests and wild plains, had brought many fauna around farming areas. The new interactions resulted in unstable eco-systems, and so crops and vegetables were damaged occasionally by sudden outbreaks of pests and plant diseases.

For crop protection, the ancients developed cultivational controls such as rotational farming and selection of resistant crops. In addition, physical controls, namely, insect-proof bags, light traps and machine oils were used as convenient technologies for pest and plant disease control.

More recently, during World War II, well known agrochemicals such as DDT (1939), BHC (1942) and parathion (1944) were discovered and developed as indispensable technologies for crop protections and to improve public health. Development of a number of synthetic agrochemicals, as well as advancements in application technologies and forcasting of pest and plant disease occurrence led to stable food production in subsequent decades.

These synthetic agrochemicals were discovered exclusively by synthesis with the exploration of combinations of specific functional groups or substituents to lead molecules. With accumulation of the knowledge of the computer assisted quantitative structure activity relationship approach and a number of trial-and-error experiences, "molecular designing" has been systematized in recent years.

More recently, the long-term use of some old synthetic agrochemicals has been circumscribed by environmental and resistant-pest concerns. In addition, it is becoming very difficult to discover new lead structures solely by synthetic means. On the other hand, there are several well known agrochemicals whose lead structures have been derived from naturally occurring substances, as shown in Fig.1.
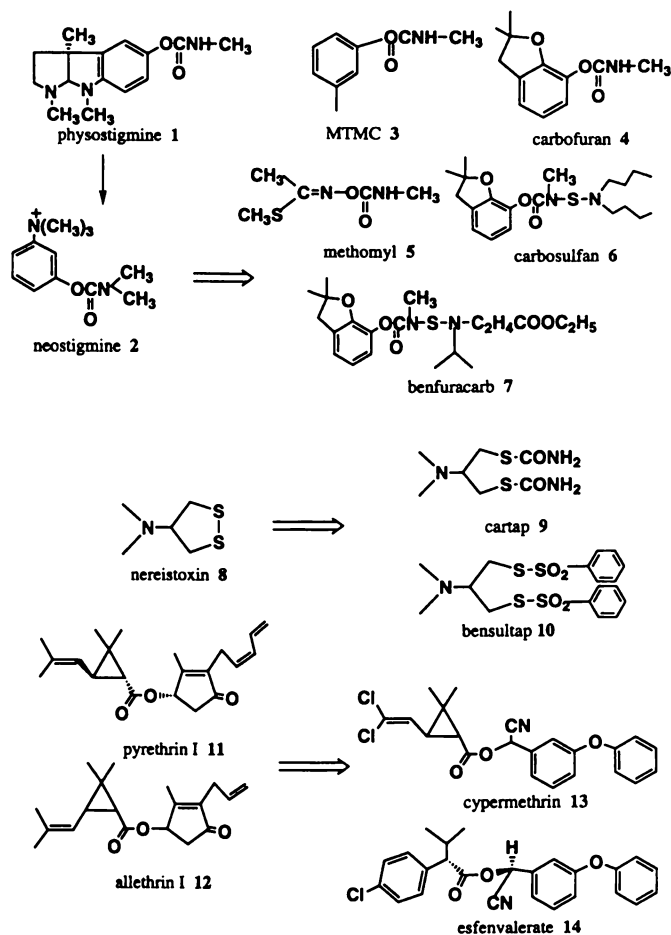
Fig. 1   Classical Insecticidal Leads of Natural Origin

Physostigmine **1**, an alkaloidal carbamate from seed of the African vine tree, *Physostigma venenosum* Balf. (*Leguminosae*), was widely used by natives as an arrow poison to catch wild animals. This alkaloid was known as a neuro-toxic substance. As an extended application, neostigmine **2**, [(2-dimethylcarbamoxyphenyl)-trimethyl-ammonium] was developed in the 1930s as a carbamate type synthetic medicine for cholinergic and para-sympathomimetic diseases. In the early 1950s, several carbamate insecticides such as MTMC **3**, whose structures were derived from natural physostigmine **1**, were developed to control rice pests, dulphacid plant-hoppers and leaf-hoppers. The mode of action of carbamate insecticides is known to be inhibiting acetylcholine esterase.

As more prominent carbamate insecticides, carbofuran **4** and methomyl **5** were developed in the 1960s, however they had rather high mammalian toxicities. In the 1970s, further structural modifications were attempted on substituents at the *N*-atom to reduce selective toxicities against mammals. Carbosulfan **6** and benfuracarb **7** were

discovered as specific insecticides against newly occurring pests, rice water weevils and thrips. As described, many useful carbamate insecticides thus have been commercialized starting from physostigmine 1 as the lead structure (1).

"Isome" (in Japanese), *Lumbrinereis heteropoda*, is an annelid worm which occurs along the southern and western seashores of Japan. This animal has long been known to contain an insect paralyzing principle, especially active against houseflies. The structure of the active principle was determined to be a 2-dithiolane (nereistoxin 8) by Japanese scientists. Nereistoxin 8 has been recognized to act as a competitive blocker of the acetylcholine receptor. The structure of nereistoxin was modified to develop cartap 9 and bensultap 10, specific insecticides against the rice stem borer, *Chilo suppressalis* (2) (Fig. 1).

The most well known and successful example of a natural product for combating agricultural and household pests is "pyrethrum". Pyrethrum, a crude extract of the flower head of *Chrysanthemum cinerariaefolium* (*Compositae*) was the most common natural insecticide in the pre-World War days for controlling agricultural and household pests. The *Chrysanthemum* originates from Persia, Dalmatian district and Yugoslavia. Chemical investigations of pyrethrum for many years had established the structure of one main insecticidal constituent as pyrethrin I 11 (3).

As a first achievement of analog synthesis, allethrin 12 was developed as a commercial synthetic pyrethroid for use in "mosquito coils". After a number of successful structural modifications as household insecticides, "pyrethrum" extended its world to a more applicable agricultural pest field. Esfenvalerate 13 and cypermethrin 14 are the best known examples of their commercial success as agricultural insecticides (3) (Fig. 1).

Even today, "pyrethrum" could still be looked upon as one of the best lead structures from natural sources for insecticide discoveries. The total performance of these analogs, such as excellent killing and paralyzing activities, selective toxicity margins, and their bio-degradable nature in the environment are still highly valued as most important leads for structural modifications to new insecticides.

2. RESEARCH PROCEDURE OF BIOLOGICALLY ACTIVE NATURAL PRODUCTS.

In order to succeed in discovering new agrochemicals, it is most important to have useful lead structures. As shown in Fig. 2, a number of biologically active natural products from plants, insects, micro-organism, marine invertebrates, and higher animals have contributed to the R & D of agrochemicals.

In general, the study of biologically active natural products consists of the following steps:

Collection of materials: plant, insect, micro-organism, or marine invertebrate
Extraction
Efficacy evaluation (bioassay)
Separation and purification
Structural elucidation
Total synthesis
Structural modification

When the structure and molecular size of an identified active ingredient is suitable for chemical synthesis, structure-activity relationship studies can be applied for further structural modification to improve biological properties as agrochemical leads.

Recent advances in purification technologies, such as high performance liquid chromatography (HPLC) and gas chromatography combined with mass spectrometry (GC-MS) have made it possible to isolate and characterize even quite unstable
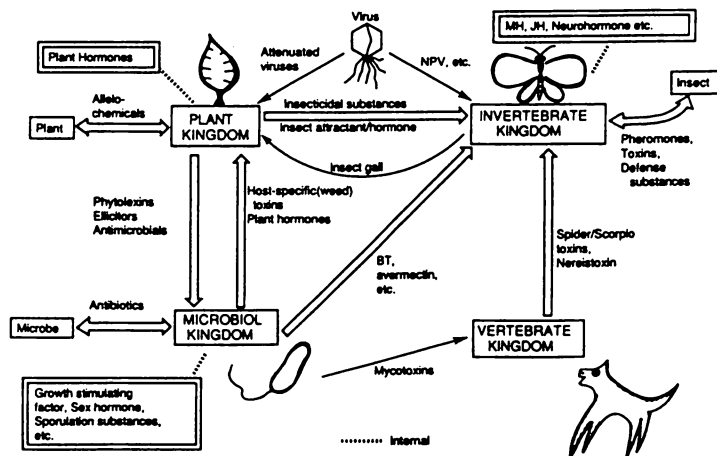
Fig. 2   Interactions between Organisms and Diversity of Biologically Active Natural Products

molecules. Progress in instrumental analysis (2D-NMR, FT-IR, LC-MS, FAB-MS, MS-MS, X-ray etc.) made it possible to carry out structural elucidation studies of complex and/or labile natural products on less than μg scale.

Number of well designed bioassay methods have also been developed, permitting the isolation of small amounts of biologically active natural products.

3. REQUIREMENTS OF A LEAD TOWARD NEW AGROCHEMICALS.

In the course of discovering new leads for new agrochemicals, the following needs must be taken into account:

3.1. Stability.

Chemicals with labile functional groups, such as a conjugated diene or polyene system, are often identified as partial structures of biologically active natural products. However, compounds with such unstable functionality cannot be handled easily, even though they exhibit excellent performance in a laboratory bioassay. In such a case, we must change the unstable partial structures to more potent and stable bio-isosters for further modification studies.

3.2. Mode of Action or Specificity.

Biologically active natural products can be classified into two types according to their activities.

Type 1 : Exhibiting activity against a wide variety of organisms, such as toxins or defensive substances (non-specific or non-selective).

Type 2 : Exhibiting activity against specific organisms and not so effective against non-target organisms, such as insect pheromones, insect hormones or plant hormones (highly specific and selective).

Since the target specificity of type 2 compounds as well as their potency is desirable as leads to potent agrochemicals, the structures of type 2 compounds have been often modified to discover a new agrochemical.

3.3. Activity under Practical Conditions.

A bioassay is one of the most important elements in natural product research aimed at isolating biologically active substances. In the laboratory, *in vitro* (including receptor assay) or *in vivo* bioassay systems are utilized during purification of active principles. In most cases, bioassay systems designed for isolation of active compounds are simplified to save sample amount and activity evaluating time. However, in many case it is difficult to evaluate the biological activity of isolated natural products under practical application. Out-door activity application might be quite far from in-house activity. In order to have a proper new lead for agrochemicals of natural origin, bioassay methods must be modified carefully to reflect practical activity.

4. NATURAL PRODUCTS INVOLVED IN BIOLOGICAL INTERACTION.

Natural product research begins with the investigating hostile or symbiotic biological interactions at the molecular level. Knowledge of many biologically interesting phenomena based on ecological observations have given opportunities to conduct natural product research. As shown in Fig. 2, biological interactions can be classified as not only those between similar organisms, such as insect-insect, plant-plant and microbe-microbe interactions, but also those between different types of organisms such as insect-plant, insect-microbe, and plant-microbe interactions.

In this section, research on biologically active natural products involved in biological interactions as well as some examples of structure modifications are described.

4.1. Insect-Insect Interactions.

Substances which bring about intraspecific insect-insect communication are insect pheromones. A pheromone is defined as a chemical substance that is produced by a member of a species which serves as a signal to other individuals of the same species producing one or more behavioral or developmental responses. There are two types of insect pheromones. One is a releaser pheromone such as a sex pheromone, an aggregation pheromone, an alarm pheromone and a trail pheromone, and so on. The other is a primer pheromone such as a class differentiating pheromone or reproduction controlling pheromone. In social insects, the order of their society is controlled by a primer pheromone. In general, insect pheromones are usually species-specific and highly potent.

Plutella xylostella

Z-11-hexadecenal **14**   Z-11-hexadecenyl acetate **15** $\overset{O}{\underset{}{\parallel}}$

Periplaneta cockroach

periplanone B **16**   periplanone D **17**
(*P. americana*)   (*P. fuliginosa & P.americana*)

Fig. 3 Typical Sex Pheromones of *Plutella xylostella* and *Periplaneta* cockroach

Fig. 4   Juvenile Hormone Mimics and Ecdysone Agonist

More than 500 insect pheromones, mainly Lepidopteran pheromones, have been identified. Many are mixtures rather than single component. Since the isolated amounts of pheromone is usually limited, activity evaluations with a synthetic specimen is essential to identify its structure. Recently, some Lepidopteran insect sex pheromones, such as Z-11-hexadecenal **14** and Z-11-hexadecenyl acetate **15** have been used practically to control the diamondback moth, *Plutella xylostella*. (4) (Fig. 3).

Sex pheromones of *Periplaneta* cockroach, periplanone B **16** (*P. americana*) and periplanone D **17** (*P. fuliginosa*) have been identified and synthesized (5). Due to their structural complexity and extremely narrow specificity, their practical application as cockroach attractants has not yet been successful (5) (Fig.3).
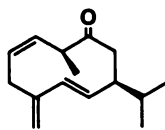
Juvenile hormones such as JH I, **18** and the molting hormone (β-ecdysone, **19**) regulate the process of insect moulting and metamorphosis. These compounds are nontoxic to mammals and also not effective against non-target organisms. Juvenile hormone analogs were investigated in search for a new insect growth regulator. Among the synthetic analogs, fenoxycarb **20** and pyriproxifen **21** have been selected and developed, since they exhibit very high activity against a wide range of insect species (6) (Fig.4).

The structure of β-ecdysone is very complex for synthetic structural modifications, however, tebufenozide **22**, one of N-t-butyl-N,N'-dibenzoylhydrazine derivatives, has been discovered as an agonist of β-ecdysone, which is useful as a selective insecticide, controlling Lepidopteran insect larvae (7) (Fig.4).

4.2. Plant-Plant Interactions.

The phenomenon that plants release toxic substances to the surrounding soil, suppressing other plant species is defined as allelopathy. Structures of allelopathic substances (allelochemicals) such as compounds **23 ~ 26** are comparatively simple (8) (Fig. 5). But, in general, the plant (weed) killing activities of allelochemicals are not particularly so strong and non-specific, in a practical sense. For this reason allelochemicals have not been probably adopted as potential leads for new synthetic herbicides, so far.

$CH_3-C{\vdots}C-C{\vdots}C-C{\equiv}C-C{=}C$-COOCH$_3$

2-cis-dehydromatricaria ester **23**
(*Solidago altissima*)

strigol **24**
(*Striga lutea*)

nagilactone A **25**
(*Podocarpus nagi*)

lepidimoide **26**
(*Lepidium sativum L.*)
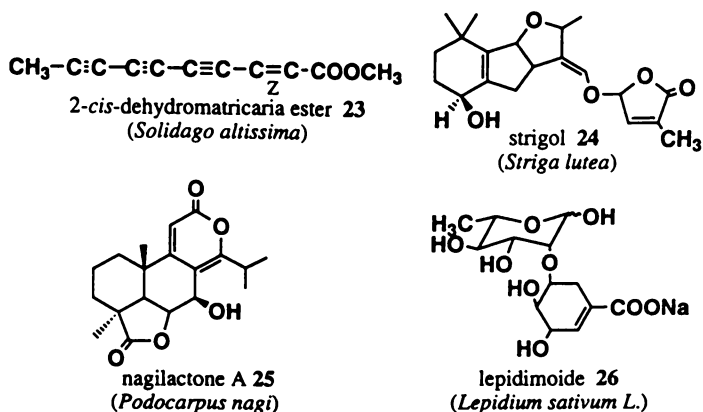
Fig. 5   Plant Allelochemicals

4.3. Microbe-Microbe Interactions.

Many antibiotics from *Actinomyces* have been developed as useful medicines. On the other hand, a number of agricultural antibiotic fungicides, such as polyoxins **27**, validamycn **28** (against powdery mildew) and kasugamycin **29**, blasticidin S **30**, (against rice blast disease) have been manufactured in Japan by large-scale fermentation from different species of *Streptomyces* (9) (Fig. 6).

Recently, methoxy-acrylate fungicides whose lead structure originates from strobilurin A **31** have been investigated. Strobilurin A, isolated from the mycelia of small basidiomycete fungi (*Strobilurus tenacellus*), exhibited excellent fungicidal activity against a wide range of plant pathogenic fungi *in vitro*. However, because strobilurin A is quite unstable photochemically, structural modification has been pursued to give stability to the molecule. As the result, ICIA-5504 **32** and several structurally related analogs, BAS-490F **33** and SSF-126 **34** have been discovered as promising fungicides (10,11) (Fig. 7) .

4.4. Plant-Insect Interactions.

There are many examples of naturally occurring plant substances which have served as leads to insecticides, insect repellents and insect growth regulators (Fig. 8 and 9). Among them, pyrethrins from *Chrysanthemum cinerariaefolium* have already been discussed with. Actually, the development of pyrethroid insecticides is one of the most successful stories in terms of the use of natural products as a lead for commercial insecticides.

"Neem", *Azadirachta indica*, is a tree native to tropical Asia and Africa. The extract of the seeds has been known to exhibit anti-feeding, repellency, toxicity and growth disruptive activities against various kinds of tenacious insect such as locusts, gypsy moths, aphids and so on (12).

The structure of the active ingredient, azadirachtin **35** is too complex to be employed as a lead for new synthetic insecticides (13). Nevertheless, owing to the intensive cultivation of this plant, crude seed extracts or purified natural azadirachtin can be utilized for insect control in a practical sense. Because of its low mammalian toxicity, "neem" products have been used as favorable commercial insecticides in tropical countries (12).

Several kinds of acetogenin type compounds such as asimicin **36** from *Annonaceae* plants are reported to have some insecticidal properties against cotton aphid and mosquito larvae (15).
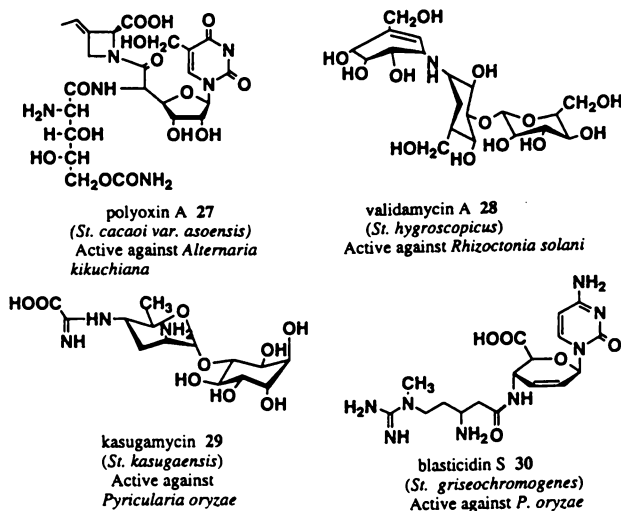
Fig. 6 Commercialized Antifungal Antibiotics
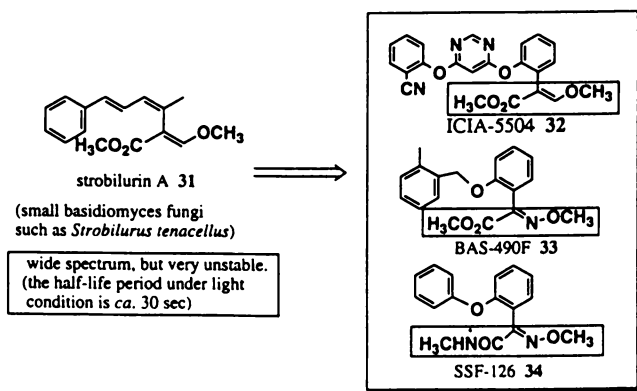
Fig. 7 Methoxyacrylate Type Fungicides

A sesqui-lignan haedoxan A **37**, from the roots of *Phryma leptostachya* is effective against not only houseflies, but also several Lepidopteran insects by ingestion (Fig. 8). The deficiency of activity of a lignan without a 1,4-benzodioxanyl group indicates that the 1,4-benzodioxanyl group is indispensable to the manifestation of insecticidal activity of haedoxan A **37** (16).

Both examples of these naturally occurring insecticides, asimicin **36** and haedoxan **37**, are yet to be modified for practical insecticides.

Unsaturated isobutylamides from *Piperaceae, Compositae* and *Rutaceae* plants are known to exhibit insecticidal properties. The insecticidal activities of pipercide **38** , dihydropipercide **39**, and guineensine **40** against adzuki bean weevil are higher than that of pellitorine **41**, which does not possess a methylenedioxyphenyl group in the molecule. The 3-bromophenyl ether analog **43** possesses a nearly 50-fold higher

azadirachtin A **35** / triterpene
(*Azadirachta indica*)

asimicin **36** / acetogenin
(*Annona squamosa*)

haedoxan A **37** / sesqui-lignan
(*Phryma leptostachya*)
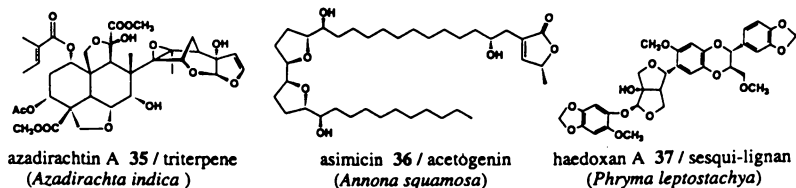
1. Typical Botanical Insecticides

pipercide **38**

dihydropipercide **39**

guinensine **40**

active against Adzuki Bean weevil

pellitorine **41**

methylenedioxyphenyl ether
derivative **42**

analog **44**

3-bromophenyl ether derivative **43**
a neary 50-fold higher activity than **42**

analog **45**
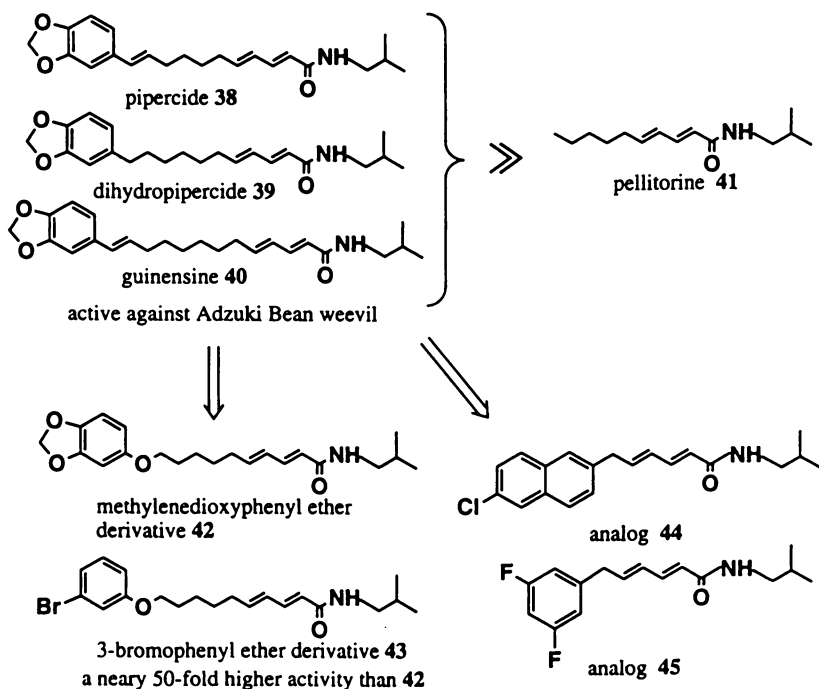
2. Insecticidal *Piperaceae* Diene Amides and Related Compounds

Fig. 8 Insecticidal Principles of Plant Origin and Their Derivatives

activity than the corresponding methylenedioxyphenyl ether derivative, **42** (17) (Fig. 8).

Structural modification trials by Elliott *et al* led to compounds **44** and **45** (Fig. 8), with shortening the chain between phenyl and carbonyl and replacing terminal methylenedioxyphenyl by 3,5-difluorophenyl or 6-chloro-2-naphthyl groups. These modified molecules exhibited potent activity against both houseflies and mustard beetles (18). Because of the rapid ability to act on the nervous system, unsaturated diene amide analogues do provide much interest as new leads for new insecticide discovery.

N,N-Diethyl-*m*-toluamide (DEET) has been used as an insect repellent against blood-sucking insects such as mosquitoes. The essential oil from *Eucalyptus citriodora* or *E. camaldulensis* exhibited significant repellent activiy against *Aedes aegypti* or *A.*

albopictus. p-Menthane-3,8-diol **46** (*trans*) and **47** (*cis*) from *E. citriodora* and eucamalol **48** from *E. camaldulensis* were identified as active ingredients and their activity were superior to DEET. Due to simplicity of the structure as well as excellent repelling activity, these compounds could be looked upon as new leads for mosquito repellents (19) (Fig.9) .

trans **46**   cis **47**   eucamalol **48**
p-menthane-3,8-diols   (*E. camaldulesis*)
(*Eucalyptus citriodora*)
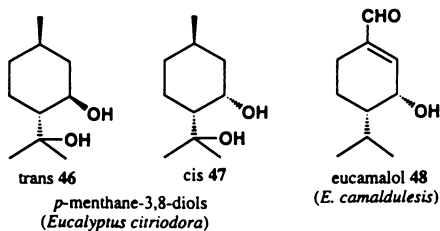
Fig. 9   Insect Repellents from *Eucalyptus* plants

Nicotine **49**, a constituent of tobacco leaf, had been used as an insecticide which binds to the acetylcholine receptors (20). Researchers at Shell discovered that SKI-71, tetrahydro-2-(nitromethylene)-2H-1,3-thiazine **50** was highly effective against lepidopteran insects, however this compound was quite unstable to light (21). Another chemical modification studied has led to the discovery of imidacloprid **51**, a novel systemic insecticide against plant-sucking insects such as aphids and leafhoppers. Imidacloprid **51** also acts as an agonist at the nicotinic acetylcholine receptor, which is classified as a neonicotinoid (22) (Fig. 10). Although, the structures of nicotine **49** and imidacloprid **51** are different, the history of structural modifications shows that both compounds are related in terms of developing a unique systemic insecticide.

neonicotinoids

nicotine **49**
(*Nicotiana tabacum*)

SKI-71 **50**

imidacloprid **51**

systemic, high residual efficacy effective against plant hopper, aphides, thrips
less acutely toxic in mammals.
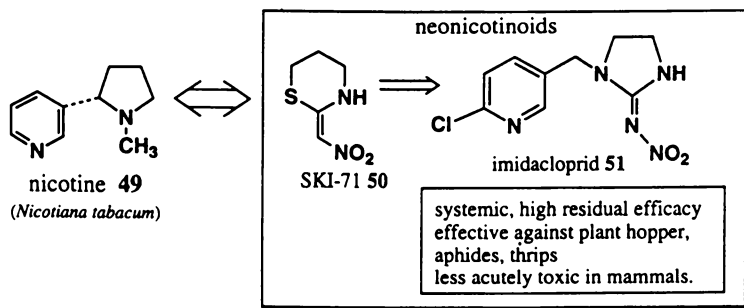
Fig. 10   Acetylcholine Receptor Inhibitor type Insecticides

4.5. Insect-Microbe Interactions.
One of the most successful examples of an insecticidal principle of microbial origin is the BT-toxin, produced by *Bacillus thuringiensis*. BT-toxins are composed of β-exotoxin (adenosine derivative) and δ-endotoxin (160 kDa protein). δ-Endotoxin has been commercialized as the first proteinaceous insecticide to control agricultural pests. Several investigations have been conducted to improve the insecticidal activity and the spectrum of activity of BT δ-endotoxin with applications of biotechnology (23).
Today, investigations of transgenic insect tolerant crops (cotton) with DNA encoding BT δ-endotoxin have proved to be successful. BT-cotton seed will be introduced into the market soon as insecticide-free cotton. This new bio-engineered product may shift future research of biologically active natural products from small organic molecules to protein.

Milbemycin **52** (Fig. 11), produced by *Streptomyces hygroscopicus*, is a 16-membered macrolide, which has been commercialized to control acarids (24). Ivermectin **53** (Fig. 11), 22,23-dihydro-derivative of avermectin B, produced by *S. avermectilis*, has also been commercialized to control not only argicultural pest but also parasites of animals such as cattle, sheep, pig, horse, dogs and cats. The mode of action of these macrolides is known to stimulate GABA action and inhibit normal neuro-transmission at neuro-muscular junction (24).

milbemycin **52**

ivermectin **53**

R= CH(CH₃)CH₂CH₃   80%
   CH(CH₃)₂   20%

dioxapyrrolomycin **54**
(*Streptomyces* sp.)

AC 303,630 **55**
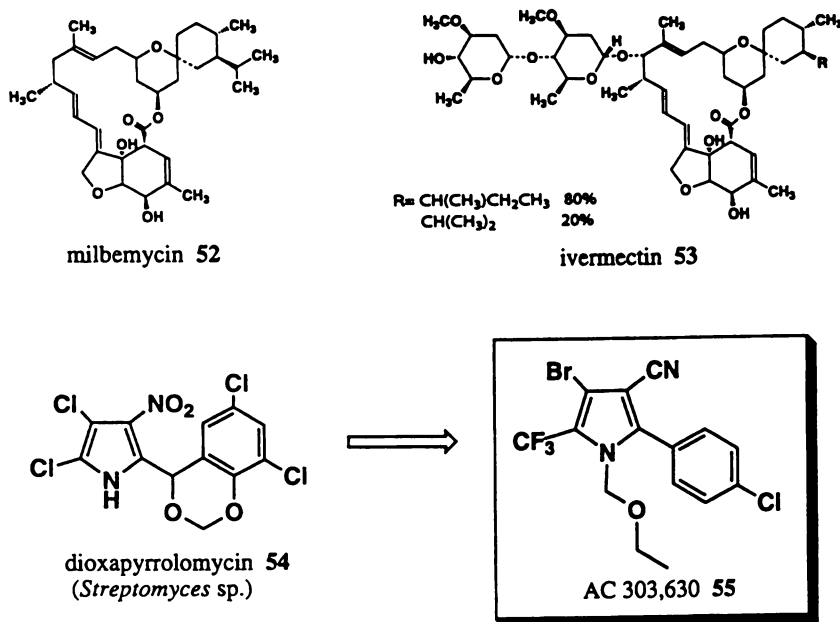
Fig. 11   New Natural Insecticides of Microbial Origin

Dioxapyrrolomycin **54**, isolated and identified from the fermentation broth of a *Streptomyces* strain, represents one of a series of halogen-containing nitropyrroles (25). This compound exhibits insecticidal and miticidal activity against tobacco budworm (*Heliothis virescens*) and two-spotted mite (*Tetranychus urticae*), however it also possesses high mammalian toxicity. This compound proved to be an uncoupler of oxidative phosphorylation. Researchers at American Cyanamid initiated chemical modification studies to improve its insecticidal activity and reduce mammalian toxicity. As its results, a broadly active compound **55** (AC 303,630) was discovered and developed as a new insecticide for controlling mainly Lepidopteran insects (Fig. 11) (26)

4.6. Plant-Microbe Interactions.
The metabolites of plant pathogenic microbes which cause disease symptoms on the host plant high selectively, are defined as host specific toxin (HST). The structure of several HST were elucidated to be a cyclic depsipeptide, AM-toxin **56** from *Alternaria mali*, (toxic to apple) and two acyloxy unsaturated epoxy carboxylic acid, AK-toxin **57** from *A. kikuchiana* (toxic to pear) and AF-toxin **58** from *A. fragariae*

(toxic to strawberry) (27) (Fig.12). These compounds can be regarded as lead candidates for structual modifications to new herbicides with excellent selectivity.

Several kinds of phytotoxic substances of microbial origin have been isolated and identified (28). Bialaphos **59** (Fig. 13), produced by *Streptomyces hygroscopicus* has been used as a novel broad spectrum and post emergent herbicide with low mammalian toxicity (29). Its active form is phosphinothricin **60**, whose structure is similar to the practical non-selective herbicide, glyphosate **61**.

AK-toxin I **57**
(*A. kikuchiana* / pear)

AM-toxin I **56**
(*Alternaria mali* /apple)

AF-toxin I **58**
(*A. fragriae* / strawberry)
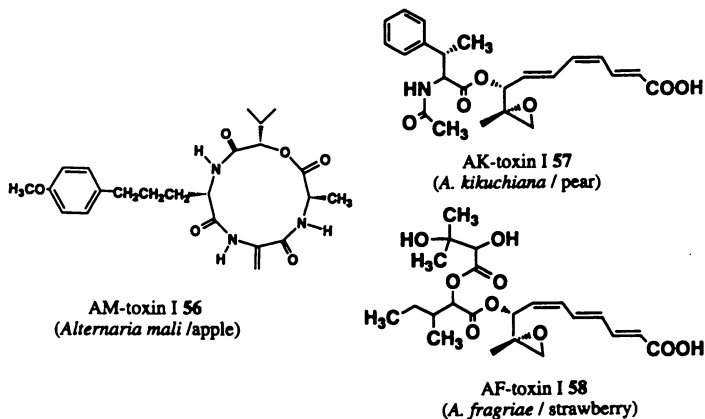
Fig.12   Structures of Typical Host Specific Toxins

bialaphos **59**
(*Streptomyces hygroscopicus*)

phosphinothricin **60**
Presumed active form of bialaphos

Non-selective herbicide on orchard weeds, Inhibitor of glutamate synthase.

glyphosate **61**
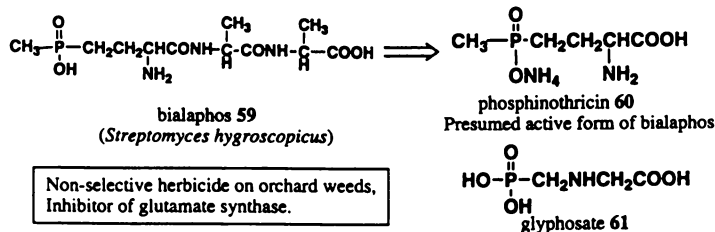
Fig.13   Structures of bialaphos and Related Compounds

A chemical substance produced by a plant to defend against attack by pathogenic microbes is called a "phytoalexin". A number of phytoalexins have been isolated and identified from many plant species (*Convolvilaceae, Solanaceae, Leguminosae* or *Rosaceae*) to be sesquiterpenes, isoflavonoids, biphenyls or furan derivatives. In general, antimicrobial activities of phytoalexins are not especially strong or specific. Probably because of this, they have not been modified for duse as synthetic fungicides.

5. CONCLUDING REMARKS.

During the past fifty years, we have made great progress in R & D of agro-chemicals in terms of structural variability and efficacy. At the same time, wide-spread concerns about the possible adverse effects of agrochemicals have increased. Thus, the chance of creating new agrochemicals with better performance on one hand, and with less enviromental impact on the other, and the probability of discovering successful compounds becomes less and less; it is commonly recognized that the probability will be less than 1 compound out of 30,000 candidates.

As one of the counter-measures to increase the probablity of finding new agrochemicals, we now draw attention with this review to the investigation of natural products for identifying better lead compounds. If successful, we may discover more desirable compounds favorable for agro-chemicals. At the same time, these biologically active substances will be an eye opener to new chemical and biological interactions among living organisms that may be utilized in increased agricultural production.

However, within the limitation of current agricultural production, including application technology of agro-chemicals, we inevitably modify the original chemical structure of these biological substances in order to obtain better compounds. As a result, in most cases our synthesized derivatives are superior to the original principles in several respects, and/or the mode of action of the original compounds may be changed, resulting in a new type of agrochemicals.

It should be recognized that the structure activity studies of the naturally occurring biologically active substances exemplified are indispensable for better agrochemicals. To be more effective in discovering and developing new agro-chemicals, we should take careful deliberation of the following points:

<1> To carefully evaluate biological activity manifested by natural products and correlate thier activity with a future plant protection method.

<2> To use as a starting material, those compounds which have ample possibility of future modification. Actually, this is quite difficult to foresee at the initial stage. Nevertheless this kind of insight is very important for the successful modification (see pyrethroids vs carbamates).

<3> To have simplified and precise bioassay systems, preferably to be correlated with *in vivo* manifestaion of the biological activity. The mode of action studies including receptor assay should be duly incorporated. Metabolism information will be quite helpful as well as computor-assisted structure design. Hopefully this information should be proactive, to be able to modify structures more easily.

<4> To correlate physicochemical properties of the synthesized products with biological activity for better efficacy.

<5> Also to correlate these physicochemical properties with their enviromental behaviors for reduced enviromental impact.

<6> To have simplified testing methods for a number of mammalian and ecotoxicity of the compounds. Actually, some most promising candidates have had to be discarded because of a longer-term toxicity including carcinogenicity. And it is well recognized that some natural products possess such negative properties.

And finally,

<7> Never to observe findings naiively, and never sticking exclusively to pre-fixed principles and ideas. Serendipity remains as an important factor.

In the above list, <3> to <7> are more or less commonly applied to current studies aimed at creating new agrochemicals, whereas <1> and <2> should be particularly emphasized in undertaking the discovery of revolutionary agrochemicals from natural products. In this sense, natural product research should take a lead in overall future plant protection strategy.

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Chapter 14

Development of Synthetic Pyrethroids with Emphasis on Stereochemical Aspects

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Extensive efforts in structure modification of alcohol and acid moieties in natural pyrethrins have led to a number of synthetic pyrethroids with diversified characteristics. Their features in chemistry, efficacy, toxicology and environmental effect are briefly discussed with emphasis on their stereochemical aspects.

The Dawn of Synthetic Pyrethroids

Natural pyrethrins have long been used as most favored household insecticides. Pyrethrum flowers are still cultivated in certain areas including Africa and Australia.
On the other hand, commercial use of synthetic pyrethroids is one of the most remarkable success stories in insecticide development stemming from natural products as a lead. The history of structural modification study of natural pyrethrins has lasted for more than half a century. It should be noted that even in 1924 Staudinger and Ruzicka (1) already reported several synthetic pyrethroids. Some of them 1 and 2 are shown in Figure 1. At that time they could easily obtain optically active *trans*-chrysanthemic acid by hydrolysis of pyrethrum extracts, whereas it was difficult to obtain alcohol components because of their instability.

Pyrethrin I

Staudinger and Ruzicka (1924)

(1)   (2)
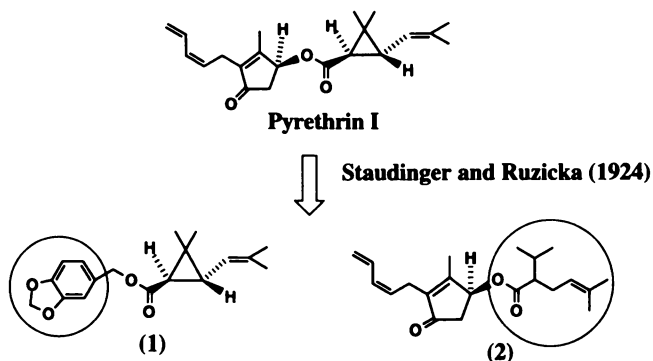
Figure 1. Structural Modification By Staudingar and Ruzicka

Although some of them were only slightly insecticidal, to note is their foresight regarding natural pyrethrins as a lead compound without knowing the real structures of alcohol moieties.

Through extensive studies during the past 50 years, natural pyethrins proved to possess ample possibility of structural modifications. Namely, just after elucidation (2) of the structures of pyrethrin I, **3** and pyrethrin II, **4** of natural pyrethrins in 1947, extensive efforts started to modify both acid and alcohol moieties. The target parts of structural modification could be divided into five moieties, **A, B, C, D** and **E**.

The side chain of the cyclopentenolone ring **A** was the first target for modification, followed by modification of the 5-membered ring **B** to other rings .The isobutenyl side chain **E** of the cyclopropane ring was the next target for modification. Although the cyclopropane ring **D** and the ester functions **C** had long been thought to be indispensable moieties, modifications at these parts, **C** and **D** enabled to find novel structures (Figure 2).

(**3**)   **Pyrethrin I**   **R = - CH₃**

(**4**)   **Pyrethrin II**   **R = - CO₂CH₃**
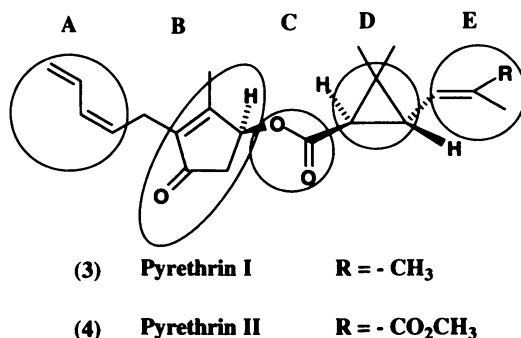
Figure 2. Structural Modification Targets of Pyrethrins

Structural Modification of Alcohol Moieties of Synthetic Pyrethroids

The modification of **A** and **B** parts is summarized in Figure 3. Compounds in frames are commercially important alcohols. Simplification of the diene moiety of pyrethrin I resulted in finding allethrin **5** by Schchter, which is the first efficacious synthetic pyrethroid (3). It has lethal and knockdown activities comparable to pyrethrins against mosquitoes. Although the compound **6** with a furfuryl side chain by Matsui was only moderately insecticidal (4), substitution of the diene portion to an aromatic ring offered an important prototype for a wide variety of further development, such as resmethrin **10** by Elliott. A propargyl analog of allethrin **7** is found to be one of the most important pyrethroid for household use. Another major breakthrough in terms of higher knock down activity was the discovery of tetramethrin **8** by Kato et. al. in 1964 (5) on the right hand side of the Figure 3. Further studies on related analogs by Itaya resulted in finding a propargyl hydantoinmethyl alcohol (6). The ester **9** with (1R)-trans-chrysanthemic acid is one of the most potent knock down agents.

The first benzyl ester was synthesized by Staudinger and Ruzicka as was mentioned before. In fact this proved to be very important model for further modifications.

A major progress along this line was the discovery of resmethrin **10** by Elliott (7). This is the first synthetic pyrethroid to have much higher lethal activity than natural pyrethrins against various insect pests. Another breakthrough in our laboratory was the invention of the first photostable alcohol components, namely 3-phenoxybenzyl alcohol and α-cyano-3-phenoxybenzyl alcohol moieties by Itaya (8) and Matsuo (9) respectively in early 70's. Their esters with chrysanthemic acid, phenothrin **11** and cyphenothrin **12** have both been commercialized by Sumitomo Chemical. An α-cyano moiety substituent on the benzylic carbon atom of 3-phenoxybenzyl ester increased the activity more than two times. The cyano substituent is a compact and straightforward moiety, which

presumably enables the phenoxyphenyl moiety to be resistant against metabolic attack and to pose an insecticidally preferred conformation. Although various α-cyano alcohols were synthesized by us, only the 3-phenoxybenzyl species was suited for modification with the α-cyano group (*9*). The dichlorovinyl analog of chrysanthemic acid were synthesized by Elliott (*10*), to lead to permethrin **13** and cypermethrin **14** , respectively with 3-phenoxybenzyl alcohol and α-cyano-3-phenoxybenzyl alcohol as alcohol moieties. These **13** and **14** are the first photostable pyrethroids, which can practically be usable under outdoor conditions.
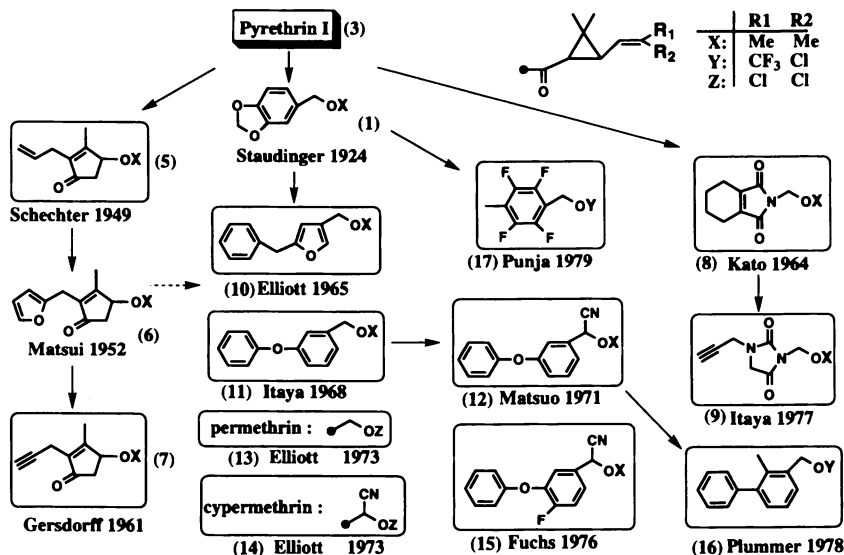
Figure 3. Development of Alcohol Moieties

It should be noted that nowadays α-cyano-3-phenoxybenzyl alcohol moiety is incorporated into a number of commercially important pyrethroids.

In addition, the 4-fluorine analog **15** of cypermethrin was invented by Fuchs and commercialized as cyfluthrin(*11*). The biphenyl compound **16** by Plummer was also a photostable pyrethroid (*12*). Tefluthrin **17** was commercialized by Zeneca for a soil insecticide (*13*), especially for control of corn root worms. In this case, the acid part is a chloro, trifluoromethyl analog of chrysanthemic acid . It is noteworthy that the alcohol part of tefluthrin **17** has no unsaturated side chain which had long been thought to be indispensable for the insecticidal activity.

A Volatile Synthetic Pyrethroid. Fission of cyclopentenolone ring was first tried by Sota, which resulted in finding an important acyclic alcohol ester **18** (*14*) (Figure 4). Although the ester was not commercialized, this became the prototype of our volatile pyrethroid. We investigated various allyl alcohol esters especially containing secondary alcohol moieties to lead finally to empenthrin **19** by Kitamura (*15*). Vapor action at room temperature in empenthrin proves to be sufficiently high to use as a fumigant in closed space to control cloth insect pests in closets. Empenthrin **19** compares well with optically active *d*-allethrin in toxicity to *M. domestica* by topical application as shown in Table I. It is to note that empenthrin has much higher knockdown activity and lethal activity at ambient temperature by vapor effect as compared with allethrin. In fact, the vapor pressure of empenthrin is 15 times as high as *d*-allethrin at 25°C.

(5)

X: Chrysanthemoyl

(Sota 1971) (18)

(Kitamura 1973) (19)
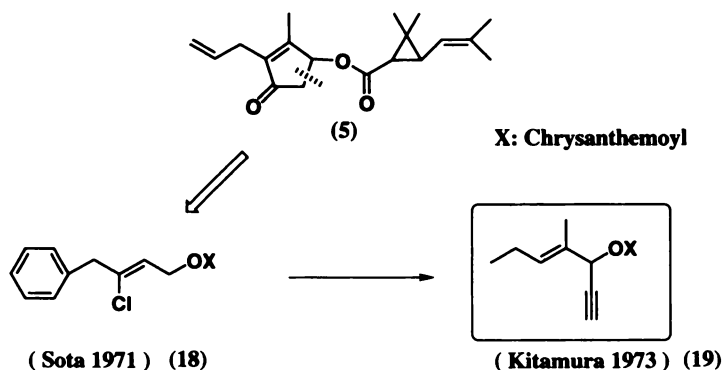
Figure 4. Development of Acyclic Alcohol Moiety

Table I. Vapor Effect of Empenthrin against *M. domestica*

	Topical Application LD_{50} (μg/female)	Vapor effect KT_{50} (min.) Mortality (%) (at 25°C)	Vapor Pressure (at 25°C)
empenthrin(18)	0.25 μg	14 min. 100%	1.1×10^{-3} mmHg
d-allethrin	0.25 μg	>120 min. 3%	7.6×10^{-5} mmHg

Efficacy and Chemistry of Prallethrin. The propargyl-analog of allethrin was first synthesized by Gersdorff in 1961 (*16*), but it did not attract much attention at that time, since the efficacy was reported to be only 60% of allethrin **5** against houseflies. The structure activity relationship between side chains and insecticidal activity of empenthrin analogs revealed that the propargyl analog of allethrin should have much more activity than allethrin (*17*). In fact, it was found that the propargyl analog was easily transformed to the allene compound in a basic medium. Actually, the ester **20** showed low insecticidal activity. Therefore, it was estimated that the propargyl analog of allethrin by Gersdorff might have had insufficient purity. We carefully prepared the propargyl analog with sufficient chemical purity (*18*) and re-examined the insecticidal activities. As a result, it was found that the insecticidal activity of compound **7** is more than twice of allethrin (Figure 5).

(5) 100

60 (Gersdorff 1961)

220 (Matsuo 1978)

(7)

(20) below 50
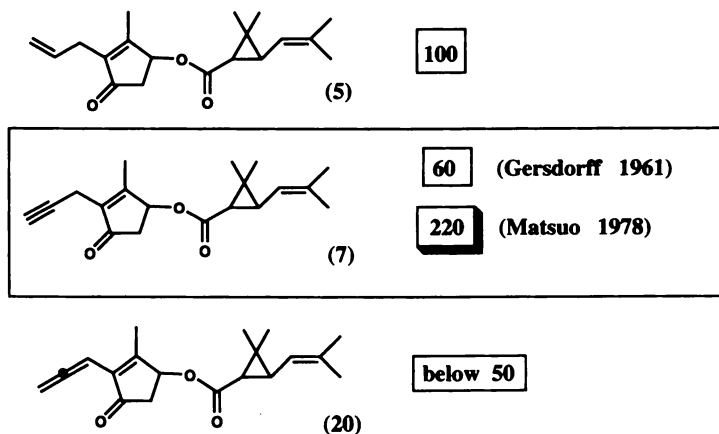
Figure 5. Relative toxicity of Cyclopentenolone Esters
against *M. domestica*

It is often encountered that biological activities reside in specific stereoisomer of the enantiomer pair. It is also the case with compound **7**. There are three asymmetric carbon atoms in the compound **7** and there exist eight stereoisomers. Figure 6 shows the insecticidal activity of eight stereoisomers of **7** against *M. domestica*. Chrysanthemic acid possesses two asymmetric centers and (1*R*)-*trans* and (1*R*)-*cis* isomers provide insecticidal esters.
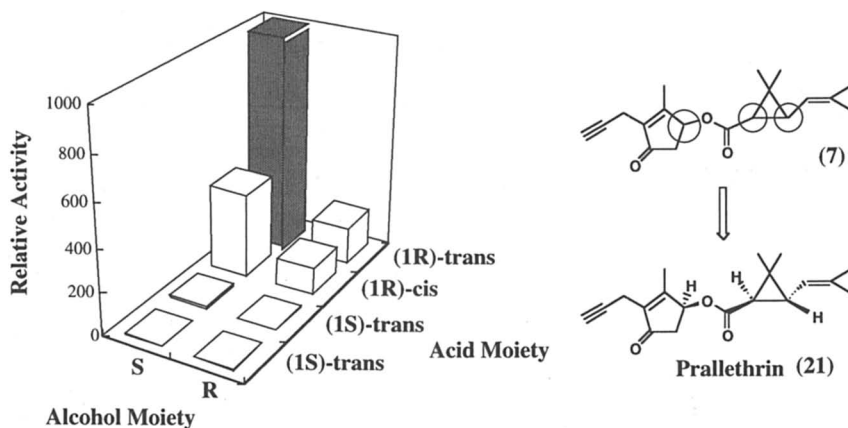
Prallethrin (21)

Figure 6. Relative lethal Activity of stereoisomers of Prallethrin
against *M. domestica*

With regard to the alcohol moiety, only (*S*)-isomer is insecticidally much more important. Thus among eight isomers, the ester of the (*S*)-alcohol with (1*R*)-*trans*-chrysanthemic acid, is the most active isomer. This isomer named prallethrin **21** has successfully been commercialized by Sumitomo Chemical. The relative lethal and knockdown activities of prallethrin in comparison with *d*-allethrin is shown in Table II.

Prallethrin is more than 4 times as active as *d*-allethrin against *M. domestica* and *B. germanica* in lethal activity, and has much better knockdown activity than *d*-allethrin against *Culex pipiens pallens*.

Table II. Insecticidal activity of prallethrin against *Musca domestica*, *Culex pipiens* and *Blattella germanica*

| Insecticide | Relative Lethal Activity * | | Relative KD** |
	M.domestica	*B.germanica*	*C.pipiens*
prallethrin	420	610	470
d-allethrin	100	100	100

*Topical application, LD50 mg/female
**Oil spray, 0.34 m³ glass chamber
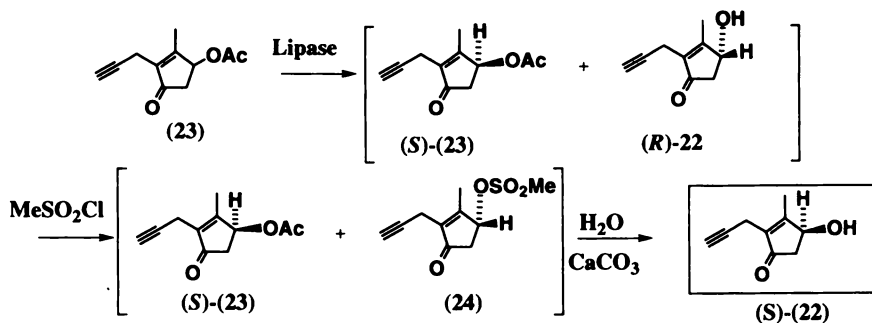
Figure 7. Synthesis of the Alcohol Moiety of Prallethrin

The synthesis of the alcohol moiety of prallethrin was conducted by Umemura (*19*) in our laboratory as shown in Figure 7. The key steps of this synthesis are the combinations of enzymatic resolution of the racemic acetate **23** and chemical inversion of the hydrolyzed alcohol (*R*)-**22**. Thus, the reaction mixture from the enzyme reaction was treated with methane sulphonylchloride to give a mixture of (*S*)-acetate, (*S*)-**23** and the (*R*)-sulfonate **24**. The two different esters were found to be smoothly hydrolyzed in a basic medium to give (*S*)-**22** in high yield as a sole product.
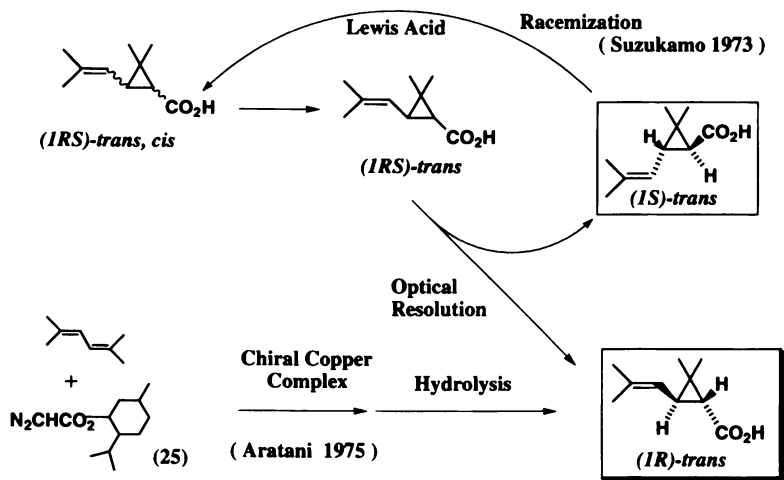
Figure 8. Synthesis of (+)-*trans*-Chrysanthemic Acid

The acid moiety of prallethrin, namely (1R)-*trans*-chrysanthemic acid, can be obtained by various methods (Figure 8). Classical optical resolution of the racemic *trans* acid with (-)-naphthylethyl amine is one of the efficient methods, although the theoretical yield is at most 50%. In this case, insecticidally useless (1S)-*trans*-chrysanthemic acid can efficiently be racemized back to give (1RS)-*cis, trans*-acid according to Suzukamo's method (20). Asymmetric synthesis of 2,5-dimethyl-2,4-hexadiene and menthol diazoacetate 25 with chiral copper complexes was successfully conducted by Aratani (21) to afford the (1R)-*trans*-ester in high optical and chemical yield.

Quite recently, Nishizawa et. al.(22) in Sumitomo Chemical have found an efficient esterase in *Arthrobacter globiformis* SC-6-98-28, which stereoselectively hydrolyzes only (1R)-*trans*-ethyl ester among four stereoisomers of chrysanthemates to give the pure (1R)-*trans* acid (Figure 9). The gene coding was cloned from *A. globiformis* and overexpressed in *Escherichia coli*. Thus, the cellular content of the enzyme reached 33 % of the total soluble protein in the recombinant *E. coli* cells. The hydrolytic activity of the recombinant *E. coli* cells for ethyl chrysanthemate attained to be 2,500 fold higher than that of *A. globiformis* cells. The optimum pH and temperature were 9.5 and 50°C respectively and more than 98 % conversion and 100 % stereoselectivity of (1R)-*trans*-chrysanthemic acid were accomplished.
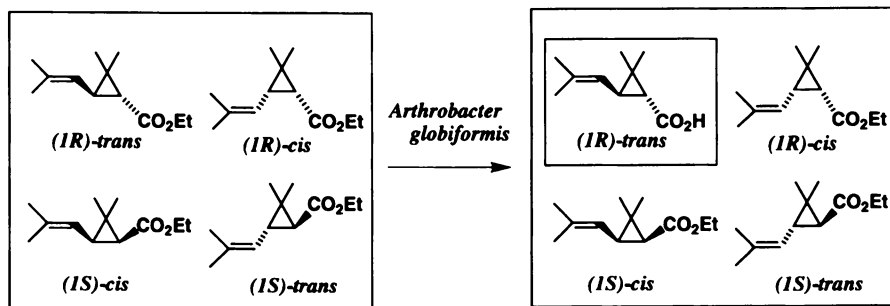
Figure 9. Synthesis of (+)-*trans*-Chrysanthemic Acid -2

Structural Modifications of Acid Moieties

Historically, structural modifications of alcohol moieties preceded the acid moieties conceivably owing to two reasons. First, because of their instability it was difficult to purify alcohol moieties of pyrethrins to be used for modification of the acid moiety.

Another reason was due to failure in early reports by Staudinger and Ruzicka of modification of acid moieties. Thus, only two modified acid moieties, namely the dichlorovinyl analog of chrysanthemic acid **26** by Farkas (23) and tetramethylcyclopropane carboxylic acid **27** by Matsui (24) in Fig.10 had been known before 1970. However, neither the dichlorovinyl acid nor the tetramethyl acid had not drawn much attention before they were esterified with photostable α-cyano-3-phenoxybenzyl alcohol.

Thus, Sumitomo Chemical commercialized fenpropathrin **28**. Elliott developed cypermethrin (**14** in Fig. 3) and the optically active dibromovinyl analog, deltamethrin **29** (25) both in early 70's. Deltamethrin is one of the most potent insecticides for agricultural use, which is the ester of (S)-α-cyano-3-phenoxybenzyl alcohol with the (1R)-cis-dibromovinyl chrysanthemic acid.

λ-Cyhalothrin **30** by ICI (26) is actually a substituted analog of cypermethrin by a trifluoromethyl group instead of one of the chlorine atoms. The modification of the acid moiety of pyrethrin II was extensively carried out by Tessier (27) to find acrinathrin **31** which is the ester of (S)-α-cyano-3-phenoxybenzyl alcohol with the (Z)-(1R)-cis-pyrethric acid derivative (Figure 10).
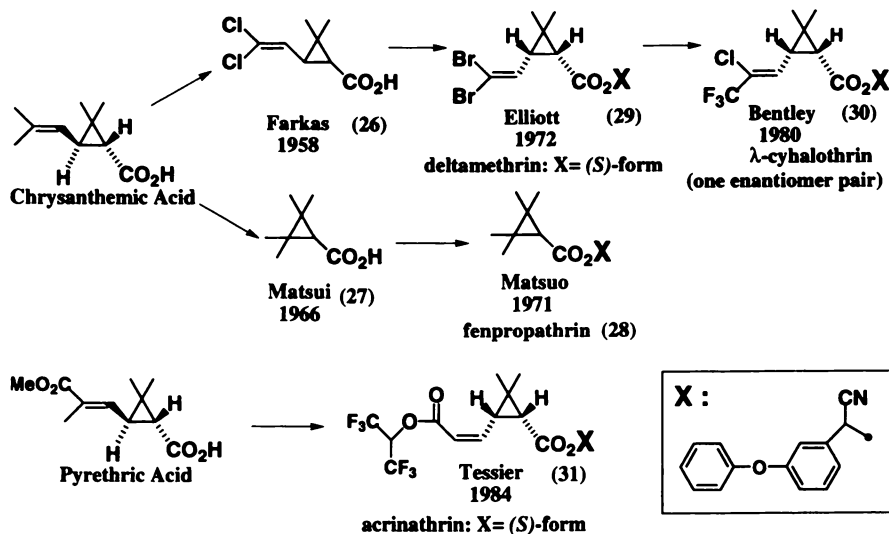
Figure 10. Development of the Acid Moiety-1

Discovery of Acid Moieties Devoid of Cyclopropane Ring.

The cyclopropane ring had long been thought to be indispensable for insecticidal activities. The extensive studies on new acid moiety devoid of cyclopropane ring was conducted by Ohno in our laboratory to find a lead compound **32**. Further exploratory work finally led to fenvalerate **33** (28). This was indeed a substantial breakthrough in the structure modification of pyrethroids lacking the cyclopropane ring in acid moiety (Figure 11). Following the discovery of fenvalerate, a number of non-cyclopropane pyrethroids were introduced as agricultural insecticides, such as fluvalinate **34** (29) and ethofenprox **35** (30).
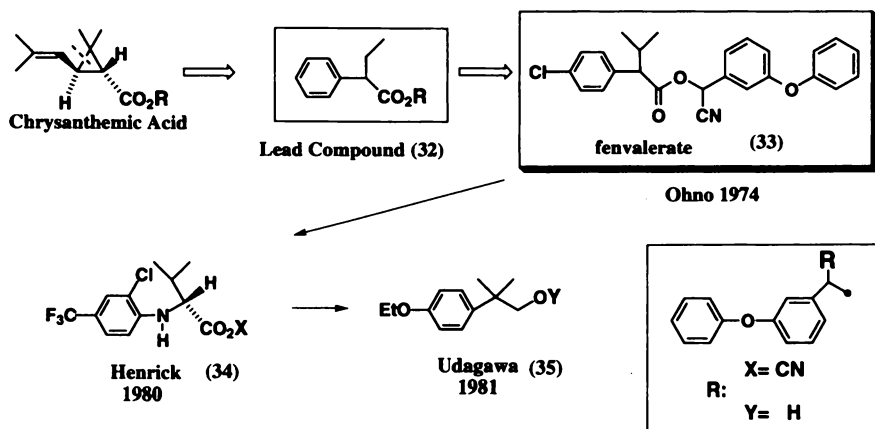
Figure 11. Development of the Acid Moiety-2

Ethofenprox is no more ester, but an ether compound, and has been developed as a rice paddy-field insecticide in Japan.

Fenvalerate **33** has two asymmetric carbon atoms in its molecule, and it has four optical isomers as shown in Figure 12. Among them, the $(2S, \alpha S)$-isomer named as esfenvalerate shows the highest insecticidal activity against houseflies, German cockroaches, diamond back moths, tobacco cutworms and green peach aphids. Relative activity against houseflies is also shown in Figure 12. Esfenvalerate shows adequate efficacy at 5 to 26 g a.i./ha against cotton pests in the field.
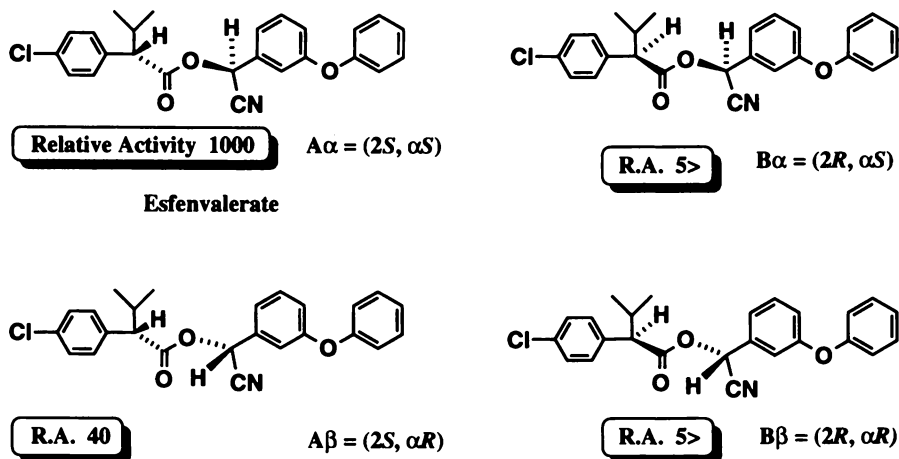
Figure 12. Four Stereoisomers of Fenvalerate and Relative Insecticidal Activity against *M. domestica*

Table III. Mammalian and Environmental Toxicities
of Pyrethroid Insecticides

Mammalian Aspect

Target Site   :   Sodium Channel in Excitatory Neurons
Acute Toxicity : Moderate (CS-Syndrome or T. Syndrome)
Teratogenicity, Reproductive toxicity, Carcinogenicity and
Mutagenicity : No Noteworthy Findings
Metabolism   : Rapid and Quite Extensive with Low Residues

Environmental Aspect

Non-biotic Degradation   (Hydrolysis and Photolysis)
        : Rapid / Moderate
Plants : Non-systemic, Biodegradable with Low Residues
Soil   : Rapid Degradation, No Leaching
Aquatic Environment : Biodegradable with Low Residues
Non-Target Organism : Practically Little Adverse Effects

Mammalian and Environmental Aspects of Synthetic Pyrethroids

Those widespread uses of pyrethroids are based on their acceptable environmental safety. Mammalian and environmental toxicities are summarized in Table III. The pyrethroids acts on sodium channel of excitatory neurons in mammals, as in the case of insects, developing CS-syndrome such as hyperexcitability, choreoathetosis, profuse salivation as acute toxic signs, or T-syndrome characterized by tremor, depending on the structure. Namely, cyanopyrethroids generally develop CS-syndrome, whereas non-cyano analogs show T-syndrome mainly. So far as are tested with regard to teratogenicity, reproductive toxicity, mutagenicity and carcinogenicity, no noteworthy findings have been reported to be related to risk assessment in humans (31). In the field, even so-called photostable pyrethroids are rapidly degraded in plants and soils with low residues (32). They are also biodegradable in aquatic organisms. Tissue residues of pyrethroids are generally quite low (33), and although the parent pyrethroids tend to be very lipophilic, sometimes similar to DDT, no bioaccumulation is observed after subacute dosing to mammals. It should be noted that their rapid biodegradation certainly contributes to least environmental contamination. Although these pyrethroids are inherently very toxic to fish and daphnia, they do not actually cause severe adverse effects thanks to strong adsorption to soil particles in water. To avian species they develop very low toxicity. Overall, as a pesticide, pyrethroid insecticides appear to have favorable features to mammals and in the environment (For detailed discussions, refer to a series of monographs of JMPR and IPCS Environmental Health Criteria Documents).

Summary and Conclusion

No one had ever thought of such diversified characteristic features of synthetic pyrethroids at the beginning of modification. Because of these extensive modifications, it is occasionally almost impossible to recognize at a glance the structural resemblance between modern synthetic pyrethroids and original natural pyrethrins. However, the result of electro-physiological studies and the computer assisted three dimensional shapes of these compounds support the close resemblance with each other. These synthetic pyrethroids have generally favorable toxicological features and enviromentally compatible properties, and actually nowadays constitute one of the most powerful tools for insect control, and we can control insect pests in houses, gardens and agricultural fields by a wide variety of application methods.

Although the discovery of new pesticide tends to be more and more difficult, newer pyrethroids with altered characteristics including wider insecticidal properties and systemic character can be forthcoming further through intensive investigations, by close collaborations between organic chemists and biologists concerned.

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Chapter 15

Structure—Activity Relationships for Insecticidal Pyrroles

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A new class of insecticides, the 2-arylpyrroles, based on a naturally occuring compound, dioxapyrrolomycin, has been prepared. Through a program of synthesis and biological evaluation, the parameters necessary for optimal insecticidal activity have been investigated. These structure-activity comparisons along with mode of action studies for this series will be discussed.

A program to identify novel sources of compounds possessing insecticidal activity has been in place in our laboratories for a number of years. As part of this program, we found that fermentation of a *Streptomyces fumanus* (Sveshnikova) culture, derived from a soil sample collected in Oklahoma, led to an extract having activity against a spectrum of agronomic pests.

Using standard isolation techniques, guided by screening for insecticidal activity, the active component was identified by Carter and co-workers in 1987 (1). The compound was named dioxapyrrolomycin. The structure, along with the insecticidal activity associated with the pure material, is shown in Figure 1. At about the same time, this pyrrole was reported by scientists at Meiji Seika and SS Pharmaceutical Company in Japan as having antibacterial and antifungal activity (2,3). Neither group reported insecticidal activity.

This natural product became the focal point for an extensive synthesis program aimed at discovering less complex analogs having improved insecticidal activity (4). This work culminated in the identification of the 2-arylpyrroles as a new class of insecticides. The general structure is shown in Figure 2. Our synthesis efforts have allowed us to define the substitutions at the various positions of the pyrrole nucleus necessary for optimal insecticidal activity (5, 6).

This paper will focus on these relationships between structural features and biological activity.

Structure - Activity Relationships

During the course of this work, it became apparent that, for optimal insecticidal activity, all of the positions on the pyrrole ring had to be substituted (5a). We

Dioxapyrrolomycin
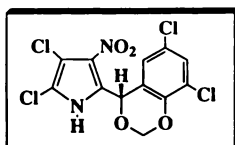
Insecticidal Activity (LC$_{50}$ - ppm)

Southern Armyworm *Spodoptera eridania* 3rd instar	Tobacco Budworm *Heliothis virescens* 3rd instar	Two-Spotted Mite *Tetranychus urticae* OP-Resistant	Western Potato Leafhopper *Empoassa abrypta* Mixed
40	32	10	<100

Figure 1. Structure and Insecticidal Activity of Dioxapyrrolomycin
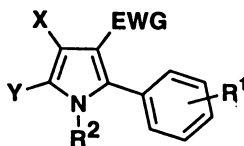
Figure 2. General Structure of the 2-Arylpyrroles

found it necessary to develop synthetic methodology that allowed access to these highly functionalized pyrroles. The synthesis of 2-aryl-3-cyano/nitro-4,5-dihalopyrroles (6), 2-aryl-3-cyano-4-halo-5-trifluoromethylpyrroles (7,8) and 2-aryl-3-trifluoromethylsulfonylpyrroles (9,10) have been described previously. The synthesis of some regioisomeric arylhalotrifluoromethylpyrrolecarbonitriles has recently been reported (11). All compounds described in this report were screened against third instar southern armyworms (SAW), *Spodoptera eridania* (Cramer), third instar tobacco budworms (TBW), *Heliothis verescens* (F), two spotted mites (TSM), *Tetranychus urticae*, and western potato leaf hoppers (WPLH), *Empoasia abrupta* (De Long), using a standard leaf dip feeding bioassay.

Variation of the electron-withdrawing group (EWG). It was found that, for good insecticidal activity, one substituent on the pyrrole ring had to be electron withdrawing. However, not all electron withdrawing groups produced active pyrroles. The results for a series of 2-aryl-4,5-dihalopyrroles are shown in Table I.

TABLE I. VARIATION OF THE 3-EWG GROUP
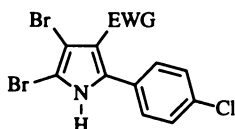
% CONTROL @ 100 PPM

	EWG	TBW	TSM	WPLH
1	CN[6]	100	0	0
2	NO$_2$[6]	100	0	0
3	COCH$_3$	0	0	0
4	CF$_3$S[9]	0	70	0
5	CF$_3$SO$_2$[10]	100	100	100

Substitution at the 3-position of the pyrrole nucleus with cyano or nitro (1 or 2) gave compounds with good activity on TBW. However, when the electron-withdrawing group was trifluoromethylthio (4) or acetyl (3), little or no activity was observed. The compound having trifluoromethylsulfonyl substitution (5) had good activity on all three species. We centered our subsequent work on preparing analogs of the 3-cyano/nitro/trifluoromethylsulfonylpyrroles.

Variation of the 4 and 5 substituents. The results for a series of pyrroles with various substituents at the 4 and 5 positions of the molecule are given in Table II. In the 3-cyano series, when both the 4 and 5 substituents are bromine (1) or chlorine (6), good activity was seen on TBW but, the compounds were inactive on mites or WPLH. Replacement of the halogen at the 5-position with trifluoromethyl resulted in a dramatic increase in the insecticidal potency. The 4-bromo-5-trifluoromethyl analog (7) gave 100% control of all three species at 100 ppm.

The 3-trifluoromethylsulfonyl substitution gave a pyrrole (9) with reduced activity on WPLH.

TABLE II. INSECTICIDAL ACTIVITY OF 4,5-DISUBSTITUTED-2-ARYLPYRROLES
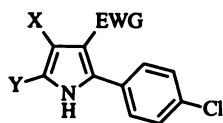
% CONTROL @ 100 PPM

	EWG	X	Y	TBW	TSM	WPLH
1	CN	Br	Br	100	0	0
6	CN	Cl	Cl	100	0	0
7	CN	Br	CF₃	100	100	100
5	CF₃SO₂	Br	Br	100	100	100
8	CF₃SO₂	Cl	Cl	100	100	100
9	CF₃SO₂	Br	CF₃	100	100	50

Variation of Substitution on the Aryl Ring. The discovery of the increase in both potency and spectrum of the 5-trifluoromethyl compound, coupled with the development of a new synthetic procedure that allows easy access to analogs in this series (8), led us to study what effect varying the substitution on the 2-aryl ring had on insecticidal activity. A portion of the results are shown in Table III.

The best activity, in terms of both potency and spectrum, was found when chlorine (7) or trifluoromethyl (13) was introduced into the 4-position of the aromatic ring.

With other halogen atoms (11, 12) good activity was found on TBW and WPLH. The compounds having electron donating groups at the 4-position of the aromatic ring (15, 16) had little or no activity.

The isomeric 2- (19), 3- (18), and 4-chloro (7) compounds were all highly active on TBW and WPLH. Only the 4-chloro isomer (7) was active on mites.
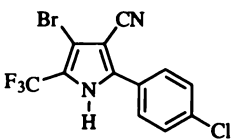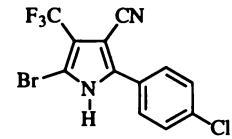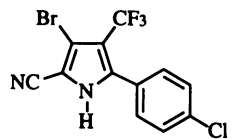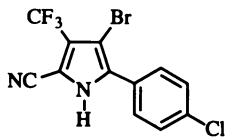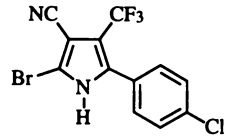
Regioisomers of arylcyanobromotrifluoromethyl pyrrole. The high activity seen for the 2-aryl-5-trifluoromethyl compounds prompted us to investigate what effect the arrangement of the groups on the pyrrole nucleus had on insecticidal activity. We utilized dipolar cycloaddition chemistry developed in our laboratories to prepare these regioisomers (11). The results of the insecticidal testing on two lepidopteran species is shown in Table IV.

TABLE III. INSECTICIDAL ACTIVITY OF 2-ARYLPYRROLES
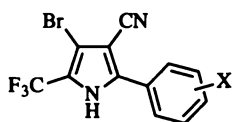
% CONTROL @ 100 PPM

	X	TBW	TSM	WPLH
7	4-Cl	100	100	100
10	H	100	0	0
11	4-F	100	0	90
12	4-Br	100	0	100
13	4-CF$_3$	100	100	100
14	4-CH$_3$	70	0	0
15	4-OCH$_3$	0	60	0
16	4-OH	0	0	0
17	4-NO$_2$	80	90	0
18	3-Cl	100	0	100
19	2-Cl	100	0	100

**TABLE IV. INSECTICIDAL ACTIVITY OF REGIOISOMERIC
2-ARYLPYRROLE CARBONITRILES.
REPRODUCED WITH PERMISSION FROM REFERENCE 5b.**

% Mortality at 10 ppm

COMPOUND	SAW SOUTHERN ARMYWORM *Spodoptera eridania* 3rd instar	TBW TOBACCO BUDWORM *Heliccoverpa virescens* 3rd instar
7	100	100
20	100	0
21	0	0
22	100	0
23	0	0

At 10 ppm, only one regioisomer, the 2-aryl-3-cyano-4-bromo-5-trifluoromethyl
pyrrole (7) gave complete control of both species. Two other regioisomers, the
2-aryl-3-cyano-4-trifluoromethyl-5-bromopyrrole (20) and the 2-aryl-3-bromo-4-
trifluoromethyl-5-cyanopyrrole (22) gave complete control of the SAW but were
inactive on third instar TBW.

Variation of the N-substituent. A major problem associated with the more
active pyrroles was the finding of high levels of phytotoxicity. Efforts to
circumvent this problem centered on the preparation of analogs substituted on

the nitrogen with chemically or metabolically labile groups. This proinsecticide concept has been utilized in the development of other insecticidal compounds (12). Table V summarizes some of our results in this area. For reference, the phytotoxic parent compound (7) controlled all three test species at 100 ppm.

TABLE V. INSECTICIDAL ACTIVITY OF N-SUBSTITUTED PYRROLES
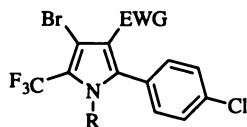
	EWG	R	TBW	TSM	WPLH
			% CONTROL @ 100 PPM
7	CN	H	100	100	100
24	CN	CH$_3$	100	0	100
25	CN	CH$_3$CH$_2$OCH$_2$	100	100	100
9	CF$_3$SO$_2$	H	100	100	50
26	CF$_3$SO$_2$	CH$_3$CH$_2$OCH$_2$	90	40	0

Simple methylation in the 3-cyano series (24) gave a compound that was inactive on mites at 100 ppm. Introduction of an ethoxymethyl group on the nitrogen (25) produced a compound that retained the high insecticidal activity of the parent pyrrole on all three insect species with none of the undesirable phytotoxic properties. Attempts to extend this methodology to the 3-trifluoromethylsulfonyl compounds (9) were less successful. The N-ethoxymethyl analog (26) was less active than the parent on mites and WPLH.

Mode of Action Studies

It was suspected that the insecticidal properties of dioxapyrrolomycin and the simpler 2-aryl pyrroles prepared in this work could be attributed to the compounds acting as uncouplers of oxidative phosphorylation in mitochondria. Disruption of this pathway results in the inability of the mitochondria to convert ADP to ATP. Inhibition of oxidative phosphorylation can be associated with two physicochemical parameters: 1) the inhibitor must be lipophilic enough to cross the mitochondrial membrane and 2) the molecule must function as both a Bronsted acid and base to disrupt the proton gradient involved in the ADP to ATP conversion. The parent pyrroles satisfy these requirements and the biological activity of the N-derivatized pyrroles support their suggested role as prodrugs.

Additional work with the N-derivatized analog, CL 303,630 (25), and it's parent pyrrole (7), as shown in Figure 3, has strengthened this hypothesis. CL 303,630 is lipophilic, but it does not possess the acidic hydrogen necessary for uncoupling activity. However, the high *in vivo* activity seen for this compound

The "Prodrug" Phenomenon - Contrasts and Comparisons

MFO
N-dealkylation

AC 303,630

- Lipophilic
- Non-acidic
- U_{50}: >1000 nM
- 13.3
- LC_{50}: 5.9 ppm

'630 treated cotton:
% of compound in body
of 3rd instar *Heliothis*
2 days postirt

- Lipophilic
- Weak Acid
- U_{50}: 2.4 nM
- 71.1
- LC_{50}: 3.3 ppm
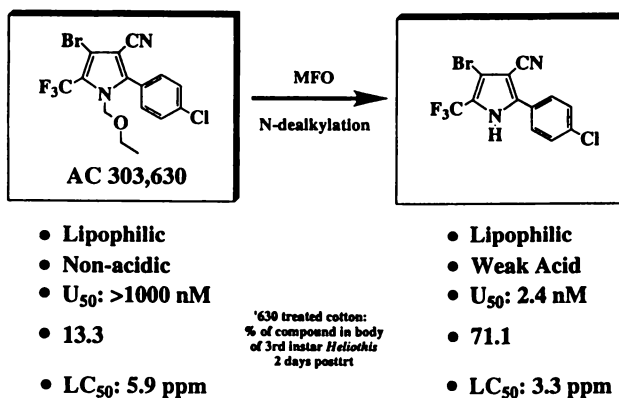
Figure 3. AC 303,630 as a Proinsecticide

COLORADO POTATO BEETLE - PBO EFFECTS
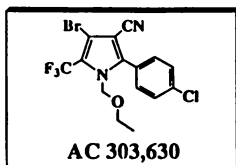
AC 303,630

Dosage	+	PBO	Level of Control (%)
10 ppm		-	100
10 ppm	+	100 ppm	8
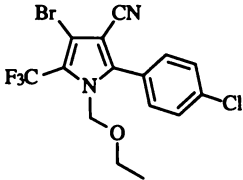
Figure 4. Activity of AC 303,630 on Colorado Potato Beetles

suggests that metabolic removal of the blocking group on nitrogen generates the active species. Standard *in vitro* uncoupling studies using rat liver mitochondria confirm that the N-derivatized material is not an uncoupler (U_{50} > 1000 nM) whereas the parent was highly active (U_{50} = 2.4 nM). In the insect, it was found that CL 303,630 (25) was converted to the parent pyrrole. Further support for the concept of CL 303,630 functioning as a proinsecticide was gained from work on Colorado potato beetles as shown in Figure 4. In this study, CL 303,630 (25) gave complete control at a dose rate of 10 ppm. Addition of piperonyl butoxide, an inhibitor of mixed function oxidases, which partially blocks the metabolic conversion of CL 303,630 (25) to the active N-H pyrrole (7), reduced the level of control to less than 10%.

This mode of action work suggests that the 2-aryl pyrroles should have activity on insects resistant to other agents. Recent work from our laboratories

confirms this. Table VI shows the results of a study utilizing pyrethroid-resistant *Plutella xylostella*. At a dose rate of 3.1 ppm, the pyrethroid fenvalerate gave 100% control of the susceptible strain of insects, as did CL 303,630 (**25**). However, at the same dosage, while CL 303,630 (**25**) continued to give 100% mortality of the resistant species, the pyrethroid failed to give any control.

Table VI. TOXICITY OF CL 303,630 TO PYRETHROID-RESISTANT *Plutella xylostella,* **THIRD-INSTARS**

	% CONTROL @ 3.1 PPM	
COMPOUND	SUSCEPTIBLE STRAIN	RESISTANT STRAIN

CL 303,630 (25)

	100	100
Fenvalerate*	100	0

* Fenvalerate @ 50 ppm did not cause mortality in R-strain

The activity of CL 303,630 (**25**) against susceptible and OP and carbamate resistant strains of mites is shown in Table VII.

TABLE VII. TOXICITY OF CL 303,630 TO SUSCEPTIBLE AND RESISTANTSTRAINS OF *Tetranychus urticae* **(LC$_{95}$ PPM)**
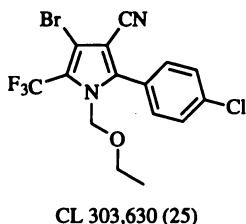
CL 303,630 (25)

COMPOUND	S-STRAIN	OP-RESISTANT	CARBAMATE RESISTANT
CL 303,630 (25)	11.3	13.0	7.0
Phosalone	56.6	>40,000	
Formetanate	13.2		>5,000

The standards in this study were phosalone, an organophosphate and formetantate, a carbamate. All three compounds were toxic to the susceptible strain of mites. CL 303,630 (25) retained activity against both strains of resistant mites while phosalone and formetanate showed no activity.

Table VIII.   STRUCTURE - ACTIVITY TRENDS
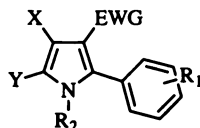
I.   X=Y=Br or Cl are approximately equal in activity. Best activity seen for X=Br or Cl and Y=CF₃.

II. Approximately the same activity seen for EWG=CN, NO₂, or CF₃SO₂.

III. R₁ requires some electron-withdrawal. The best groups are Cl, Br, or CF₃.

IV. Best activity seen when R₁ is in the 4-position.

V.   N-Derivatized pyrroles (R₂≠H) act as pro-insecticides with the highest activity seen for R₂=alkoxyalkyl.

VI. The parent pyrroles (R₂=H) are uncouplers of oxidative phosphorylation. They show correlations between pKa, log P, and insecticidal activity.

Conclusions

The 2-aryl pyrroles, a new class of insecticides that exert their activities through uncoupling of oxidative phosphorylation, have been investigated in our laboratories. Through an extensive synthesis program coupled with biological screening, we have developed the structure-activity correlations described in Table VIII.

These trends, and more extensive physicochemical studies correlating pKa and log P with insecticidal activities, are currently being used to design more potent, insect specific molecules (13). Currently, one compound CL 303,630 is undergoing full scale development.

Acknowledgements

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Chapter 16

Structure–Activity Study and Conformational Analysis of RH–5992, the First Commercialized Nonsteroidal Ecdysone Agonist

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In the early 1980's, the first compound from a class of 1,2-diacyl-1-substituted hydrazines was synthesized and discovered to have insecticidal activity. RH-5849, which was synthesized subsequently in the analog synthesis program, demonstrated lethal and unusual effects on the development of Lepidopteran insects. A thorough mode of action study further demonstrated that RH-5849 was the first non-steroidal ecdysone agonist. These early results triggered a tremendous amount of laboratory and field research around the world, in order to understand the full impact of this new class of environmentally friendly insecticides. This paper will discuss: (a) the structure activity study that led to the discovery and eventually the commercialization of RH-5992, bearing the trade name: CONFIRM® (in USA) and MIMIC™ (outside USA), (b) summary of the mode of action of RH-5992 as an ecdysone agonist, and (c) conformational analysis of RH-5992.
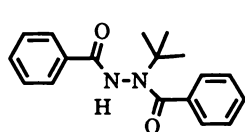
RH-5849   RH-5992
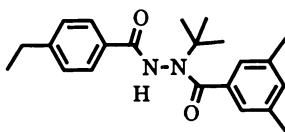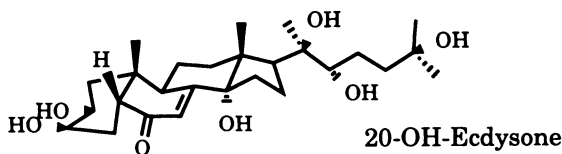
20-OH-Ecdysone

Soon after the first patent (1) of 1,2-diacyl-1-substituted hydrazines as insecticides was published, Rohm and Haas disclosed RH-5849 as the first non-steroidal agonist (mimic) of the insect molting hormone, 20-hydroxyecdysone (20E) (2, 3). The structure and initial field test results of RH-5849 (1-t-butyl-1,2-dibenzoylhydrazine) and the discovery synthesis of the first compound in this series were also reported in a pesticide conference (4) and an ACS agrochemical meeting, respectively (5). Several years later, RH-5992, 1-t-butyl-1-(3,5-dimethylbenzoyl-2-(4-ethylbenzoyl) hydrazine, was announced (6) to be the first commercially viable insecticide in this chemistry. It not only mimics the insect molting hormone, but also very selectively and effectively controls Lepidopteran insect larvae. Because the discovery of this new type of compound represents a breakthrough in chemical insect control, RH-5849 and RH-5992 have since been cited in more than one hundred scientific publications to date.

The first commercial introduction of RH-5992 as MIMIC[TM] Insecticide occurred in 1993 in Japan and Europe. In August 1994, the US EPA granted an emergency use permit for RH-5992 (CONFIRM® Insecticide) to be sold for controlling beet armyworm (*Spodoptera exigua*) on 750,000 acres of cotton in Mississippi. This year (1995), more permits have been granted for RH-5992 for emergency use in several states in the US for the protection of cotton. In addition to use on cotton, RH-5992 is being developed for control of various lepidopteran larval pests on crops as diverse as apples, sugarcane, citrus, rice, vegetables, sugar beets, and forests.

In May 1995, CONFIRM® insecticide was first registered for the protection of walnuts in the US. Numerous tests have demonstrated that RH-5992 is safe to predatory mites, wasps, spiders, lacewings and beetles which naturally control other insect pests. In other words, RH-5992 can reduce the need and the amount of total insecticide applications for crop protection and therefore qualifies as a very effective tool in an integrated pest management (IPM) program. Because of the above properties and its safe mammalian toxicological data, RH- 5992 is also the first pesticide to become part of the fast track review by the US EPA under its Reduced Risk Pesticide Program.

Synthesis

A general synthetic method for the synthesis of 1,2-diacyl-1-substituted hydrazines had been reported previously (5). We will focus on the synthesis of 1,2-dibenzoyl-1-t-butyl hydrazines which are a subclass of analogs for the SAR study reported in this paper.

In the discovery synthesis, **1** was the first target molecule as an intermediate for the synthesis of another chemistry which might manifest some unexpected biological activity (5). For example, when one equivalent of 4-chlorobenzoyl chloride was reacted with one equivalent of t-butylhydrazine hydrochloride as shown in Figure 1, a very small amount of "undesired" 1,2-di-(4-chlorobenzoyl)-1-t-butylhydrazine **2** (A = 4-Cl) could not be totally avoided.

Ironically, this "undesired" reaction by-product turned out to be a key compound in the discovery of this new class of insecticides. Without the chemist's diligent effort
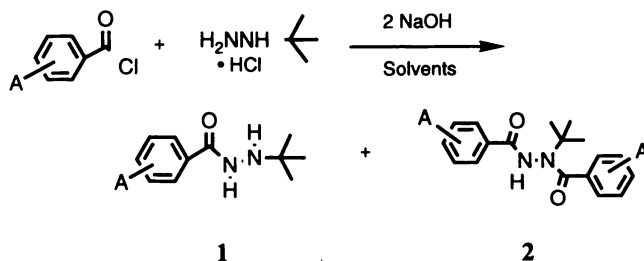
Figure 1: Discovery Synthesis

in the isolation and identification of this minor byproduct from the reaction mixture and the biologists' careful testing and keen observation from the test results, an important discovery of this class of new insecticides might have been many years away. Furthermore, its reaction sequence has been a key step for the synthesis of the majority of analogs represented by **3**, where A and B are different substituents. Without this specificity, compounds for the structure-activity relationship study for the optimization from the first lead compound could not be prepared. By treating another equivalent of different benzoyl chloride with **1**, unsymmetrically substituted analogs **3** can be prepared as depicted in Figure 2:
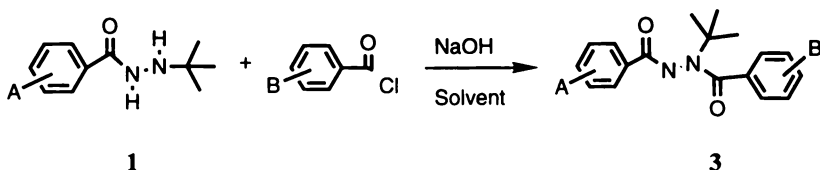
Figure 2: Synthesis of Unsymmetrical Dibenzoyl Hydrazines

Symmetrically substituted analogs **1** can be prepared in one step by simply treating two equivalents of acyl chloride with one equivalent of t-butylhydrazine under basic reaction conditions.

Empirical Structure-Activity Relationship led to RH-5992

It was found that **4**, the first lead compound, selectively controlled southern armyworm (*Spodoptera eridania*) at a spray concentration of 600 ppm. At this high concentration, no activity had been observed against other tested species including Mexican bean beetle (*Epilachna varivestis*), boll weevil (*Anthonomus grandis*), green peach aphid (*Myzus persicae*), and two-spotted spider mite (*Tetranychus urticae*). A dose response study of **4** indicated a lead level activity with an LC_{50} (lethal concentration for 50% of the population) of 19 ppm against the same species. This result also implied that the lead compound selectively controlled lepidopteran species represented by southern armyworm.

4   **5**
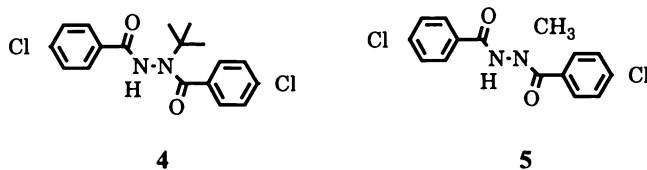
It was immediately realized that t-butyl group in structure **4** played an important role in the insecticidal activity, because the corresponding methyl analog **5** had shown no activity against any organisms tested at 600 ppm. Several other symmetrical analogs with different substitutions on phenyl rings, including RH-5849 which has no substitution on the phenyl rings, were prepared. RH-5849 was found to be more active than the original lead with LC_{50} of 12 ppm . Furthermore, it was also found that this compound had profound effect on insect molting. At this time, a more rigorous study of the mode of action was begun and an extensive analog synthesis program was implemented for the optimization of this molecule. It should be noted here that the discussion of SAR in this paper has been based on the activity of analogs against southern armyworm, which is considered as a reliable test organism for lepidopteran activity. The tests are typically conducted with at least three concentrations, from which an approximate LC_{50} value can be extrapolated. The LC_{50} value is calculated at 96 hours after application (SAW96) and expressed in ppm of active ingredient in a given spray solution.

For convenience, 1,2-diacyl-1-substituted-hydrazines may be dissected into four regions, namely A, B, R, C regions and the backbone dicarbonyl hydrazine bridge, as shown in Figure 3.
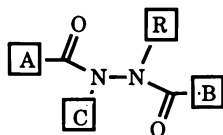
Figure 3: General Structure of 1,2-Diacyl-1-substituted-hydrazines

Like for most of the commercial pesticides available on the market, hundreds of analogs have been synthesized and evaluated. Careful analysis of data indicated that the most active compounds are generally composed of the following groups.

R Region: a bulky alkyl group; particularly a t-butyl group.

A and B Regions: phenyl or heterocyclic rings. Usually, the most active analogs are those which have different substituents on two phenyl rings. Analogs derived from symmetrically substituted phenyl rings on both A and B (except RH-5849) are generally less active than unsymmetrically substituted compounds.

C Region: Hydrogen is necessary for the activity. When it was substituted with an unhydrolyzable group, the compound tends not to be active.

The backbone dicarbonyl hydrazine bridge is essential to the activity. A partial or total replacement of this backbone with other moieties, for example, a sulfoxide or

sulfone instead of carbonyl, a thiocarbonyl instead of carbonyl, or one of the nitrogen atoms replaced with another atom such as carbon, results in either drastically reducing the activity or total loss of activity.

In this paper the SAR will be focused on discussing analogs in which the R is a t-butyl, A and B are phenyl rings; especially unsymmetrically substituted phenyl rings, and C is a hydrogen as shown by the structure **3**. Even under this simplified condition, the SAR study is still quite complex, because there are almost an unlimited number of compounds which can be made by just modifying the substituents in both phenyl rings. However, based on the following stepwise analysis, we have been able to identify RH-5992 as one of the best candidates in terms of its insecticidal activity against lepidopteran species. In addition, the simple two step reaction procedure as shown in Figure 1 and Figure 2 also helped us, in the early stage of this analog synthesis program, to quickly synthesize a large number of analogs in a reasonably short time for empirical SAR study. It is noted that in each case we have used the activity of RH-5849 (SAW96 = 12 ppm) as a base point for the comparison. In structure **3**, the two phenyl rings which are substituted by A and B will be called A-ring and B-ring, respectively.

Step one: When A is hydrogen (i.e.H on the A-ring) in structure **3**, Table I shows the effect of monosubstitution on B-ring, compared with RH-5849 against southern armyworm:
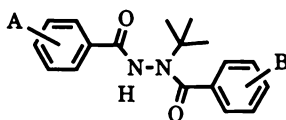
3

Table I: Effect of Monosubstitution On B-ring

| | B Substituent | |
| | Compounds More | Compounds Less |
Position on B-Ring	Active Than RH-5849	Active Than RH-5849
ortho	Et, I, Br, NO_2, Cl	OMe, Me, NH_2
meta	Br, Et, Me, Cl	CF_3, OMe, NO_2, NH_2
para	Cl, F	NO_2, Me, OMe

From Table I, it seems that a small to medium sized hydrophobic group in the meta position is favorable for the southern armyworm activity, while polar groups are not. It also shows that a large electron withdrawing group such as halogen (I, Br, or Cl) and NO_2 on the ortho position is favorable for the activity. Since Et group is better than Me group and both of them are electron donating groups, it could mean that the effect of size is a more dominant factor than the electronic effect on the ortho position. The effect of the substituent on para position, on the other hand, favors a small electron withdrawing group (Cl or F), but not a electron donating group (Me or OMe) or a larger electron withdrawing group such as NO_2. It is noted that recently, a similar discussion based on a QSAR study of a similar set of compounds tested against another lepidopteran species, rice stem borer (*Chilo suppressalis*) was reported (7, 8).

Step two: When B is H (no substituent on B-ring) in structure **3**, Table II shows the effect of mono-substitution in the A-ring against southern armyworm, compared with RH-5849 (A = H) :

<table>
<tr><th colspan="3">Table II: Effect of Monosubstitution On A-ring</th></tr>
<tr><th></th><th colspan="2">A Substituent</th></tr>
<tr><th>Position on A-Ring</th><th>Compounds More
Active Than RH-5849</th><th>Compounds Less
Active Than RH-5849</th></tr>
<tr><td>ortho</td><td>Cl, Me</td><td>Br, NO$_2$, CF$_3$, NH$_2$,
OMe</td></tr>
<tr><td>meta</td><td>Cl, OMe</td><td>Me, Et, NO$_2$</td></tr>
<tr><td>para</td><td>Et, Br, I, Me, CF$_3$, OMe</td><td>Cl, i-Pr, t-Bu</td></tr>
</table>

From Table II, it appears that a small hydrophobic group is favorable in both of the ortho and meta positions, while at para position a bigger hydrophobic group is well tolerated. Even a para-t-butyl group still exerts some activity, although it is much less active than RH-5849. And, the activity of compound with B = 4-chloro is very close to that of RH-5849 against southern armyworm. Table II also indicates that the electronic effect could not compete with the effect of the hydrophobicity of the substituents, because both sets of compounds, either more active than or less active than RH-5849, contain electron donating and electron withdrawing groups.

Examination of both Table I and Table II together reveals that the SAR on the A-ring is different from the SAR on the B-ring. In other words, a good substituent on the A-ring relative to RH-5849 may not have the same SAR trend as demonstrated by the same group on B-ring.   Furthermore, it was found that a compound designed by combining a good substituent on the A-ring (e.g. 2-Cl) and a good substituent on the B-ring (e.g. 2-Br) did not necessarily demonstrate an activity better than that of RH-5849. As a matter of fact, the compound with 2-Cl on A and 2-Br on B is about 3-fold less active than RH-5849 against southern armyworm. Therefore, a more systematic and complete set of compounds had to be synthesized and the results of the biological activities analyzed.

6   **7**

Figure 4: Lepidopteran Activity Optimization

Step 3: When compound **6** (where A = 4-Me and B = H in Table II) is chosen as a lead for the lepidopteran activity optimization (Figure 4), the resulting SAR pattern may be illustrated by listing a set of analogs ranging from less active than **6** to more active than **6**, as shown below in Table III:

Table III: Effect of Mono- and Di-substitutions on B-Ring of Structure 10 Against Southern Armyworm

Less Active Than 6	Equal or Slightly Better Than 6	More Active Than 6	4-fold or More Active Than 6
2-Me	4-Cl	4-F	3-Me
3-Et	4-Me	3,4-Cl$_2$	3,5-Cl$_2$
4-CF$_3$	2-Cl		3,5-Me$_2$
	3-Cl		
	4-Et		

The results shown in Table III indicate that, although the SAR demonstrated in Table I and II by monosubstitution does not hold for the disubstitution, a synergistic effect is demonstrated by certain substituents, such as those three compounds which are 4-fold more active than the lead compound **6**. Furthermore, these three compounds still retain their high level of activity seven days after treatment of southern armyworm (seeTable IV).

Table IV: *In vivo* and *In vitro* Insecticidal Activity

A	B	SAW96 LC$_{50}$(ppm)	SAW0DR* LC$_{50}$(ppm)	SAW7DR** LC$_{50}$(ppm)	*Plodia* IC$_{50}$(nM)[†]
H	H	12	12	27	
4-Me	H	10	14	19	-
4-Me	3-Me	<2.5	2.7	5	40
4-Me	3,5-Cl$_2$	<2.5	3.3	7	-
4-Me	3,5-Me$_2$	<2.5	2.5	4.6	11
4-Et	3,5-Me$_2$	<2.5	0.9	3.3	1.0-2.4

* Zero day residual activity against southern armyworm (*S. eridania*).
** Seven day residual activity against southern armyworm.
[†] IC$_{50}$ is concentration of test compound which inhibits 50% of binding of radio-labelled ligand to ecdysteroid receptor extracted from *Plodia interpunctella* cells.

In the above Table IV it also shows that, when we further optimized **8** (i.e. B is 3,5-Me in **7**) by replacing 4-methyl group on the A-ring with a bigger hydrophobic group such as an ethyl group (Figure 5), the resultant analog, RH-5992, was much more active than RH-5849 both in its toxicity to southern armyworm larvae and at the receptor level..
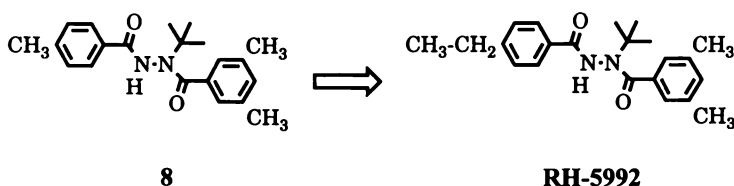
8   **RH-5992**

Figure 5: Lepidopteran Activity Optimization Leading to RH-5992

RH-5992 has 0-day residual activity less than 1 ppm and 7-day residual activity of about 3 ppm (see Table IV). This has confirmed the previous hypothesis based on results in Table II that the para position of the A-ring can tolerate a bigger hydrophobic group. In addition to the whole insect southern armyworm activity, specific receptor binding data has been included for the purpose of comparison.

In summary, the SAR study of RH-5992 against southern armyworm discussed above serves only one of many possibilities to optimize activity and potency to get potential candidates for new insecticide development. In other words, if one chooses another test organism (e.g. bean beetle) for SAR analysis of analogs synthesized in this synthesis program, the outcome of activity optimization would probably be very different from what we are discussing in this paper. For example, a subclass of analogs in this series, including RH-5849, not only has lepidopteran larvicidal activity, but also has coleopteran larvicidal activity (4). The SAR study of a series of compounds based on their coleopteran activity has resulted in the identification of a potential candidate for commercialization to protect crops mainly infested by beetle pests. The SAR analysis of this class of compounds may be the subject of future report.

Mode of Action

It has been known that in almost all insect species, 20E plays a crucial role in regulating molting from one developmental stage to another. It is also critical in regulation of specific gene expression in various tissues during the inter-molt period (9). During the molting period, in synchrony with rising 20E titers, the larvae stop feeding and synthesis of a new cuticle is initiated. As the 20E titers decrease, eclosion hormone is released. Eclosion hormone triggers a behavioral response in the molting larva which allows it to escape from its old cuticle into a new one (10). Hence, the success of a molt depends upon the rise and decline of 20E titers and release of other hormones in a regulated manner. At the cellular level the action of 20E is manifested via interaction with the ecdysteroid receptor (EcR). However, the interaction of 20E with EcR takes place only after EcR heterodimerizes with another protein, ultraspiracle (11). Both EcR and USP are products of members of the steroid hormone receptor superfamily of genes (12, 13, 14). A liganded EcR-USP complex upon binding to ecdysone response elements of a 20E responsive gene results in the expression of that gene. By use of *in vitro* synthesized EcR and USP from cloned DNA sequences coding for these proteins, it has been demonstrated that the ecdysteroids bind to EcR in the EcR-USP complex (11). No ligand for USP has been found to date. In order for the non-steroidal diacylhyadrazine analogs like RH-5849 and RH-5992 to qualify as true ecdysone agonists or mimetics, not only should their effects mimic those produced by 20E at the whole insect, tissue and cellular level, but also at the molecular level. Also, the ecdysone agonists should compete with 20E and other ecdysteroids for binding to EcR in the EcR-USP heterodimer complexes.

Over the last 7 years results of numerous investigations, including two review articles (15, 16), have provided compelling evidence that RH-5849 and RH-5992 act via the same mode of action as 20E, not only at the whole insect, tissue and cellular level, but also at the molecular level. They induce lethal effects due to their high

binding affinity for the EcRs, superior metabolic stability than ecdysteroids and long half life in the target insects. Hence, susceptible larvae intoxicated with RH-5849 and RH-5992 enter a state of "hyperecdysonism" (17) resulting in a lethal molt.

The first demonstration that RH-5849 competes with ^{3}H-Ponasterone A for binding to EcR in cytosolic extracts of Kc cells or nuclear extracts of cells derived from imaginal wing discs of *Plodia interpunctella* was demonstrated by Wing (3) and Wing and Aller (18). Since then several other investigators have compared the relative binding affinities of RH-5849, RH-5992, 20E, and Ponasterone A to EcRs in various tissues or tissue extracts from various insect species. In Table V the relative equilibrium binding constants (Kd) of Ponasterone A, 20E, RH-5849 and RH-5992 to EcR extracts from dipteran (*Drosophila)* and lepidopteran (*P. interpunctella)* cell lines have been compared (19).

Table V: Equilibrium Constants for binding of various ligands to ecdysteroid receptor extracts from a dipteran and a lepidopteran cell lines

	Kd (nM)			
Insect Source	Pon. A	20E	RH-5849	RH-5992
*Drosophila**	0.7	60	2000	192
*P. Interpunctella***	2.0	210	230	2.0

*, *D. mellanogaster* Kc cells; **, *P. interpunctella* imaginal wing disc cell line.

In Table V, the Kd values for Ponasterone A were determined from saturation isotherm experiments. The Kd values for the other ligands were calculated from IC_{50} values for displacing ^{3}H-Ponasterone A in competitive ligand binding assays using the Cheng-Prusoff equation (20).

Several interesting conclusions can be drawn from results in Table V. Comparison of the relative binding affinities of the two ecdysteroids to the dipteran and lepidopteran EcR in cell extracts confirm the earlier results that Ponasterone A binds with much greater affinity to EcR than 20E. However, the binding affinities of each of the two ecdysteroids to the two EcRs are very similar. On the other hand, both the non-steroidal ecdysone agonists bind with significantly greater affinity to the lepidopteran EcR extract than to the dipteran EcR extract. The binding affinity of RH-5992 to the two EcR extracts is not only about 10-100 fold better than RH-5849, but it also binds to the lepidopteran EcR extract with similar affinity as Ponasterone A does. The high binding affinity of RH-5992 to the lepidopteran EcR extract correlates very well with its specific toxicity to the lepidopteran pests. Similar results have been obtained for binding of the ecdysteroids, RH-5849 and RH-5992 to EcR in extracts from *C. tentans* cells (21), integument tissue extracts from *G. mellonella* larvae (22) and intact imaginal discs of *S. exigua* and *L. decemlineata* (16). In addition, it has also been demonstrated by competitive ligand binding experiments that RH-5992 does interact with EcR as does 20E using *Drosophila* and mosquito (*Aedes aegypti*) EcR and USP proteins produced by *in vitro* transcription and translation of cDNA sequences (23). In these experiments specific binding of ^{3}H-Ponasterone A was obtained only when *Drosophila* EcR or *Aedes* EcR proteins were present in the binding reactions along with USP protein from either one of the two insects. These results confirm the model for 20E action proposed by Yao, et.al. (11).

Moreover, ^{3}H-Ponasterone A bound to the EcR/USP heterodimers could be competed with increasing concentrations of RH-5992. RH-5992 bound to the mosquito EcR with about ten fold greater affinity than to the *Drosophila* EcR.

It is now very evident that both RH-5992 and RH-5849 act as true agonists of 20E in inducing the expression of 20E inducible genes. However, unlike 20E, due to the greater metabolic stability and high binding affinity of RH-5992 to EcR of susceptible insects, release of eclosion hormone (and presumably bursicon as well) and expression of genes which normally occur following the decline of 20E do not take place (24). Therefore, the sum total effect of RH-5992 in susceptible insects is a premature lethal molt.

Conformational Analysis of RH-5992

In contrast to the empirical SAR approach, mode of action studies quite clearly demonstrate that RH-5992 interacts at the same receptor as 20E. This has allowed us to apply a second strategy for optimizing the chemical structure toward the desired biological result. If indeed both compounds interact with the same receptor at the same site, an understanding of how the binding mode of these two compounds superimpose onto each other could lead to an effective model for designing novel active analogs. Though the actual conformations at the receptor site is unknown, it is usually assumed that for any molecule to be recognized by the same receptor, there must be some commonality in the 3 dimensional structure of each molecule in solution. (25, 26) While, on first inspection, RH-5992 and 20E have no discernible common features, two such superpositions have been proposed recently (27, 28).

Our process was begun by first understanding the allowed conformations of RH-5992. We have previously obtained the X-ray structure of RH-5849 (29) which was confirmed by the work of T.H. Chan et.al.(30). The X-ray structure of RH-5992 is very similar to that of RH-5849, showing that the aryl ring attachments do not appear to influence the intrinsic structure to any great extent.. The key conformational feature is the nearly orthogonal twist to the OC-N-N-CO bond, the central hydrazine bond. The X-ray structures for RH-5849 and RH-5992 are shown in Figure 6.

While the X-ray structure is generally assumed to be a low energy conformation for a molecule, crystal packing forces may lead to conformations in a crystal which may not be the lowest energy conformer, and solvation effects in solution may also rearrange the energies of conformers. To validate the X-ray structure as the lowest energy conformer, conformational analysis was performed on RH-5992. The analysis was done using the MM2 force field in MACROMODEL 4.5 (31) Built into MACROMODEL is the ability to simulate solvent using a continuum model (32). Since solution conformations were desired, this GB/SA option with water as the solvent was also used. Estimates for the C2-N2-N2 C2 and C2-N2-N2-C3 torsional constants and the N2-N2 bond length and force constant where made in the MM2 force field so that calculations were possible. Torsional force constants were added which created a 2 fold symmetric torsion with a barrier height of 20Kj ($\sim$ 5Kcal).

RH-5849   RH-5992
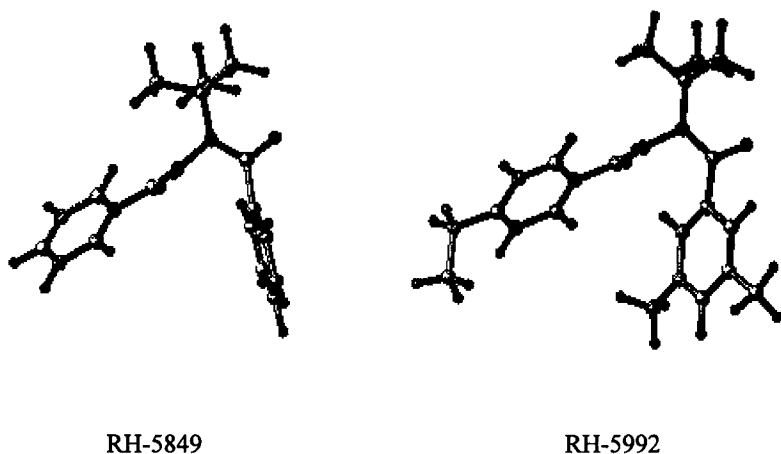
Figure 6: X-ray Structures of Ecdysone Agonists

The X-ray structure was minimized and served as the starting conformer for a Monte Carlo conformational search (33). Only the central backbone bonds of the hydrazide were allowed to rotate; the ethyl group was assumed to achieve a reasonable orientation upon minimization. Each cycle of the search consisted of a random series of torsional changes followed by complete minimization of the resulting structure. One thousand cycles were performed and all conformers within 50 Kj of the perceived lowest energy conformer were retained. This resulted in a list of 100 conformers. Using the Xcluster utility of MACROMODEL (34) the conformers were clustered based on the backbone torsional angles. The conformers appeared to group themselves into 5 domains (Figure 7). These domains can be described as appearing, folded, T-shaped, extended, and hooked shaped. The conformational domains can be described based on the geometry of the two amide bonds, where the first descriptor is for the amide bond of the p ethyl benzamide, and the second descriptor is for the 3,5 dimethyl benzamide side of the diacylhydrazine. Using this nomenclature, the folded conformer 1 is Z, E. The X-ray structure 2 is then an E,E conformer, the extended conformer 3 is E,Z and the two hooked conformers are Z,E 4, and Z,Z 5 with a twist. Based on the MACROMODEL derived energies, the X-ray structure, conformation 2, and the completely folded conformer, conformation 1 are equivalent in energy. The extended and hooked conformations, conformation 3, 4 and 5 are calculated to be 26, 32, and 36 Kj higher in energy (see Table VI). It is also interesting to note that the greatest solvation stabilization is achieved in the X-ray structure domain, conformer 2, while the least is gained in the fully extended conformer 3.

Since conformational interconversions between the first 4 domains involve the rotation of an amide bond which has a two fold barrier to rotation of ~65 Kj (35) or more, equilibration between conformational domains should be somewhat restricted.

(5)

(2)

(1)

(4)

(3)
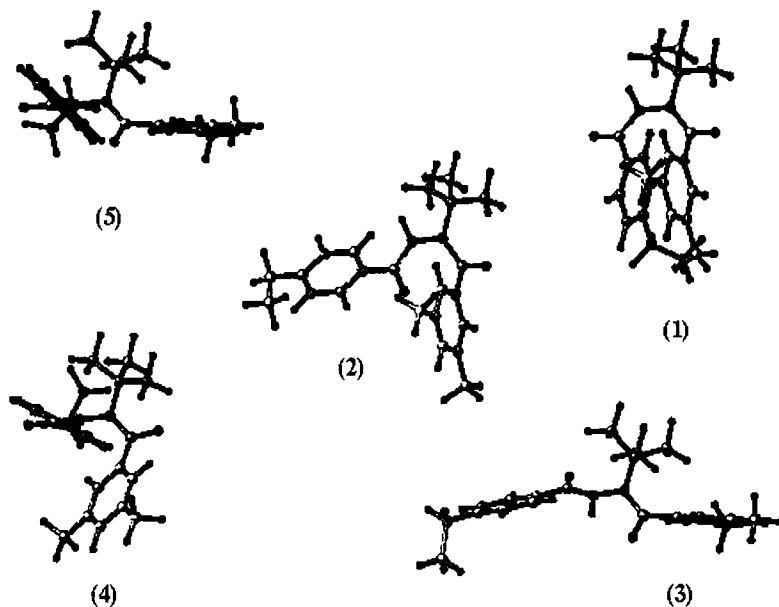
Figure 7: Conformational Domains of RH-5992

Table VI: Energies of Conformers
Representing RH-5992 Conformational Domains

Conf	Total Energy	Solvation Energy
1	142.68	-16.40
2.	142.95	-23.56
3	168.71	- 7.20
4	174.09	-14.32
5	178.83	-13.83

These results validate the use of the X-ray structure as the receptor recognition conformer. However, they also indicate that at least one other conformation needs to be considered in developing binding models, the Z,E conformer. The extended conformations of RH-5992, the E,Z and Z,E conformers which more closely match the molecular dimensions of 20E appear, however, to be higher in energy and thus should not contribute significantly to the conformer population for this structure. Development of the superposition models will be reported at a later date.

Conclusion

We have demonstrated that SAR studies are effective for lead optimization to quickly arrive at the best candidate for product development. Understanding the mode of action of this class of compounds and using receptor-ligand based *in vitro* assays

further allowed us to obtain relative binding affinities of both natural and synthetic agonists. This provided very useful information for synthetic chemists to optimize the SAR of the toxophore. Furthermore, information generated by the conformational analysis of RH-5992 and the molecular mapping of both the steroidal and non-steroidal agonists may give clues to scientists for designing novel structures for new synthesis programs. Finally, with the availability of "molecular probes" like RH-5992 and 20E, it would be possible to analyze salient features of various insect ecdysteroid receptors. This infomation could be potentially useful for the discovery of new ecdysone agonists selective for different group of insects.

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Chapter 17

Structure–Activity Relationships
of the Avermectins and Milbemycins

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In 1976 scientists at Merck & Co. Inc. discovered a complex of eight
closely related natural products, subsequently named avermectins, in a
culture of *Streptomyces avermitilis* MA-4680 (NRRL8165)
originating from an isolate by the Kitasato Institute from a soil sample
collected at Kawana, Ito City, Shizuoka Prefecture, Japan. They are
among the most potent anthelmintic, insecticidal and acaricidal
compounds known. The avermectins are structurally related to another
group of natural products, the milbemycins, the first examples of
which were described by Japanese workers. Avermectin B_1, under
the non-proprietary name abamectin, is widely used as an agricultural
miticide and its 22,23-dihydro derivative, ivermectin is used world
wide as a broad spectrum parasiticide in animals and in man. More
recently, a new avermectin, doramectin, has been prepared by directed
biosynthesis and developed for similar use in animals. Three
milbemycins are used for animal health, milbemycin D, milbemycin
oxime and moxidectin. This presentation describes some of the
structure-activity relationships between the avermectins and
milbemycins and an intensive synthetic program on 4"-deoxy-4"-
epiamino avermectins culminating in two novel compounds in
development, emamectin benzoate as an agricultural insecticide and
eprinomectin as a broad spectrum endectocide for use in animals.
Mode of action studies have revealed a glutamate-gated chloride
channel from *C. elegans* proposed to be the target site for both
avermectins and milbemycins.

In 1976 scientists at Merck & Co. Inc. discovered a complex of eight closely
related natural products, subsequently named avermectins, in a culture of
Streptomyces avermitilis MA-4680 (NRRL8165) originating from an isolate by
the Kitasato Institute from a soil sample collected at Kawana, Ito City, Shizuoka
Prefecture, Japan. The structures of the avermectin natural products and semi-
synthetic derivatives are shown in Figure 1. (*1*).
    The avermectins are closely related to another group of pesticidal natural
products, the milbemycins, the first examples of which were described by
Japanese workers, but later were found to be more abundant in nature than the
avermectins *(2-7)*. Milbemycin structures are shown in Figure 2.

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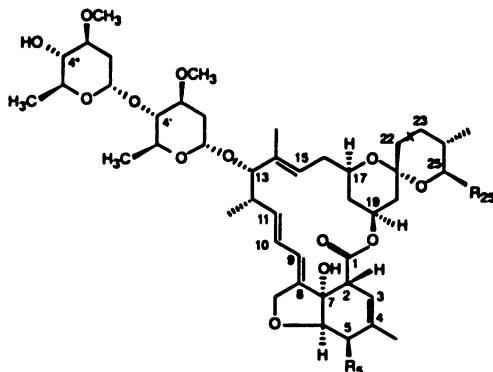
Avermectin A: R_5 = OCH$_3$ B: R_5 = OH

1: X = –CH=CH–   2: X = –CH$_2$–CH–

a: R_{25} =   b: R_{25} =

Ivermectin: R_5 = OH   X = –CH$_2$–CH$_2$– R_{25} =   and

Doramectin: R_5 = OH   X = –CH=CH– R_{25} = Cyclohexyl

Figure 1. Avermectin Structures
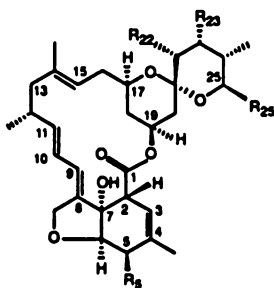
Milbemycin Alfa Series
R_5 = OH, OCH$_3$
R_{22} = H, OH
R_{23} = H, OCOCH(CH$_3$)(CH$_2$)$_3$CH$_3$
R_{25} = CH$_3$, C$_2$H$_5$

Anthelmintic F-28249
Antibiotic S 541
R_5 = OH, OCH$_3$
R_{22} = H R_{23} = OH
R_{25} =

Milbemycin Alfa$_1$ (A$_3$):	R_5 = OH	R_{22} = R_{23} = H	R_{25} = CH$_3$
Milbemycin Alfa$_3$ (A$_4$):	R_5 = OH	R_{22} = R_{23} = H	R_{25} = CH$_2$CH$_3$
Milbemycin D:	R_5 = OH	R_{22} = R_{23} = H	R_{25} = CH(CH$_3$)$_2$
Anthelmintic F-28249-Alpha:	R_5 = OH	R_{22} = R_{23} = H	R_{25} = C(CH$_3$)CHCH(CH$_3$)$_2$
Moxidectin:	R_5 = –OH	R_{23} = =NOCH$_3$	R_{25} =
Milbemycin Oxime:	R_5 = =NOH	R_{25} = CH$_3$ and C$_2$H$_5$	
Milbemycin D:	R_5 = –OH	R_{25} = –CH(CH$_3$)$_2$	

Figure 2. Milbemycin Structures

Avermectins and milbemycins are among the most potent anthelmintic insecticidal and acaricidal compounds known.

Avermectins and Milbemycins in Animal Health and Man

Structure-activity relationships of these compounds are extremely complicated because they are active against such a broad spectrum of helminths and arthropods. Table I, for example shows the parasites of cattle for which ivermectin has efficacy claims. In the interest of accuracy, our laboratory prefers to present activity data as the 95% effective dosage. As can be seen in Table II the ED_{95} values for ivermectin against nine helminth parasites of cattle are quite variable. A similar study in horses (Table III) gave equally variable results but more importantly gave the first indication that ivermectin had activity against filarial parasites of the genus Onchocerca. Table IV shows a titration of ivermectin against the dog heart worm *Dirofilaria immitis* where ivermectin shows 97.2% efficacy at 2 µg/kg (8). In comparing three milbemycins with ivermectin there is a wide range of efficacious dose against *D. immitis* in dogs even though they are much more equivalent against other helminths.

The activity against *Onchocerca cervicalis* in the horse and *D. immitis* in dogs prompted a trial against *Onchocerca volvulus*, the causative agent of river blindness in man. The data show that a single dose of approximately 200 µg/kg is effective for one year (Table V)(9). Table VI shows the activity of ivermectin against another human filarial parasite *Wuchereria bancrofti*, the causative agent of lymphatic filariasis. In this case the effective duration of ivermectin is approximately one month (10).

In the area of crop protection, avermectin B1 (abamectin) was selected for commercial development. Table VII indicates the breadth of its spectrum of activity against mites and insects. Table VIII presents the data obtained from an examination of the activities of avermectin disaccharides, monosaccharides, aglycones and deoxy aglycones against six nematodes in sheep (11). Potency decreases from the disaccharide through the monosaccharide to the aglycone but is regained as the deoxyaglycone. When the 13-deoxy-22,23-dihydroavermectin B1a and B1b aglycones were compared at dose levels of 200, 100 and 50 µg/kg in sheep they were found to have almost equivalent activities as shown in Table IX. 13-Deoxy-22,23-dihydroaveremctin B1b aglycone is the same compound as milbemycin-D, which although equivalent to ivermectin against sheep helminths, was 500 fold less potent against *D. immitis* and as we will see later, less active in the field against agricultural pests.

The most productive modification in all of our studies, for both animal health and crop protection, was the installation of an amino group in the 4" -position. Table X shows the results of testing a variety of 4"α and 4"β-amino and acylated aminoavermectins in sheep (12). The 4"α and 4"β derivatives have similar activities, with the 4"β compounds being slightly more active and since they are produced in much greater yield it was decided to concentrate on them. 4"-deoxy-4"-lower acylamino derivatives were consistently the most potent when tested against seven parasites of sheep. From these, 4"-deoxy-4"-epiacetylamino avermectin B1 was selected as the all around most promising compound for development as a novel broad spectrum endectocide under the non-proprietary name eprinomectin. It's structure is shown in Figure 3. Table XI documents the ED_{95} values for eprinomectin delivered orally against adult nematodes in experimentally - infected sheep.

Table I. Parasites of Cattle for Which Ivermectin Has Efficacy Claims

- Gastrointestinal Nematodes:
  - *Haemonchus placei* (Adults, L_3 & L_4)
  - *Ostertagia ostertagi* (Adults, L_3 & L_4)
  - *O. lyrata* (Adults & L_4)
  - *Trichostrongylus axei* (Adults & L_4)
  - *T. colubriformis* (Adults & L_4)
  - *Cooperia oncophora* (Adults & L_4)
  - *C. punctata* (Adults & L_4)
  - *C. pectinata* (Adults & L_4)
  - *Nematodirus helvetiania* (Adults & L_4)
  - *N. spathiger* (Adults)
  - *Strongyloides papillosus* (Adults)
  - *Oesophagostomum radiatum* (Adults & L_4)
  - *Bunostomum phlebotomum* (Adults & L_4)
- Other Nematodes:
  - *Dictyocaulus vivparus* (Adults & L_4)
  - *Parafilaria bovicola*
  - *Thelazia* spp. (Adults)

- Grubs:
  - *Hypoderma bovis* – Warble Fly (1st, 2nd, & 3rd Instars)
  - *H. lineatum* (1st, 2nd & 3rd Instars)
  - *Dermatobia hominis*
- Lice:
  - *Damalinia bovis*
  - *Linognathus vituli*
  - *Haematopinus eurysternus*
  - *Solenopotes capillatus*
- Mites:
  - *Chorioptes bovis*
  - *Psoroptes ovis (P. communis)* – Mange Mite
  - *Sarcoptes scabiei*
- Ticks:
  - *Boophilus microplus*
  - *B. decoloratus*
  - *Ornithodoros savignyi*

Table II. Dose Response Calculation of 95% Effective Dosage for Ivermectin in Experimentally Infected Cattle (mg/kg)

| Parasite Species | Treatment Route and Parasite Stage | | | |
| | S.C. | | P.O. | |
	L_4	Adult	L_4	Adult
Haemonchus placei	0.061	<0.05	0.037	0.020
Ostertagia ostertagi	0.014	<0.04	0.019	0.018
Inhibited *O. ostertagi*	0.037	–	0.133	–
Trichostrongylus axei	0.029	0.031	0.050	0.031
Trichostrongylus colubriformis	0.126	0.285	0.070	0.201
Cooperia oncophora	0.076	0.148	0.050	0.090
Cooperia punctata	0.024	0.148	0.051	0.056
Oesphagostomum radiatum	0.022	<0.05	<0.05	<0.25
Dictyocaulus viviparus	<0.05	<0.05	<0.05	<0.25

Table III. Observed Efficacy of Ivermectin Injected Subcutaneously in Naturally Infected Horses

Parasite	% Efficacy of:			Calculated ED₉₅, (mg/kg)
	0.02 mg/kg	0.1 mg/kg	0.5 mg/kg	
Strongylus				
S. vulgaris	93	100	100	<0.1
S. edentatus	100	99	100	<0.02
S. equinus	100	100	94	<0.02
Gastrophilus				
G. intestinalis	95	96	100	0.043
G. nasalis	100	100	100	<0.02
Cyathostomum	(64)	(99)	(>99)	(0.060)
C. pateratum	90	99	99	0.022
C. catinatum	67	99	>99	0.056
Cylicocyclus	(86)	(>99)	(>99)	(0.026)
C. nassatus	89	>99	>99	0.020
C. leptostomus	83	99	99	0.044
Cylicostephanus	(13)	(>99)	(>99)	(0.066)
C. minutus	34	99	99	0.073
C. longibursatus	40	>99	>99	0.042
"Small Strongyles", L₄	66	96	>99	0.080
"Small Strongyles", Adult	50	>99	>99	0.051
Oxyuris equi, L₄	41	94	98	0.163
Onchocerca cervicalis, m.f./26 cm²	83	>99	>99	<0.1

Table IV. Efficacy of Ivermectin on Developing Larvae of *Dirofilaria immitis* in Experimentally Infected Dogs

Dose µg/kg	Treatment Date Days	Number of Dogs	% Efficacy
0.3	30	7	0
1.0	30	7	53.2
2.0	30	7	97.2
2.0	45	7	63.8
3.3	30	7	98.1
0		7	0

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Activity of Avermectins and Milbemycins on Developing Stages of *Dirofilaria immitis* in Dogs

Ivermectin	6 µg/kg
Milbemycin D	1000 µg/kg
Milbemycin Oxime	500 µg/kg
Moxidectin	3 µg/kg

Table V. Double-Blind Study of Ivermectin in Patients with *Onchocerca volvulus* Infections

	Skin Density of Microfilariae	
Study Day	Placebo	Ivermectin
-1	99.4	130.4
2	108.2	38.8
4	99.7	14.1
8	105.1	6.6
14	125.9	2.2
28	102.6	0.6
90	84.5	1.0
180	65.3	2.9
270	80.8	5.0
360	93.0	11.8

10 Patients received a single oral dose of ivermectin 12 mg
10 Patients received Placebo

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Table VI. Ivermectin in the Treatment of *Wuchereria bancrofti Filariasis*

	Efficacy Geometric Mean Microfilariae/mL						
	Day						
Single Oral Dose	0	1.5	5	12	30	90	180
25 μg/kg	761	2.9	<1	<1	5.2	42.9	98
50 μg/kg	1154	3.3	<1	<1	3.5	103.6	92.3
100 μg/kg	610	3.0	<1	<1	<1	19.9	95.9
200 μg/kg	478	<1	<1	<1	1.5	43.7	70.8

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Table VII. Agricultural Activities of Avermectin B_1

- Red Imported Fire Ant *(Solenopsis invicta)*
- Citrus
    - Citrus Rust Mite *(Phyllocoptruta oleivora)*
    - Broad Mite *(Polyphagotarsonemus latus)*
    - Citrus Red Mite *(Panonychus citri)*
    - Citrus Thrips *(Scirtothrips citri)*
- Ornamentals
    - Two-Spotted Spider Mite *(Tetranychus urticae)*
    - Leafminer *(Liriomyza trifolii)*
    - Thrips Species
- Cotton
    - Spider Mites *(Tetranychus urticae, T. turkestani, T. pacificus, T. cinnabarinus)*
    - Lepidoptera
    - Cotton Aphid *(Aphis gossypii)*
- Vegetables (Celery, Tomato, Pepper)
    - Leafminers *(Liriomyza trifolii, L. sativae)*
    - Two-Spotted Spider Mite and Tomato Russet Mite *(T. urticae and Aculops lycopersici)*
    - Armyworms *(Spodoptera exigua and S. eridania)*
    - Tomato Pinworm *(Keiferia lycopersicella)*
- Pears
    - Pear Psylla *(Psylla pyricola)*
    - Two-Spotted Spider Mite, European Red Mite, Pear Rust Mite *(T. urticae, Panonychus ulmi, Epitremerus pyri)*

Table VIII. Efficacy and Spectrum of Avermectin Derivatives against 6 Species of Nematodes in Sheep

Structure	Dose (mg/kg)	Efficacy					
		H.c.	O.c.	T.a.	T.c.	C.s.	Oe.c.
Avermectin A_1	0.1	2	2	0	0	2	0
Avermectin A_2	0.1	3	3	3	3	0	3
Avermectin B_1	0.1	3	3	3	3	3	3
Avermectin B_2	0.1	3	3	3	3	2	3
22,23 Dihydro Avm B_1	0.1	3	3	3	3	3	3
22,23 Dihydro Avm B_1 Monosaccharide	0.3	3	3	3	3	2	3
22,23 Dihydro Avm B_1 Aglycone	3.0	1	2	3	3	1	3
22,23 Dihydro-13-Deoxy Avm B_1 Aglycone	0.1	3	3	3	3	3	3

H.c. = *Haemonchus contortus*, O.c. = *Ostertagia circumcincta*, T.a. = *Trichostrongylus axel*,
T.c. = *Trichostrongylus colubriformis*, C.s. = *Cooperia* spp., Oe.c. = *Oesophagostomum columbianum*
Efficacy as % reduction from control: 0 = <50%, 1 = 51-75%, 2 = 76-95%, 3 = >95%

Table IX. Activity of 13-Deoxyavermectin Aglycones in Experimentally Infected Sheep

	mg/kg P.O.	*Haemonchus contortus*	*Ostertagia circumcincta*	*Trichostrongylus axei*	*Trichostrongylus colubriformis*	*Cooperia curticei*	*Cooperia oncophora*	*Cooperia* spp., L$_4$	*Oesophagostomum columbianum*
					Percent Efficacy				
13-Deoxy-22,23-Dihydro-Avermectin B$_{1a}$ Aglycone	0.2	>99	>99	98	98	98	51	99	>99
	0.1	>99	87	95	95	81	94	93	99
	0.05	99	95	94	91	52	44	96	99
13-Deoxy-22,23-Dihydro-Avermectin B$_{1b}$ Aglycone	0.2	>99	>99	>99	>99	98	94	99	100
	0.1	>99	>99	>99	>99	88	94	99	>99
	0.05	>99	93	99	91	52	39	93	98
Ivermectin	0.2	>99	>99	>99	>99	>99	94	99	100

Table X. Biological Activities of Aminosubstituted Avermectin Derivatives

Avermectin Structures			Dose	Sheep Anthelmintic Test						
R$_{4'\alpha}$=	R$_{4'\beta}$=	–C$_{22}$–C$_{23}$–	(mg/kg)	H.c.	O.c.	T.a.	T.c.	C.c.	C.o.	Oe.c.
	Ivermectin		0.10	3	3	3	3	–	3	3
	Avermectin B1		0.10	3	3	3	3	–	3	3
H	NH$_2$	–CH=CH–	0.10	3	3	0	1	3	–	3
NH$_2$	H	–CH=CH–	0.10	3	3	0	0	3	–	3
H	NH$_2$	–CH$_2$–CH$_2$–	0.10	3	3	1	2	3	–	3
H	NHCH$_3$	–CH=CH–	0.10	3	3	3	2	3	–	3
H	N(CH$_3$)$_2$	–CH=CH–	0.04	3	3	0	1	1	–	3
H	NHCH(CH$_3$)$_2$	–CH=CH–	0.05	2	0	0	1	1	1	3
H	NH(CH$_2$)$_7$CH$_3$	–CH=CH–	0.10	0	0	0	0	0	–	0
H	NHCH$_2$C$_6$H$_5$	–CH=CH–	0.20	3	3	0	3	3	3	3
H	NHCH$_2$CH$_2$OH	–CH=CH–	0.10	3	3	0	2	3	–	3
H	NHCOCH$_3$	–CH=CH–	0.05	3	3	3	3	3	–	3
NHCOCH$_3$	H	–CH=CH–	0.05	3	0	3	0	2	3	3
H	NHCOCH$_3$	–CH$_2$–CH$_2$–	0.10	3	3	3	3	3	3	3
H	N(CH$_3$)COCH$_3$	–CH=CH–	0.05	3	3	3	3	3	3	3
H	NHCHO	–CH=CH–	0.05	3	3	3	3	3	3	3
H	NHCOCH$_2$CH$_3$	–CH=CH–	0.05	3	3	3	3	3	3	3
H	NHCOCH$_2$OCH$_3$	–CH$_2$–CH$_2$–	0.10	3	3	3	2	3	–	3
H	NHCOC(CH$_3$)$_3$	–CH=CH–	0.10	3	2	2	2	3	–	3
H	NHCOC$_6$H$_5$	–CH=CH–	0.05	2	2	0	0	3	3	3
H	NHSO$_2$CH$_3$	–CH=CH–	0.05	3	3	3	3	3	3	3
H	NHCOOCH$_3$	–CH=CH–	0.10	3	3	3	3	3	3	3
H	NHCONHCH$_3$	–CH=CH–	0.10	3	1	3	2	3	–	3
H	NHN(CH$_3$)$_2$	–CH=CH–	0.10	3	3	3	3	3	3	3

H.c. = *Haemonchus contortus*, O.c. = *Ostertagia circumcincta*, T.a. = *Trichostrongylus axei*, T.c. = *Trichostrongylus colubriformis*, C.c. = *Cooperia circumcincta*, C.o. = *Cooperia oncophora*, Oe. c. = *Oesophagostomum columbianum*, * Efficacy as % reduction from control: 0 = <50%, 1 = 51-75%, 2 = 76-95%, 3 = >95%
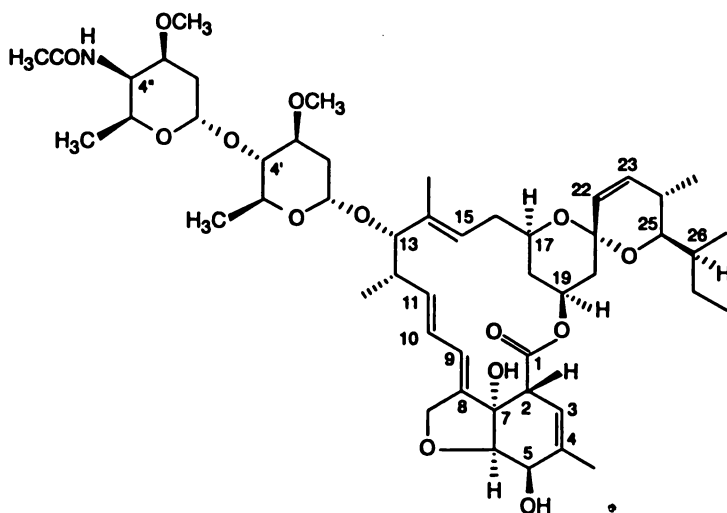
Figure 3. Eprinomectin (MK-397)

Table XI. ED_{95} Values for Eprinomectin Delivered Orally against Adult Nematodes in Experimentally-Infected Sheep

Parasite	ED_{95} (mg/kg)
Haemonchus contortus	<0.025
Ostertagia circumcincta	0.033
Trichostrongylus axei	<0.025
T. colubriformis	<0.025
Cooperia oncophora	0.033
C. curticei	0.042
Oesophagostomum columbianum	<0.025
Immature Stages of Above	<0.025

Avermectins and Milbemycins in Crop Protection

Table VII lists the various mites and insects on crops against which avermectin B1 (abamectin) has activity. Although abamectin is extremely effective as both an acaricide and an insecticide it is clear from Table XII that its most consistent activity is as a miticide. When avermectin disaccharides, monosaccharides, aglycones and deoxyaglycones were tested against the two-spotted spider mite on bean plants, a pattern of activity similar to that against helminths was shown. Activity decreased in the order disaccharides, monosaccharides and aglycones but was regained as the 13-deoxyaglycones (Table XIII). However a persistence study, comparing avermectin B1 (abamectin) to 13-deoxy-22,23-dihydroavermectin B1b aglycone (milbemycin D) against the two-spotted spider mite and tobacco budworm, on bean plants, showed that the initial activity of milbemycin D was lower and diminished more rapidly than that of abamectin. Furthermore, the activity of milbemycin D against the root knot nematode was considerably lower at three-fold higher application rates (Table XIV). Recognizing that the loss of bioactivity from disaccharides to aglycones could be regained with the 13-deoxyaglycones, a series of 13-substituted aglycones was synthesized and tested against the two-spotted spider mite and the southern armyworm. (Table XV). Certain 13-substituents including α and β-methoxyethoxymethoxy provided compounds with activity at least as good as the disaccharide. 13-α and β-fluoro derivatives were also highly active.

It has long been recognized that avermectins and milbemycins, in thin films, are sensitive to oxidative degradation both under light and in the dark. Figure 4 shows the rate of degradation of avermectin B1 in the dark and with exposure to a KRATOS model LH153 solar simulator. It is clear that after 24 hours of exposure very little avermectin B1 remained *(13.)*. In the field its half-life is also less than 24 hours, so that its utility against certain crop pests is limited. Since the early events of photodecomposition are probably related to the diene portion of the molecule, it was decided to synthesize some diene derivatives and test their activity against two-spotted spider mites. Table XVI shows the activity of the compounds synthesized. Interestingly, although the 8,9-oxide was highly active, the 8,9-cyclopropane was practically devoid of activity. From this group, the 8,9-oxide, the structure of which is shown on Figure 5, was selected for further study. Figure 6 shows the comparative decomposition rates of avermectin B1 and its 8,9-oxide derivative, as thin films on glass plates either in the dark or under exposure to the solar simulator *(13)*. Clearly the 8,9-oxide showed improved stability under both conditions. Table XVII shows the foliar residual activity of four derivatives against the two-spotted spider mite. In three of the four compounds examined, when the diene chromophore was destroyed residual activity improved. Table XII presents an overview of the activity of avermectin B1 against mites and insects. While its activity against mites is universally excellent, activity against insects, especially lepidoptera, is two orders of magnitude lower. This level of activity was insufficient to justify commercial development of a new compound for these uses. Thus a program of synthetic chemistry and biological screening was initiated with the southern armyworm selected as the target species. Several 22,23-dihydroavermectin B1 monosaccharides and aglycones showed a sixteen-fold improvement in insecticidal activity, as shown in Table XVIII, but no further enhancement of activity could be achieved with these types of derivative. An important breakthrough came with the discovery of the 4''-aminoavermectins, the synthesis of which are shown in Figure 7. The synthetic route yields two epimers which have similar biological activity and since the 4''-epiamino derivatives are the major products of reductive amination, they were selected for further study. Table XIX shows the activity of a series of 4''-aminoavermectins against the two spotted spider mite and the southern armyworm. While these derivatives, as a whole, had reduced activity against mites, compared to avermectin B1, they had vastly

Table XII. Activity of Abamectin against Mites and Insects

Mite Species (Contact Effect against Adult Mites)	LC_{90} (ppm)
Phyllocoptruta oleivora (Citrus Rust Mite)	0.02
Tetranychus urticae (Two-Spotted Spider Mite)	0.03
Tetranychus turkestani (Strawberry Mite)	0.08
Panonychus ulmi (European Red Mite)	0.04
Panonychus citri (Citrus Red Mite)	0.24
Polyphagotarsonemus latus (Broad Mite)	0.03
Insect Species (Foliar Residue Bioassay)	**LC_{90} (ppm)**
Leptinotarsa decemlineata (Colorabo Potato Beetle)	0.03
Manduca sexta (Tomato Hornworm)	0.02
Epilachna varivestes (Mexican Bean Beetle)	0.20
Acyrthosiphon pisum (Pea Aphid)	0.40
Trichoplusia ni (Cabbage Looper)	1.0
Heliothis zea (Corn Earworm)	1.5
Spodoptera eridania (Southern Armyworm)	6.0
Keiferia Lycopersicella (Tomato Pinworm)	0.03

Table XIII. Activity of Avermectins against the Two-Spotted Spider Mite
(Tetranychus urticae)

	LC_{90} (ppm)
Avermectin B_1	0.03
Avermectin B_2	0.5
Avermectin A_1	ND
Avermectin A_2	0.5
Avermectin B_1 Monosaccharide	0.03
Avermectin B_1 Aglycone	1.0
Ivermectin	0.1
Ivermectin Monosaccharide	>0.5
Ivermectin Aglycone	>6.25
13-Deoxy Avermectin B_1 Aglycone	0.01
13-Deoxy Ivermectin Aglycone	0.01

Table XIV. Residual Activity of Avermectin B_1 and Milbemycin D

	Two-Spotted Spider Mite (Tetranychus urticae) % Mortality – Adults			Tobacco Budworm (Heliothis virescens) % Mortality			Root Knot Nematode (Meloidogyne incognita) 10 = Complete Control 0 = No Control	
	ppm	5 Days	12 Days	ppm	5 Days	12 Days	lb/acre	Control
Avermectin B_{1a}	0.06	99	96	0.6	97	100	1	8.0
	0.015	98	83	0.2	77	60	0.3	4.25
							0.1	3.5
							0.03	3.75
13–Deoxy–22,23– Dihydroavermectin B_{1b} Aglycone	0.06	84	38	0.6	53	55	3	5.5
	0.015	30	34	0.2	30	45	1	3.5
							0.3	3.25
							0.1	2.75

Table XV. 13-Substituted 22,23-Dihydroavermectin B_{1a} Aglycones Miticidal and Insecticidal Activity

C-13-Substituent	*T. urticae* EC_{90}, (ppm)	Southern Armyworm EC_{90}, (ppm)
Avermectin B_1	0.03	6.0
Ivermectin	0.1	6.0
H_2	0.01	0.5
12,13–Dehydro	>6.25	>1.0
α–OH	0.5	>6.25
β–OH	0.05	>8.0
=O	6.25	>0.5
α–$OCH_2OCH_2CH_2OCH_3$	0.05	<1.0
β–$OCH_2OCH_2CH_2OCH_3$	0.01	>0.25
α–OMe	1.00	–
α–Cl	>0.1	<1.0
β–Cl	0.25	0.5
α–F	.05	>0.5
β–F	.01	0.5
β–I	1.25	–
α–NH_2	>0.1	>1.0
=$NOCH_3$	0.05	0.5
Δ–13,14–15–Morpholinyl	6.25	–
Δ–13,14–15–$N(CH_3)COCH_3$	>6.25	–
Δ–13,14–15–OH	>6.25	–
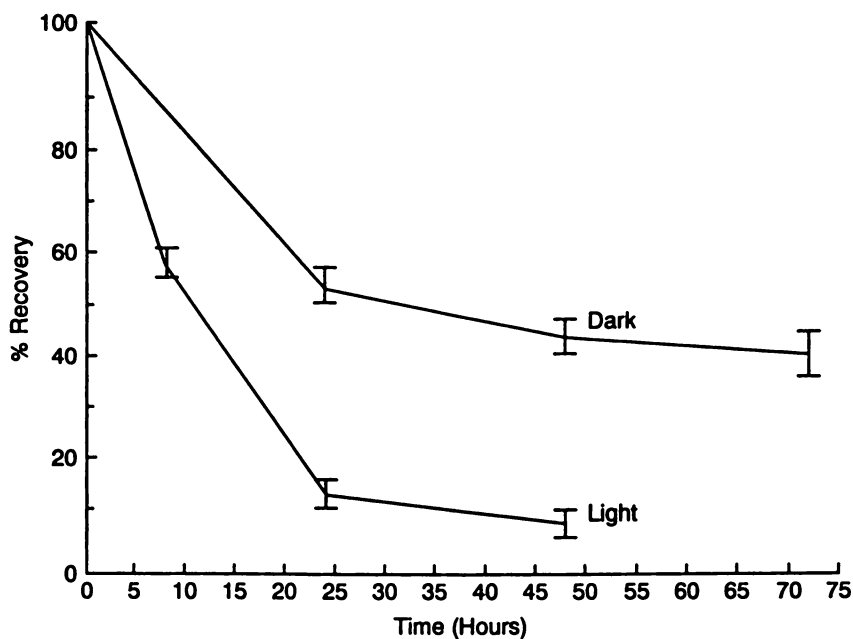
Figure 4. Photodecomposition of Avermectin B_1. (Reproduced from reference 13. Copyright 1989 American Chemical Society.)

Table XVI. Contact Activity of Avermectin Derivatives against Two-Spotted Spider Mite Adult Females

Compound	Percent Mortality at 96 Hours (0.05 ppm)
Avermectin (AVM B_1)	100
AVM B_1 8,9-Oxide	100
AVM B_1 8,9-Cyclopropane	15
AVM B_1 3,4-Cyclopropane	20
10,11-Dihydro AVM B_1	100
22,23-Dihydro AVM B_1 (Ivermectin)	92
10,11,22,23-Tetrahydro AVM B_1	100
3,4,10,11,22,23-Hexahydro AVM B_1	11
3,4,8,9,10,11,22,23-Octahydro AVM B_1	18
10-Fluoro-10,11-Dihydro AVM B_1	100
10-Hydroxy-10,11-Dihydro AVM B_1	72
Milbemycin (25-Sec-Butyl) 8,9-Oxide	20
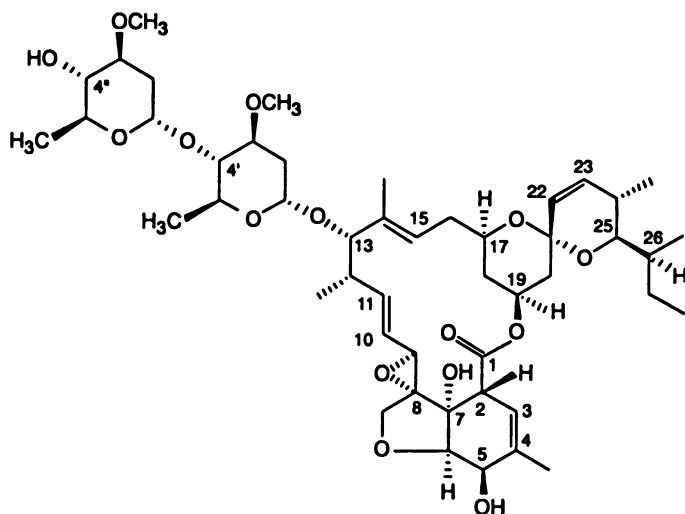
Figure 5. Avermectin B₁-8,9-Oxide
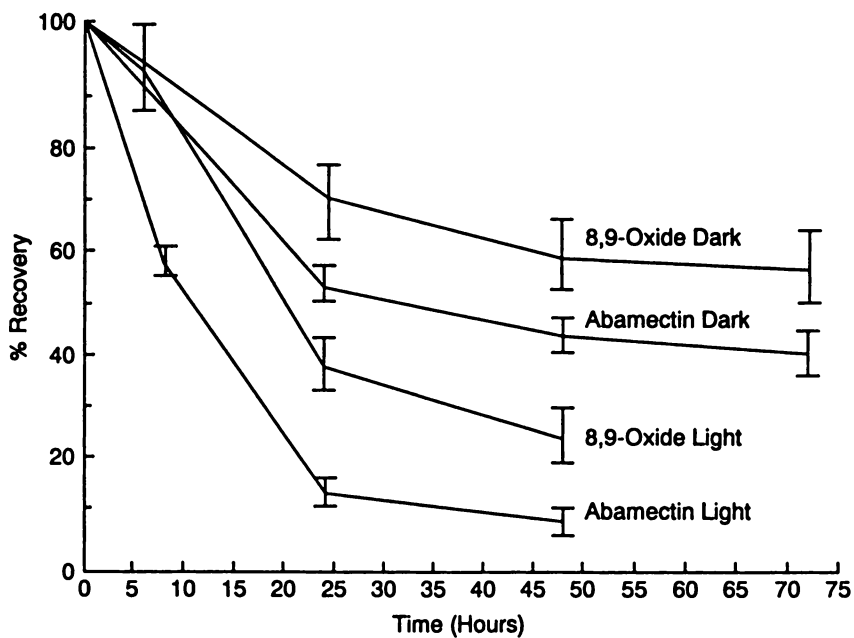
Figure 6. Comparative Photodecomposition of Avermectin B_{1a} and its 8,9-Oxide. (Reproduced from reference 13. Copyright 1989 American Chemical Society.)

Table XVII. Foliar Residual Activity of Avermectin Derivatives against Two-Spotted Spider Mites Adult Females

Compound	Percent Mortality at 0.1 ppm	
	0 DAT*	15 DAT*
Avermectin B_1 (AVM B_1)	96.2	16.9
AVM B_1 8,9-Oxide	99.5	70.7
10,11-Dihydro AVM B_1	98.0	67.0
10,11-22,23-Tetrahydro AVM B_1	95.1	<5
10-Fluoro-10,11-Dihydro AVM B_1	92.3	60.2

* 0 DAT and 15 DAT = 0 and 15 days after treatment spider mites placed onto foliage; mortality counts made 96 hours after infestation

Table XVIII. Activity of Avermectin Derivatives against Southern Armyworm Neonates on Sieva Beans

Compound	EC_{90} ppm
Avermectin B_1	8.0
Ivermectin	8.0
Avermectin B_1 Monosaccharide	8.0
Ivermectin Monosaccharide	0.5
Ivermectin Aglycone	>0.5
13-Deoxy IVM Aglycone	0.5
13-β-Cl-13-Deoxy IVM Aglycone	0.5
13-β-F-13-Deoxy IVM Aglycone	0.5
13=NOCH$_3$-13-Deoxy IVM Aglycone	0.5

1) t-Butyldimethylsilyl Chloride

2) Oxalyl Chloride – DMSO

3) H_3CNH_3OAc – NaBH4

4) p-TsOH – H_2O – MeOH
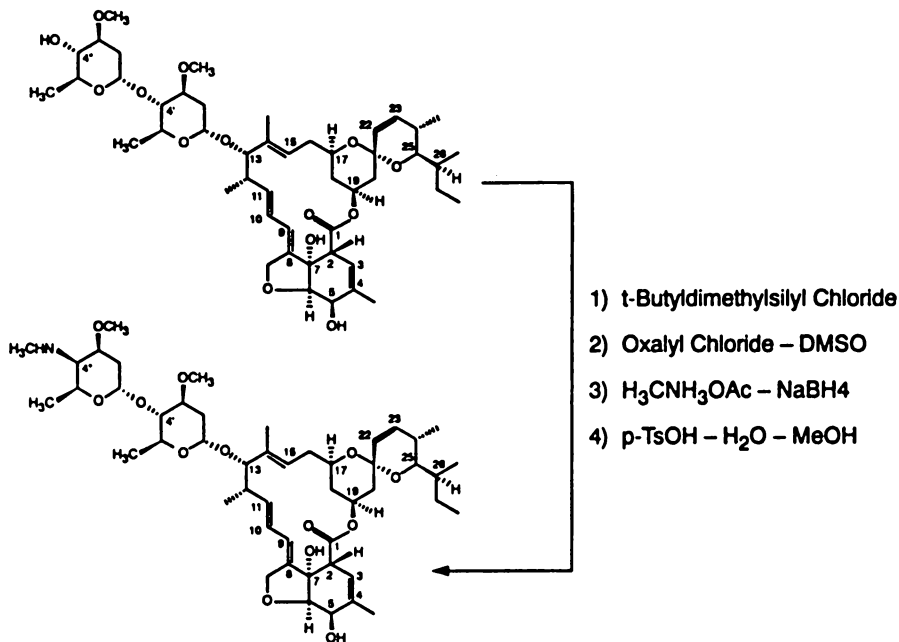
Figure 7. Synthesis of 4"Amino Avermectins

Table XIX. Activity of Aminoavermectins against Mites and Insects

	Two-Spotted Spider Mite Assay LC_{90} (ppm)	Southern Armyworm Assay LC_{90} (ppm)
B_1 (Abamectin)	0.03	8.000
4"–Amino–4"–Deoxy B_1	0.25	0.100
4"–Epi–Amino–4"–Deoxy B_1	0.25	0.020
4"–Epi–Amino–4"–Deoxy–22,23–Dihydro B_1	1.25	0.500
4'–Epi–Amino–4'–Deoxy B_1 Monosaccharide	>0.25	>0.500
4'–Epi–Amino–4'–Deoxy–22,23–Dihydro B_1 Monosaccharide	>0.05	0.100
13–Amino–13–Deoxy–22,23–Dihydro–B_1 Aglycone	>0.10	>1.000
4"–Epi–MeNH–	0.25	0.004
4"–EPI–(Me)$_2$N–	>.05	0.020
4"–Epi–(Me)$_2$CHNH–	0.25	0.020
4"–Epi–C$_6$H$_5$CH$_2$NH–	0.25	0.020
4"–Epi–H$_3$C(CH$_2$)$_7$NH–	0.25	0.100
4"–Epi–CH$_3$CO–NH–	0.25	0.500
4"–Epi–CH$_3$CO–MeN–	0.50	0.050
4"–Epi–C$_6$H$_5$CO–NH–		
4"–Epi–(Me)$_2$NNH–	0.05	0.100
4"–Epi–CH$_3$SO$_2$–NH–	0.05	0.1
4"–Epi–(CH$_3$)$_2$NCH=N–	0.25	0.100
4"–Epi–HN–CN	>.05	0.500
4"–Epi–N$_3$	>.05	2.000
4"–Epi–CH$_3$O–CO–NH–	0.05	0.1
4"–Epi–NHCONHCH$_3$–	1.25	0.5
4"=N–NHCONH$_2$	0.05	0.5

increased activity against the southern armyworm. From these compounds, 4"-deoxy-4"-epimethylaminoavermectin B1 was clearly the most potent against the southern armyworm and it was selected for commercial development as an agricultural insecticide (14,15). The benzoate salt was selected for its excellent stability and was assigned the non-proprietary name emamectin benzoate. Its structure is shown in Figure 8. Table XX shows its activity against a spectrum of mites and insects.

The mode of action of the avermectins and milbemycins has been investigated in several laboratories for many years. A glutamate-gated chloride channel recently has been identified in *C. elegans* and is proposed to be the target
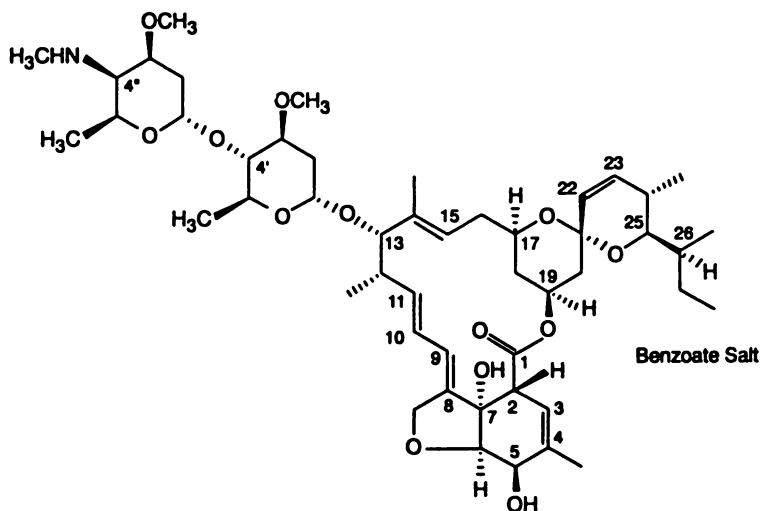
Figure 8. Emamectin (MK-244)

Table XX. Foliar Ingestion Activity of
4"-Epi-Methylamino-4"-Deoxyavermectin B$_1$
Against Insect Larvae and Adult Spider Mites and Aphids

Species (Common Name)	LC$_{90}$ (ppm) at 96 Hours
Manduca sexta (L.) (Tobacco Hornworm)	0.003
Trichoplusia ni (Huebner) (Cabbage Looper)	0.014
Spodoptera exigua (Huebner) (Beet Armyworm)	0.005
Spodoptera frugiperda (J.E. Smith) (Fall Armyworm)	0.01
Leptinotarsa decemlineata (Say) (Colorado Potato Beetle)	0.032
Epilachna varivestis (Mulsant) (Mexican Bean Beetle)	0.20
Tetranychus urticae (Koch) (Two-Spotted Spider Mite)	0.29
Aphis fabae (Scopoli) (Bean Aphid)	19.9
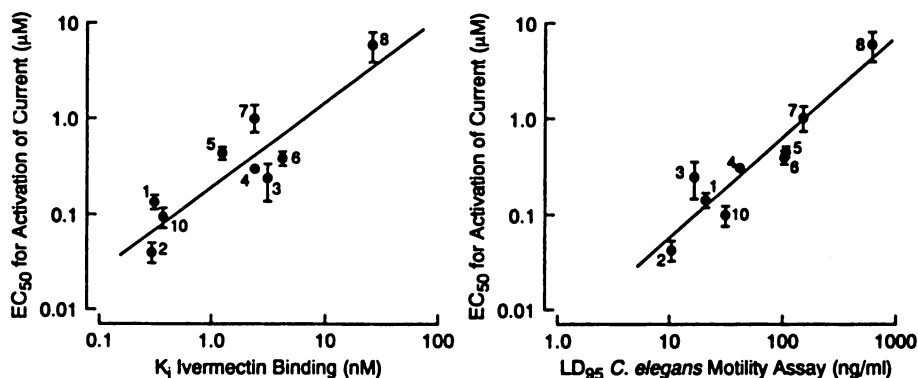
Figure 9. Mode of Action of Avermectins and Milbemycins

● Directly Activate and Potentiate the Activity of Glutamate on *C. elegans* Glutamate Gated Chloride Channel Expressed in Xenopus Oocytes

● Current Activation, Nematocidal Activity and Ivermectin Binding Correlate for a Series of Avermectin Derivatives

site of both the avermectins and milbemycins (*16*). When expressed in *Xenopus oocytes*, avermectins and milbemycins directly activate and potentiate the action of glutamate on this channel. Furthermore, as shown in Figure 9, current activation, nematocidal activity and ivermectin binding correlate for a series of avermectin derivatives.

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Chapter 18

A New Biorational Approach to the Development of Herbicides from Fungal Metabolites

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Because host-specific toxins (HST) are extremely potent and possess a high degree of specific phytotoxicity, as a result of their unique structures, they represent a promising class of compounds which could lead to the preparation and development of selective herbicides. Based on this concept, structure-phytotoxicity relationship of a series of AK-toxins (HSTs produced by *Alternaria kikuchiana*) have been examined, in order to determine structural requirement(s) for activity.

Plant pathogenic fungi represent interesting subjects that have great potential for the development of pest control strategies. A number of bioactive substances that are produced by these fungi can be included in this area. In this regard, we have been dealing with several host specific phytotoxic metabolites produced by *Alternaria.* species, and have concluded that a better understanding of the mechanism for their high potency and selectivity could lead to the development of new types of herbicides. In this paper, we present a brief review of host-specific toxins which play a key role in some selected plant-pathogen systems, and, in addition, describe our investigations on AK-toxin, an HST produced by a Japanese-pear fungal pathogen, *Alternaria kikuchiana*. Our major interest is developing an understanding of the molecular features essential for its selective phytotoxicity. It is our belief that the cumulative findings obtained in this and similar investigations can be used to develop a new biorational method for designing selective herbicides.

Host-specific Toxins as Host-recognition Determinants of Pathogens

One of the most attractive and exciting subjects regarding host-parasite interactions in plant diseases involves the host-selection mechanism by pathogenic fungi. A key to understanding this mechanism is host-specific (or host-selective) toxins(HSTs) (*1*) produced by the pathogens. HSTs are secreted from the spores and mycelia of the pathogens on the host plant, and are thought to play a primary role in determining parasite specificity by blocking the defense responses of the host plant.

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Historically, the first example of HST was reported in 1933 by Dr. Tanaka of Kyoto University (2). His report suggested that a culture filtrate from the pathogen causing black spot disease in Japanese pear, *A. kikuchiana*, contains phytotoxic metabolite(s), which gives rise to typical necrotic symptoms caused by inoculation of the parasitic fungus only in the cultivar susceptible to this disease. Later similar phenomena were discovered in other *Alternaria* and *Helminthosporium* diseases (3), and the causal factors were extensively investigated.

At present, HST is defined as the microbial metabolite which satisfies the following 6 criteria (4-6):
1) The production of a host-specific phytotoxic metabolite by a pathogen.
2) After spore-germination, the release and secretion of the metabolite from hyphae.
3) The reproduction of biochemical responses to the diseases.
4) Inhibition or blocking of induced resistance.
5) A parallelism between pathogenicity and toxin-production by the causal fungal strains.
6) A parallelism between disease susceptibility and toxin sensitivity in host plants.

Host-Specific Toxins Produced by *Alternaria* Pathogens. The causal principle(s) of leaf spot disease in Japanese pear, or HST(s) in the culture filtrate of A. kikuchiana was not chemically characterized until 50 years after its discovery by Tanaka. On the other hand, in 1967, Sawamura reported that *A. mali*, the causal pathogen of apple leaf spot disease, also produces HST(s) named AM-toxin(s), which are found in the culture medium (7). This fungus attacks limited types of apple cultivars, such as Indo and Delicious. Susceptibility to AM-toxin was consistent with the host range of this pathogen. More recently, 5 additional HSTs were reported for 5 types of diseases caused by the fungi belonging to the *Alternaria* genus(3). Our research has focused on the isolation and structural elucidation of AM- and AK-toxins, since both of the producer pathogens cause wide-spread and economically important diseases in Japan. The structures of the AM-toxins I (alternariolide), II and III [**1a, 1b** and **1c**] (8-10) and AK-toxins I and II [**2a** and **2b**] (11, 12) were elucidated and are shown in Fig. 1. These structures were confirmed by total synthesis (13-15).

The structural features of these two types of *Alternaria* HSTs are quite different. AM-toxins are cyclic depsipeptides which contain unusual amino acids such as dehydroalanine and 1-amino-5-(p-methoxy- and p-hydroxy-)phenylpentanoic acid, while AK-toxins are esters composed of (β-methyl-)N-acetyl-phenylalanine and (8R,9S)-9,10-epoxy-8-hydroxy-9-methyl-(2E,4Z, 6E)-decatrienoic acid. Interestingly, AK-toxins are structurally related to two groups of HSTs, namely AF-toxin I, II and III (produced by *A. fragariae*, or *A. alternata* strawberry pathotype, **3a, 3b, 3c**) and ACT-toxin I and II (*A. citri* , or *A. alternata* tangerine pathotype, **4a** and **4b**) , the structures of which were established by Nakatsuka et al. (16, 17) following the determination of AK-toxins. These toxins share (8R,9S)-9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid as a common component (see Fig. 1), although the geometry at the 2, 4 and 6 positions of the decatrienoic acid is different. AF-and ACT toxins are also toxic to AK-toxin-susceptible Japanese pear cv. Nijisseiki, a typical host plant of A. kikuchiana, while AK-toxins are not toxic to the host plants of AF- and ACT-toxin producing fungi (16, 18).

[1a] AM-toxin I :R = OCH₃
[1b] AM-toxin II :R = H
[1c] AM-toxin III:R = OH

[2a] AK-toxin I :R = CH₃
[2b] AK-toxin II:R = H

[3a] AF-toxin I :R =OCOCH(OH)C(CH₃)₂OH
[3b] AF-toxin II :R = H
[3c] AF-toxin III:R = OCOCH(OH)CH(CH3)₂

[4a] ACT-toxin Ib :R = OH
[4b]ACT-toxin IIb:R = H
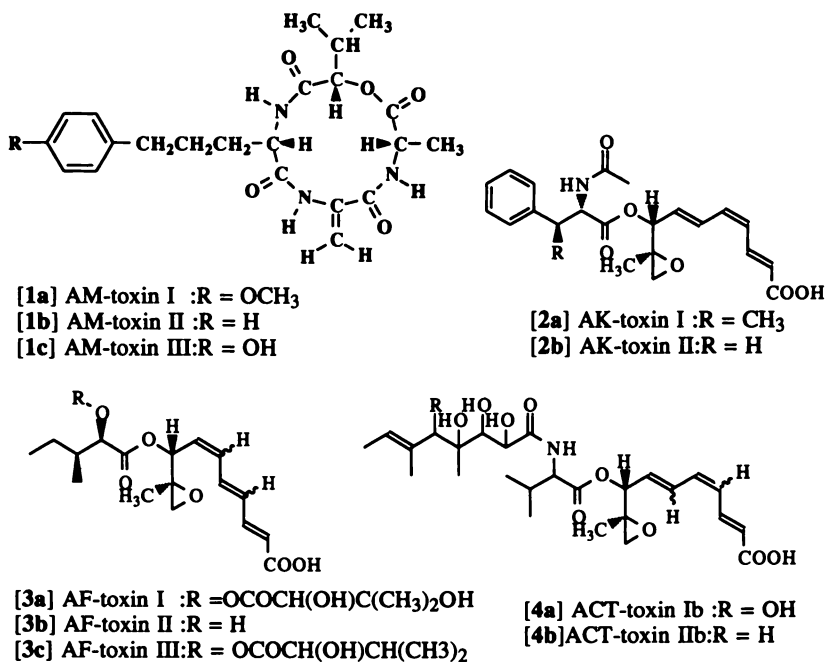
Figure 1. Host-specific toxins from *Alternaria* species

Biological Activity of AM- and AK-Toxins. As shown in Table I, the toxicities of AM- and AK-toxins correlate with the host ranges of the causal fungi, respectively. The mode of action of these HSTs has been extensively investigated, and for the case of the AK-toxin, the data suggest that the primary site of action involves the membrane system (*19, 20*). Based on the electron-microscopy, specific invagination of the plasma membrane is observed only in the susceptible cultivar (*16*). Concomitantly, abnormal electrolyte (K^+) leakage from the cells of susceptible cultivar occurs after the toxin treatment, and this leakage increases with time. However, the presence of the AK-toxin receptor in the membrane fraction has not yet been confirmed.

Structure-Activity Study

Syntheses of AK-toxins and analogs. In order to investigate the structural requirements of AK-toxin for host-specific toxicity, we performed a stereoselective synthesis of AK-toxin II methyl ester, together with AF-toxin II, starting from Vitamin C as a chiral synthon (*15, 21, 22*). Vitamin C is easily converted to the aldehyde 5 according to the reactions shown in Scheme 1(*21, 22*). Treatment of 5 with methyl 4-triphenylphosphoniumcrotonate gave the *trans-cis-trans* (6a) and the all *trans*-trienoic acid ester (6b) in 85% in 3:1 ratio. Oxidation of 6a with *m*-chloroperbenzoic acid gave two diastereomeric epoxides 7a and 7b in 38% and 43%

**Table I.   Pathogenicity of *A. mali, A. kikuchiana* and *A. fragariae*
and Host-specific Toxicity of AM- and AK-toxins**

Pathogenic Fungi	Pathogenicity and Toxicity	Apple		Japanese Pear	
		Indo (s)	Jonathan (r)	Nijisseiki (s)	Chojuro (r)
Alternaria mali	Pathogenicity	+++	+	+	-
	AM-toxin I (10^{-5}M)	+++	+	+	-
	AM-toxin I (10^{-8}M)	+	-	-	-
	AM-toxin II(10^{-6}M)	+	-	-	-
	AM-toxin III(10^{-7}M)	+	-	-	-
Alternaria kikuchiana	Pathogenicity	-	-	+++	-
	AK-toxin I (10^{-7}M)	-	-	+++	-
	AK-toxin I (10^{-4}M)	-	-	+++	-
	AK-toxin II(10^{-5}M)	-	-	+++	-

The marks + and - indicate positive and negative response respectively. Induced
necrosis rating scale is as follows: +++, very severe; ++, severe; +, moderate; and -,
no necrosis. The characters (s) and (r) are abbreviation of "susceptible" and
"resistant" respectively.

yield, respectively (scheme 1). The structures of these epoxides were determined by
comparing their ^{1}H-NMR spectra with those of the natural AK-toxins. De-protection
of the silyl group in epoxide 7a with tetrabutylammonium fluoride gave the alcohol in
good yield. The alcohol was esterified with *N*-acetyl-L-phenylalanine and
dicyclohexylcarbodiimide (DCC) in the presence of 4-pyrrolidinopyridine to give a
mixture of AK-toxin II methyl ester and its epimer at the α-carbon of the amino acid
moiety. This was a result of inevitable racemization during the condensation reaction
due to the *N*-acetyl group of the phenylalanine moiety. The stereochemistry of the α-
carbon of the phenylalanine moiety in the obtained esters was determined by
hydrolysis followed by the analysis of the amino acids (*23*). The synthetic AK-toxin
II methyl ester was identical with the natural one in all respects. Synthesis of AK-
toxin II has also been performed starting from D-fructose by Shibuya's group (*24*).
Syntheses of the unusual acid component of AK-toxin, (8*R*,9*S*)-9,10-epoxy-8-
hydroxy-9-methyl-decatrienoic acid, were reported by other two independent groups
(*25, 26*).

   Since the synthesized AK-toxin II methyl ester was toxic to the AK-toxin
susceptible cultivar of Japanese pear, this suggests that the presence of the free
terminal carboxylic acid is not essential for the activity. This finding can be applied to
the design of an affinity ligand for the isolation of the putative AK-toxin binding
protein in the toxin-sensitive plant.
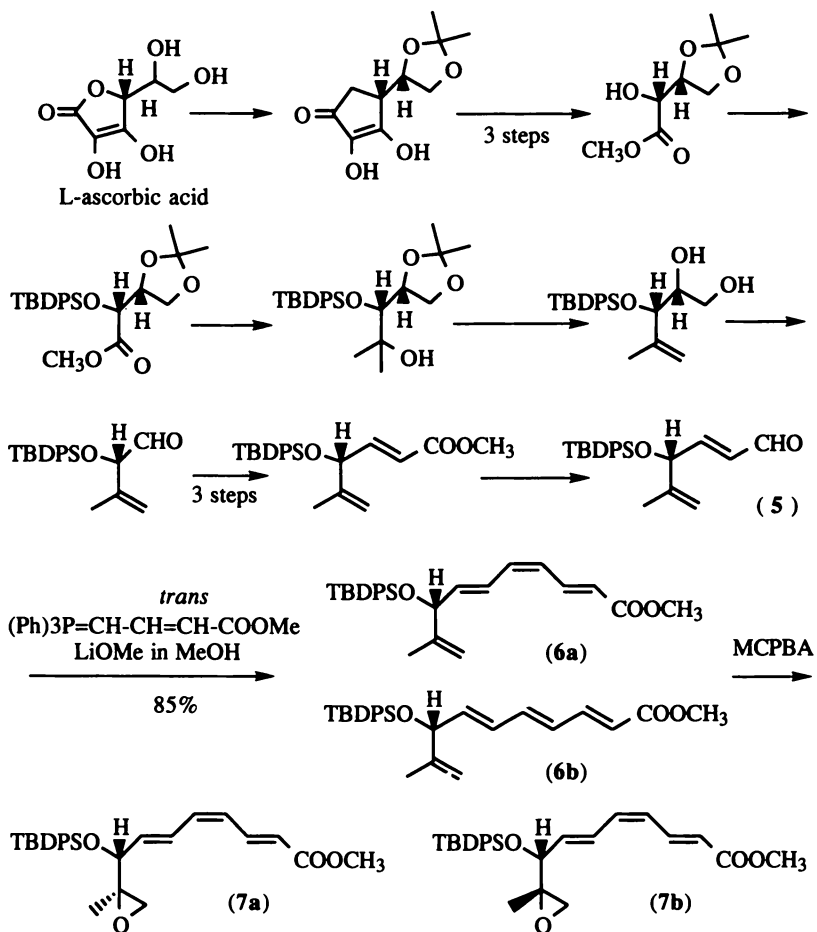
Scheme 1.

Table II. Phytotoxicity of Synthesized AK-Toxin II Analogs against Japanese Pear cv. Nijisseiki
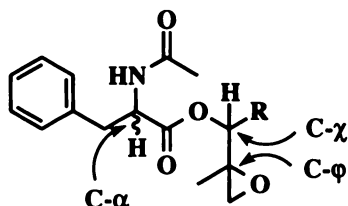
R	Configuration			Toxicity		
	C-α	C-χ	C-φ	10^{-2}M	10^{-3}M	10^{-4}M
-(CH=CH)₃-COOCH₃	S	R	S	+	+	+
(trans, trans, trans)	R	R	S	+	+	+
	S	R	R	-	-	-
	R	R	R	-	-	-
-(CH=CH)₂-COOCH₃	S	R	S	+	+	+
(trans, trans)	R	R	S	+	+	+
	S	R	R	-	-	-
	R	R	R	-	-	-
-(CH=CH)-COOCH₃	S	R	S	+	+	-
(trans)	R	R	S	+	+	-
	S	R	R	-	-	-
	R	R	R	-	-	-
	S	S	S	-	-	-
	R	S	S	-	-	-
	S	S	R	-	-	-
	R	S	R	-	-	-

In addition to AK-toxin itself, our synthetic method allowed the preparation of some AK-toxin analogs, in which the hydroxy acid moieties were 7,8-epoxy-6-hydroxy-7-methyl-2,4-octadienoic acid (diene analog) and 5,6-epoxy-4-hydroxy-5-methyl-2-hexenoic acid (monoene analog) (22). Bioassay results using Japanese pear cv. Nijisseiki showed that, although the phytotoxicities fell with a decrease in the number of double bonds, the monoene analog was still active (Table II). Subsequently, the enantiomeric effects of the asymmetric sites in AK-toxin on phytotoxicity were examined by preparing all of the possible monoene isomers (see Table II) (27). The data in Table II show that the *R* and *S* configurations at the hydroxyl group of the unsaturated acid and the epoxy moiety, respectively, are essential for the expression of toxicity. The absolute configuration of the phenylalanine moiety appeared to have little or no effect on activity. Flexibility with respect to the stereochemistry in the phenylalanine moiety can be related to the fact that AF- and ACT-toxins are also toxic to Japanese pear cv. Nijisseiki. In this regard, compound **9**, in which the *N*-acetylphenylalanine moiety in the monoene analog of the AK-toxin II was replaced with *N*-acetylvaline, was synthesized and found to be active against cultivar Nijisseiki (unpublished data). Thus the minimum structural features of AK-toxin necessary for the expression of toxicity appears to be structure **8**, shown in Fig. 2.

In addition, the preparation of ^{14}C, ^{3}H and/or ^{125}I labeled AK-toxin are in progress, using the synthetic procedures described above. These labeled materials will be quite useful for mode-of-action studies.

Trials to Develop Phytotoxic Compounds that are Biologically Isosteric to AK-Toxin. Although the conjugated triene carboxylic acid moiety of AK-toxin significantly contributes to enhanced the phytotoxicity, its preparation is rather complicated and time-consuming, requiring multi-step chemical reactions. We therefore initiated a search for a compound which has simpler structural features but retains selective phytotoxicity against Japanese pear cv. Nijisseiki, namely a biologically isosteric compound. Modification of the structure was first performed by replacing the conjugate system with aromatic structures, as shown in compounds **10** and **11** in Fig. 2. These compounds were synthesized as shown in scheme 2 and were obtained as a mixture of racemates and diastereomers with respect to all of the asymmetric carbons denoted with asterisks (unpublished data). Interestingly, phytotoxicity assays using cultivar Nijisseiki showed that each of the compounds **10a, 10b, 11a** and **11b** was more or less active, although the absolute potency appeared somewhat weak. Purification of each mixture is now in progress in order to evaluate the activities of the isomers. These results provide encouragement for future investigations of structure-activity relationships of the AK-toxin analogs, with the object of obtaining a biological isoster that can be easily prepared and be used as a reliable probe for mode-of-action studies.

[8] R: Alkyl, R': Alkyl or Benzyl
R":CH₃ , X: NH or O

[9a] : (2'R), [9b] : (2'S)

[10a] : R=H, [10b] : R=CH₃

[11a] : R=H, [11b] : R=CH₃

[12]
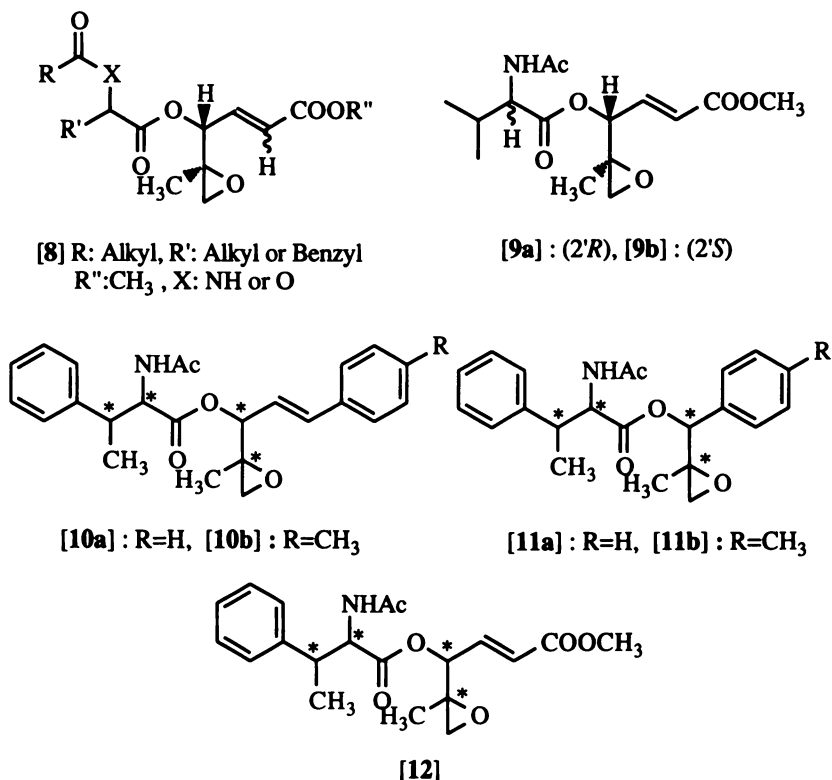
Figure 2. The minimum structural feature for the AK-toxin activity [8] and the synthesized compounds [9 -12]

Conclusions

Selectivity is one of the most challenging problems in developing new herbicides. It is, however, extremely difficult to design a compound with proper selectivity. By contrast, there are considerable selective relationships in nature such as host-parasite interactions, which have been established through the long history of co-evolution. Host-specific toxins produced by plant pathogens constitute concrete objects representing such natural selectivities. Elucidating the mechanism and the structural requirements of HSTs can surely reveal some of the yet-unknown weak points of plants and provide strategies for dealing with them, namely biorational methods to develop excellent herbicides.

Acknowledgments
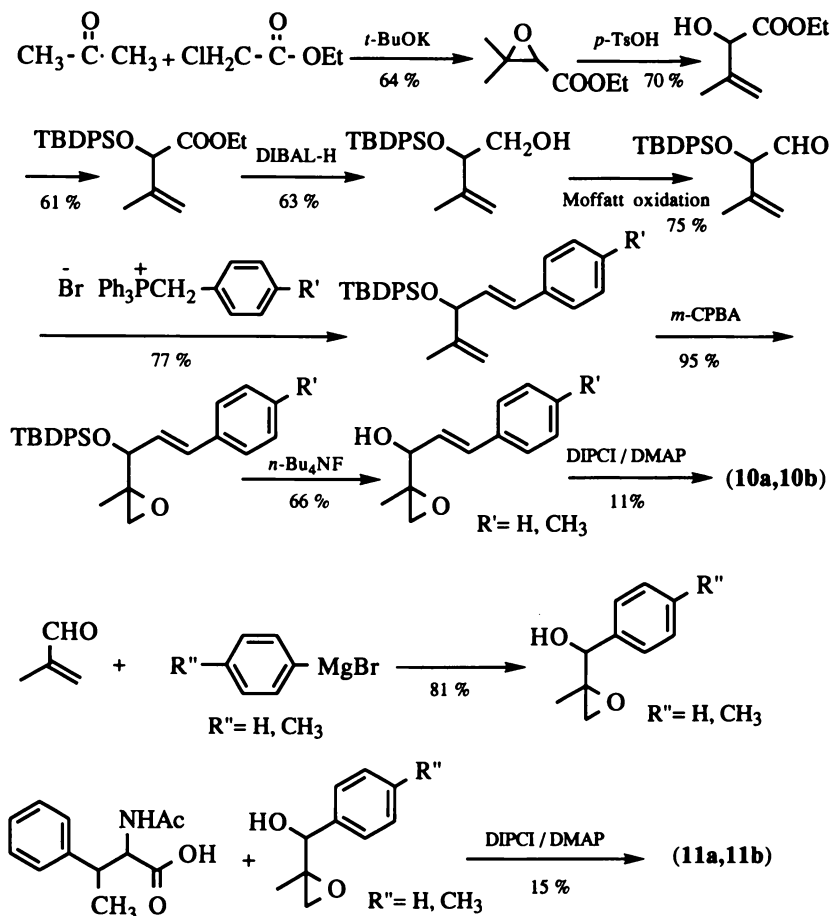
Scheme 2.

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Chapter 19

Chemistry and Fungicidal Activity of Soraphen $A_{1\alpha}$ Derivatives

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The macrolide soraphen $A_{1\alpha}$ 1 was isolated from the myxobacterium *Sorangium cellulosum* (strain So ce 26). It shows potent and broad fungicidal activity. The molecule was derivatised at various positions and many analogs were found with excellent fungicidal activity, some more potent than soraphen A itself. The fungicidal activity of soraphen A derivatized at positions 2, 5, 11, and 12 is described here.

During the study of myxobacterial metabolites by Höfle and Reichenbach at the Gesellschaft für Biotechnologische Forschung in Germany ,[1,2] Soraphen $A_{1\alpha}$ 1 was isolated from *Sorangium cellulosum*.[3,4] It was tested in the Ciba greenhouses against a battery of pests in greenhouse trials, and was found to possess excellent activity against fungal pathogens on plants.[5] In common with many agrochemical companies, Ciba has a policy of screening natural products as potential agrochemicals. In Table 1 soraphen $A_{1\alpha}$ is compared with several commercial and in-development fungicides. It is apparent that it has potent and broad activity against many important fungal pests. Only the recently introduced strobilurin type fungicides azoxystrobin and kresoxim have such a broad spectrum, but with a different mode of action.[6] This breadth of fungicidal activity prompted an intensive study of soraphen both at the GBF and Ciba. Finally, however, the compound was not commercialised because of its teratogenic effects in animals.

Soraphen $A_{1\alpha}$ exerts its action through inhibition of acetylcoenzyme A carboxylase,[7,8] which converts acetate to malonate in the fatty acid synthesis pathway. As implied by its macrolide structure, biosynthetic studies have shown it to be a polyketide, assembled from acetate and propionate with two C-2 units arising from glycerol by an unknown pathway.[9] The polyketide synthase gene cluster

Table 1. Comparison of the fungicidal activity of soraphen $A_{1\alpha}$ with some standards

Compound	Puccinia recondita on Wheat Soil	Erysiphe graminis on Barley Soil	Pyricularia oryzae on Rice Soil	Cercospora arachidicola on Peanut Foliar	Phytophthora infestans on Tomato Foliar	Plasmopara viticula on Grape Foliar	Venturia inequalis on Apple Foliar
Soraphen A (1)	2	0.6	2	2	60	20	2
Azoxystrobin	2	2	60	0.02	2	2	0.02
Kresoxim	2	0.06	200	0.02	2	2	0.02
Difenoconazole	0.6	0.06	-	0.6	-	-	0.2
Epoxyconazole	6	0.02	200	0.02	-	-	20
Flusilazole	2	0.2	-	0.2	-	-	0.6
Fenpropimorph	0.6	0.2	20	6	-	-	20
Metalaxyl	-	-	-	-	0.6	0.6	-

responsible for this biosynthesis has been identified and partially sequenced.[10] The total synthesis of soraphen A has been reported,[11] as well as a number of reports of the synthesis of substuctures.[12-17]

When the producing strain was reexamined, more than 20 congeneric metabolites were isolated, forming a soraphen family.[18] At the same time a collaborative derivatization program was initiated starting from the major metabolite soraphen A$_{1\alpha}$ 1. Some of the results of this work are presented here.

Tautomers

1 contains a hemiacetal group, which readily enters an equilibrium with its hydroxy-ketone tautomer 2 on dissolution in water (Figure 1).[19,20] This compound is a β-keto ester which in turn tautomerises readily to its enol form 3. *E/Z* isomerism of the double bond of the enol 3 and epimerisation at C(2) in 1 and 2 make possible a large number of tautomers. Three of these tautomers 1, 2, and 3 were prepared separately, and their interconvertion to the same equilibrium mixture was followed in aqueous solution.[19] The tautomers 1 and 2 show different reactivity and stereoselectivity in their reactions even at functionalities distant from the hemiacetal / hydroxy-ketone moieties. The selectivity of the reactions of these tautomers has been described.[21]
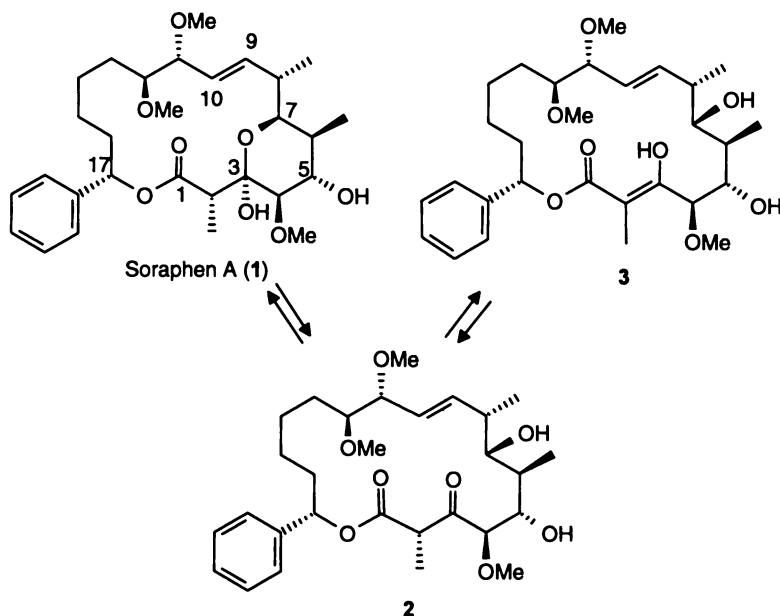
Figure 1. Tautomerisation of Soraphen A.

Synthesis.

In the course of the collaborative studies on the derivatization of soraphen A_{1_α}, virtually the whole molecule was subject to modification. Apart from the work described here, replacement of the C(17)-phenyl ring with other groups has been accomplished.[22] Furthermore extensive derivatisation of the south east ring encompassing positions 2-7 has been described.[20,23,24] In addition the 9,10 double bond was functionalised.[21] As with the derivatisation of other macrolides, the observation was continually made that substitution reactions involving S_N2 reactions failed, whereas S_N1 reactions often led to the desired products.[25]

Position 2. Starting from the hydroxy-ketone **2**, the enol **3**, the enolate of **3** or protected analogs of these compounds, electrophiles were introduced into position 2.[20,24] By these means the compounds delineated in Figure 2 and Table 2 were prepared. It was not possible to determine the stereochemistry of the two epimeric fluoro derivatives.

Of the compounds in Table 2 the halo substituted derivatives are fungicidally most active. The most potent of the fluoro compounds has fungicidal activity approaching that of soraphen A_{1_α} itself. From this result it is clear that the attainment of the enol **3** or enolate tautomers is not neccessary for soraphen A_{1_α} to manifest fungicidal activity.
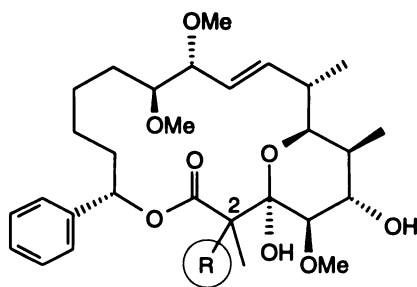
Figure 2. Structure of the compounds shown in Table 2

Position 5. Derivatives at C(5) were prepared directly from soraphen A_{1_α} **1**.[20] The hydroxy group was alkylated, forming the ethers shown in Table 4, and acylated with conventional reagents to the esters listed in Tables 5 and 6 (Figure 3). The carbonates and carbamates in Table 6 were prepared analogously. Oxidation yielded the 5-ketone, which was transformed further to the oximes and hydrazones shown below (Table 3). The βOH compound (Table 3) was obtained by reduction of the ketone and its mesylate was formed (Table 3) by further treatment of this alcohol with mesyl chloride. Treatment of soraphen A_{1_α} with PCl5 yielded the 5α-chloride (Table 3). The 5-O-deoxy compound (Table 3) was prepared by radical reduction of the thionocarbonate (Table 4).[26] Silylation and sulfonation was also successful (Table 4).

Table 2. The fungicidal activity of soraphen A derivatives modified at position 2.[a]

R (Fig. 2)	Puccinia recondita on Wheat Foliar	Puccinia recondita on Wheat Soil	Erysiphe gramanis on Barley Foliar	Erysiphe gramanis on Barley Soil	Pyricularia oryzae on Rice Foliar	Pyricularia oryzae on Rice Soil	Cercospora arachidicola on Peanut Foliar	Venturia inequalis on Apple Foliar	Botrytis cinerea on Apple Fruit	Botrytis cinerea on Bean Foliar	Rhizoctonia solani on Rice Soil
F	20	c	20	c	nt	nt	6	2	2	6	c
F	20	c	2	2	b	c	2	0.6	0.6	2	c
βCl	60	6	2	6	b	c	6	2	2	20	c
βBr	b	6	b	c	b	c	6	20	0.6	6	c
αBr	20	6	6	2	b	6	6	6	6	2	c
βN₃	b	c	20	c	20	c	20	60	6	b	nt
αN₃	b	c	6	c	b	c	20	b	6	20	nt
(S-CH₂C(CH₃)₂CN)	60	c	6	2	60	c	60	6	2	20	c
(S-C₆H₄-Cl, para)	b	6	6	0.6	b	6	20	60	2	20	c
Me	20	c	6	6	b	c	20	20	0.6	6	c
βCD₃	60	c	6	6	60	6	6	6	6	2	c
αEt	b	c	b	c	b	c	20	b	b	b	c
βAllyl	b	c	b	c	b	6	20	b	b	b	c
αAllyl	b	c	60	2	b	c	b	6	2	20	c

a. Values are EC_{80} in mg litre^{-1}; b. = >60 mg litre^{-1}; c. = >6 mg litre^{-1}; nt = not tested

Table 3. The fungicidal activity of soraphen A derivatives modified at position 5[a]

R	Puccinia recondita on Wheat Foliar	Puccinia recondita on Wheat Soil	Erysiphe gramanis on Barley Foliar	Erysiphe gramanis on Barley Soil	Pyricularia oryzae on Rice Foliar	Pyricularia oryzae on Rice Soil	Cercospora arachidicola on Peanut Foliar	Venturia inequalis on Apple Foliar	Botrytis cinerea on Apple Fruit	Botrytis cinerea on Bean Foliar	Rhizoctonia solani on Rice Soil
H	60	c	6	c	b	c	20	60	0.6	6	c
αCl	60	c	b	c	b	c	b	b	2	20	c
βOH	b	c	b	c	b	c	60	60	20	b	c
βOSO₂Me	b	c	b	c	b	c	b	b	b	b	c
αNHCOMe	20	c	b	c	nt	nt	b	60	b	b	c
=O	b	c	b	c	b	c	b	nt	b	b	c
=NOH	b	c	b	c	b	c	b	b	b	b	c
=NOMe	b	c	b	c	b	6	b	b	b	b	c
=NNH₂	20	c	20	6	b	c	6	6	6	20	c
=NNMe₂	b	c	b	c	b	c	60	b	b	b	c
=NNHTs	b	c	b	c	b	c	60	b	20	60	c
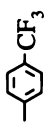
a. Values are EC_{80} in mg litre^{-1}; b. = >60 mg litre^{-1}; c. = >6 mg litre^{-1}; nt = not tested

Table 4. The fungicidal activity of soraphen A derivatives modified at the 5α-OH.[a]

αR (Fig. 3)	Puccinia recondita on Wheat Foliar	Puccinia recondita on Wheat Soil	Erysiphe gramanis on Barley Foliar	Erysiphe gramanis on Barley Soil	Pyricularia oryzae on Rice Foliar	Pyricularia oryzae on Rice Soil	Cercospora arachidicola on Peanut Foliar	Venturia inequalis on Apple Foliar	Botrytis cinerea on Apple Fruit	Botrytis cinerea on Bean Foliar	Rhizoctonia solani on Rice Soil
OMe	b	c	b	c	b	c	b	b	b	b	c
OAllyl	20	c	60	c	b	c	60	20	20	6	c
(propargyl)	20	6	6	c	nt	nt	6	20	20	20	c
OCH₂CH₂OMe	b	c	60	c	b	c	60	b	b	b	c
OMEM	b	c	b	c	b	c	60	b	b	b	c
(THP)	b	c	b	c	b	c	20	b	20	b	c
OBn	b	c	b	c	b	c	b	b	b	b	c
(benzyl-OMe)	60	c	b	c	b	c	b	b	b	b	c
(benzyl-NO₂, O₂N)	b	c	b	c	60	c	60	20	b	60	c
OSiMe₃	b	c	b	c	b	6	b	b	b	b	c
OSiOHtBu₂	b	c	b	c	b	c	b	b	b	b	c
OCSOPh	b	c	b	c	b	c	20	60	60	b	c
OSO2Me	b	c	60	c	b	c	20	nt	20	b	c
OSO2Tol	b	c	b	c	b	c	b	b	b	b	c

a. Values are EC_{80} in mg litre^{-1}; b. = >60 mg litre^{-1}; c. = >6 mg litre^{-1}; nt = not tested

Table 5. The fungicidal activity of soraphen A derivatives esterified at the 5-OH.[a]

X=RCOO (Fig. 3)	Puccinia recondita on Wheat Foliar	Puccinia recondita on Wheat Soil	Erysiphe gramanis on Barley Foliar	Erysiphe gramanis on Barley Soil	Pyricularia oryzae on Rice Foliar	Pyricularia oryzae on Rice Soil	Cercospora arachidicola on Peanut Foliar	Venturia inequalis on Apple Foliar	Botrytis cinerea on Apple Fruit	Botrytis cinerea on Bean Foliar	Rhizoctonia solani on Rice Soil
H	b	0.6	2	0.6	b	c	0.2	nt	0.6	2	6
Me	b	2	20	2	20	2	2	nt	6	6	c
Et	b	c	6	2	b	6	nt	6	6	6	c
nPr	b	c	20	6	b	2	nt	20	60	20	c
iPr	b	c	60	c	b	2	20	nt	60	b	c
tBu	b	c	b	c	60	c	b	b	b	b	c
$CHCl_2$	6	2	6	2	b	c	6	6	0.6	0.6	6
Vinyl	20	c	60	c	b	c	6	6	6	20	c
(branched alkene)	60	c	b	c	b	c	2	6	6	2	c
Ph	b	c	b	c	b	c	60	nt	b	b	c
⟨aryl⟩–CF_3	b	c	b	c	b	c	b	b	b	b	c
CH_2OMe	20	2	20	6	60	6	6	6	2	6	6

a. Values are EC_{80} in mg litre^{-1}; b. = >60 mg litre^{-1}; c. = >6 mg litre^{-1}; nt = not tested

Table 6. The fungicidal activity of soraphen A derivatives esterified at the 5-OH.[a]

X=RCOO (Fig. 3)	Puccinia recondita on Wheat Foliar	Puccinia recondita on Wheat Soil	Erysiphe gramanis on Barley Foliar	Erysiphe gramanis on Barley Soil	Pyricularia oryzae on Rice Foliar	Pyricularia oryzae on Rice Soil	Cercospora arachidicola on Peanut Foliar	Venturia inequalis on Apple Foliar	Botrytis cinerea on Apple Fruit	Botrytis cinerea on Bean Foliar	Rhizoctonia solani on Rice Soil
CH$_2$CH$_2$CO$_2$H	b	2	60	0.6	b	2	6	20	6	20	c
COOH (cyclopropyl)	b	c	6	2	b	c	6	6	2	b	c
CONMe$_2$ (cyclopropyl)	6	0.6	0.6	0.6	b	6	0.6	2	0.6	0.6	0.6
CH$_2$NHBoc	b	c	b	c	b	c	6	6	6	60	c
CH$_2$NH$_2$	6	2	6	0.6	b	6	<2	6	<2	2	6
CH(iPr)NHBoc	b	c	b	c	b	c	b	b	b	b	c
CH(iPr)NH$_2$	b	6	6	2	b	2	6	20	0.6	2	6
(ring structure)	b	c	60	c	b	c	b	20	60	6	c
NH2	b	c	20	c	b	c	b	60	b	60	c
NHPh	b	c	b	c	b	c	b	nt	b	b	c
OtBu	b	c	b	c	b	c	60	60	b	b	c

a. Values are EC$_{80}$ in mg litre^{-1}; b. = >60 mg litre^{-1}; c. = >6 mg litre^{-1}; nt = not tested

Most of the 5-O-alkyl, 5-O-silyl, and 5-O-sulfonyl derivatives had greatly reduced activity (Table 4). Similarly the 5-keto, 5-oximo, and hydrazono analogs were very weak. However many of the esters showed excellent activity comparable with that of soraphen $A_{1\alpha}$ 1 itself (Tables 5 and 6). One notices that the hydrolytically labile formate, dichloroacetate, and glycinate esters are among the most active compounds, indicating that some of the compounds may be acting as pro-drugs. Most interesting is the fact that the deoxygenated compound still shows significant fungicidal activity demonstrating that the hydroxy group in this position is not completely essential.
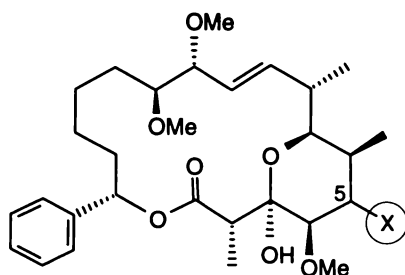
Figure 3. Structure of the compounds shown in Table 3 (X = R),
Table 4 (X = αR), and Tables 5 and 6 (X = αOCOR).

Position 11. The C(11) derivatives were prepared from soraphen C,[20,27] which bears an αOH group at C(11) and is also produced by *Sorangium cellulosum*. In order to derivatize it selectively it was necessary to protect the C(5)-OH group. Silylation blocked both C(5)-OH and C(11)-OH. Subsequent treatment with mild acid selectively removed the C(11)-O-silyl group. The free C(11)-OH group was then derivatized in the usual manner to form the compounds in Tables 7-9 (Figure 4). The desoxy compound was prepared by radical reduction of the 11-chloro compound.
The esters at C(11)-OH (Table 8) were weakly active, apart from the rather labile methoxyacetate and phenylalanylate esters which showed very good activity. The deoxygenated compound had much reduced activity (Table 7), but the C(11) ethers (Table 7) and in particular the C(11)-acetals (Table 9) were extremely potent fungicides. Several of the acetals were even more active than soraphen $A_{1\alpha}$ 1 itself. One of them, the methoxyethoxymethoxy (MEM) ether, was tested in field trials, and was found to be also somewhat more effective than soraphen $A_{1\alpha}$ in this environment. However, as the increase in activity did not make up for the increase in difficulty of preparation and thus the cost, the compound was not pursued further.

Position 12 The key intermediate for the preparation of 12-substituted soraphen derivatives was a 5-O-silylated 12-hydroxy compound (= 5-O-silylated soraphen V), which was obtained by a two-step demethylation of the methoxy group at the 12 position of soraphen $A_{1\alpha}$.[21] With a silyl group protecting the C(5)-OH moiety the 12 hydroxy group was manipulated in the usual manner to the compounds shown in Tables 10 and 11 (Figure 5).[28] The epimeric compound was prepared by reduction of the 12-ketone and the 12-iodo derivative by substitution of the triflate.

Table 7. The fungicidal activity of soraphen A derivatives modified at position 11.[a]

X = R (Fig. 4)	Puccinia recondita on Wheat Foliar	Puccinia recondita on Wheat Soil	Erysiphe gramanis on Barley Foliar	Erysiphe gramanis on Barley Soil	Pyricularia oryzae on Rice Foliar	Pyricularia oryzae on Rice Soil	Cercospora arachidicola on Peanut Foliar	Venturia inequalis on Apple Foliar	Botrytis cinerea on Apple Fruit	Botrytis cinerea on Bean Foliar	Rhizoctonia solani on Rice Soil
OH	60	6	6	2	b	2	nt	6	0.6	6	c
βOH	b	c	b	c	b	c	60	b	b	b	c
H	b	c	60	c	b	c	6	60	60	20	c
Cl	b	c	20	6	nt	nt	6	6	b	20	c
=O	b	6	b	c	nt	nt	b	b	60	b	c
=NOMe	b	c	60	2	nt	nt	60	60	20	b	c
OEt	20	2	20	2	b	2	2	2	0.6	2	2
OnBu	20	c	6	c	nt	nt	6	<0.6	0.6	6	6
OiPr	20	2	2	2	nt	nt	2	0.2	0.2	2	0.6
OtBu	60	6	2	6	b	c	2	2	0.6	6	2
OAllyl	60	6	2	0.6	b	c	20	0.6	2	0.2	6
OBn	60	c	6	c	nt	nt	6	0.6	2	0.6	c
OSiMe2tBu	b	c	b	c	b	c	b	b	b	b	c

a. Values are EC_{80} in mg litre^{-1}; b. = >60 mg litre^{-1}; c. = >6 mg litre^{-1}; nt = not tested

Table 8. The fungicidal activity of soraphen A esterified at position 11.[a]

X = OCOR (Fig. 4)	Puccinia recondita on Wheat Foliar	Puccinia recondita on Wheat Soil	Erysiphe gramanis on Barley Foliar	Erysiphe gramanis on Barley Soil	Pyricularia oryzae on Rice Foliar	Pyricularia oryzae on Rice Soil	Cercospora arachidicola on Peanut Foliar	Venturia inequalis on Apple Foliar	Botrytis cinerea on Apple Fruit	Botrytis cinerea on Bean Foliar	Rhizoctonia solani on Rice Soil
H	60	2	20	2	b	2	2	6	2	6	c
CH₂OMe	60	c	6	20	b	2	6	6	0.6	2	c
COOH	b	c	6	6	b	c	20	20	6	b	c
NHBoc / iPr	b	c	6	6	b	c	20	20	20	b	c
NH₂ / iPr	b	c	20	6	b	c	6	20	6	60	c
NHBoc / SMe	b	c	20	6	b	c	6	20	20	b	c
NH₂ / SMe	b	c	6	2	b	c	6	6	6	20	c
NHBoc / Ph	b	c	20	6	b	6	20	60	b	b	c
NH₂ / Ph	b	c	20	0.6	60	2	2	20	20	20	c
OMe	6	6	6	2	b	c	6	6	2	6	c
NHMe	20	c	b	c	b	c	20	6	6	6	c
NHPh	b	c	60	c	b	c	b	b	20	b	c

a. Values are EC_{80} in mg litre^{-1}; b. = >60 mg litre^{-1}; c. = >6 mg litre^{-1}; nt = not tested

Table 9. The fungicidal activity of 11-acetal derivatives of soraphen A.[a]

X = R (Fig. 4)	Puccinia recondita on Wheat Foliar	Puccinia recondita on Wheat Soil	Erysiphe gramanis on Barley Foliar	Erysiphe gramanis on Barley Soil	Pyricularia oryzae on Rice Foliar	Pyricularia oryzae on Rice Soil	Cercospora arachidicola on Peanut Foliar	Venturia inequalis on Apple Foliar	Botrytis cinerea on Apple Fruit	Botrytis cinerea on Bean Foliar	Rhizoctonia solani on Rice Soil
OCH_2OMe	60	2	0.6	0.2	20	6	0.6	0.2	0.6	0.6	2
OCH_2OEt	60	c	2	2	b	c	2	0.6	2	0.2	6
OCH_2OiPr	20	c	2	6	20	0.2	0.6	2	0.6	0.2	6
OCH_2OnBu	60	c	2	c	60	c	0.6	2	2	2	c
OCH_2OnOct	b	c	20	c	b	c	20	6	20	b	c
OCH_2OBn	b	c	20	c	b	c	2	6	20	6	2
$OCH_2OCH_2CH_2OMe$	6	c	2	6	b	6	0.6	0.6	0.6	2	6
OCH_2SMe	60	6	2	2	b	c	2	2	2	2	6
OCH_2OAc	60	6	6	0.6	b	c	2	0.6	6	20	c
OTHP	60	c	2	6	2	0.6	2	6	2	0.2	6
OCHMeOMe	20	6	2	0.6	2	2	0.6	2	2	2	6
OCHMeOMe	20	6	2	0.6	<2	0.6	0.2	2	2	0.6	c
(sugar structure)	b	c	60	2	b	c	60	20	20	b	c

a. Values are EC_{80} in mg litre^{-1}; b. = >60 mg litre^{-1}; c. = >6 mg litre^{-1}; nt = not tested

Figure 4. Structure of the compounds shown in Tables 7 and 9 (X = αR), and Table 8 (X = αOCOR).
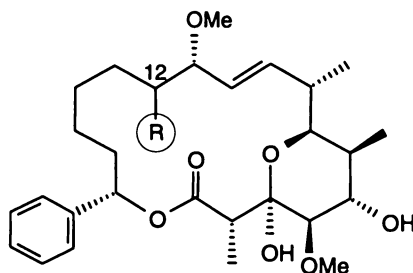
Figure 5. Structure of the compounds shown in Table 10 and 11.

Nearly all of the compounds in this series described here were very weak fungicides. However, the 12β-iodo compound showed a high level of fungicidal activity, approximately equal to that of soraphen A itself. This is particularly surprising as there is no longer an oxygen-containing substituent at this position. A further distinctive feature of this compound is that on soil application no control at all was observed with the highest dose, but on foliar application it showed activity as potent as that of soraphen A_{1_α}. We can offer no explanation for this observation.

Model Compounds.
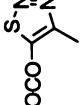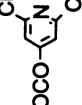
It is difficult to draw conclusions from structure-activity data derived from such a complex target system as a fungus infecting a plant embedded in the earth. On trying to decide which parts of the molecule were important it was thought initially that the bottom-half was more relevant than the top-half. This was because the 11-OMe could be extensivly modified without loss of activity, and the 12-OMe could be replaced with iodide. The 9,10 dihydro compound (soraphen F) is also quite active, so that it appeared to us that the functionality present in the top half of the molecule is non-essential, and that the role of the top-half may be to maintain the bottom-half in the appropriate conformation.

Table 10. The fungicidal activity of soraphen A derivatives modified at position 12.[a]

R (Fig 5)	Puccinia recondita on Wheat Foliar	Puccinia recondita on Wheat Soil	Erysiphe gramanis on Barley Foliar	Erysiphe gramanis on Barley Soil	Pyricularia oryzae on Rice Foliar	Pyricularia oryzae on Rice Soil	Cercospora arachidicola on Peanut Foliar	Venturia inequalis on Apple Foliar	Botrytis cinerea on Apple Fruit	Botrytis cinerea on Bean Foliar	Rhizoctonia solani on Rice Soil
βOH	b	c	b	c	b	c	b	b	60	60	c
αOH	b	c	60	c	b	c	60	b	60	b	c
H	b	c	b	c	b	c	60	60	2	20	c
αI	b	c	b	c	b	c	60	20	b	b	c
βI	6	c	6	c	2	c	6	2	<2	<2	2
=NOH	b	c	b	c	b	c	b	b	6	b	c
αOH βMeO	b	c	60	c	b	c	20	b	b	b	c
βOH αMeO	b	c	b	c	b	c	b	b	b	b	c
OCONHPh	b	c	b	c	b	c	b	b	60	b	c
OSO₂Me	60	c	b	c	b	c	b	b	6	60	c
OCSOTol	b	c	b	c	b	c	b	b	b	b	c

a. Values are EC_{80} in mg litre⁻¹; b. = >60 mg litre⁻¹; c. = >6 mg litre⁻¹; nt = not tested

Table 11. The fungicidal activity of soraphen A derivatives modified at position 12.[a]

R (Fig 5)	Puccinia recondita on Wheat Foliar	Puccinia recondita on Wheat Soil	Erysiphe gramanis on Barley Foliar	Erysiphe gramanis on Barley Soil	Pyricularia oryzae on Rice Foliar	Pyricularia oryzae on Rice Soil	Cercospora arachidicola on Peanut Foliar	Venturia inequalis on Apple Foliar	Botrytis cinerea on Apple Fruit	Botrytis cinerea on Bean Foliar	Rhizoctonia solani on Rice Soil
$OCH_2OCH_2CH_2OMe$	20	c	b	c	b	c	b	b	<2	20	c
OCH_2OMe	6	c	b	c	b	c	b	b	<2	20	c
OEt	20	6	b	c	b	c	60	60	6	60	c
OBn	20	c	6	c	nt	nt	60	60	2	2	c
OAllyl	20	c	6	c	nt	nt	60	60	b	6	c
OtBu	b	c	b	c	b	c	b	b	60	b	c
OCOMe	b	c	b	c	b	c	b	b	b	b	c
OCOtBu	b	c	b	c	b	6	b	b	b	b	c
$OCOCH_2OMe$	b	c	b	c	b	c	b	b	b	b	c
OCO-(thiazole)	b	c	b	c	b	6	b	b	b	b	c
OCOPh	b	c	b	c	b	2	b	b	b	b	c
OCO-(dichloropyridine)	b	c	b	c	b	c	b	b	b	b	c

a. Values are EC_{80} in mg litre^{-1}; b. = >60 mg litre^{-1}; c. = >6 mg litre^{-1}; nt = not tested

To examine this possibility many compounds embodying bottom-half substructures of soraphen $A_{1\alpha}$ were prepared including compounds containing the full bottom-half functionality (Fig. 6).[12-16] Unfortunately none of these compounds showed fungicidal activity at application rates more than a thousand times higher than the EC_{80} value of soraphen A. We thus have no model to explain the activity of the various compounds.
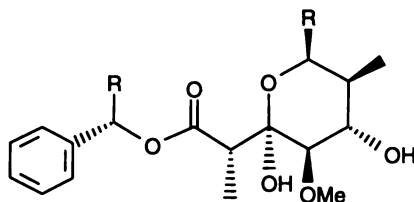
Figure 6. Soraphen bottom-half substuctures

Conclusion

Using the appropriate strategies it was possible to derivatize selectively many parts of the soraphen molecule. Most of the compounds prepared were less fungicidally active than the parent soraphen A. This is perhaps understandable and typical for the case of when the structural integrity of a highly potent natural product is altered. Nevertheless a number of very active compounds were prepared comparable with soraphen A, and in the case of the 11-acetals, a series of compounds were prepared with improved fungicidal activty.

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Chapter 20

Bombyx Prothoracicotropic Hormone: Chemistry and Biology

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Prothoracicotropic hormone (PTTH) is a brain neuropeptide that activates prothoracic glands to produce ecdysone, thereby playing a central role in the endocrine control of postembryonic development in insects. PTTH was first isolated from heads of the adult silkworm, *Bombyx mori*, and its amino-terminal sequence was determined. Subsequently, the entire amino acid sequence and dimeric structure of PTTH were elucidated. *Bombyx* PTTH is a homodimeric peptide composed of two identical chains which are held together by disulfide bond(s). Each chain consists of 109 residues and contains a carbohydrate chain bound to an asparagine at position 41. Furthermore, we developed methods of expressing *Bombyx* PTTH in *Escherichia coli* and in a baculovirus system, and succeeded in producing biologically active recombinant PTTH. The location of disulfide bonds in *Bombyx* PTTH was determined in recombinant PTTH produced by transgenic *E. coli*. The structure of the carbohydrate chain in *Bombyx* PTTH produced by the baculovirus system and that in the native hormone were determined. Furthermore, we isolated a cDNA of *Bombyx* PTTH homologue from the saturniid moth, *Samia cynthia ricini*.

The life cycle of insects is characterized by metamorphosis and ecdysis. These dramatic events have long been the subject of biological investigation. In 1922, Kopec, a Polish scientist, first documented that metamorphosis in gypsy moth was under the control of "the factor" in the brain (*1*). This was the first report that the brain could function not only as a center of the nervous system, but also as an endocrine organ. Since then much effort by entomologists and biochemists has been focused on these subjects, establishing that "the classical scheme"--namely the brain factor, which is currently called prothoracicotropic hormone (PTTH), stimulates the prothoracic glands to synthesize and release the molting hormone, which regulates metamorphosis and ecdysis in combination with juvenile hormone released from the corpora allata.

In the study of the chemistry of insect hormones, remarkable success was first achieved by isolation and structure determination of ecdysone in 1965 and juvenile hormone in 1967. Although many researchers have made efforts to purify PTTH from various insects for chemical characterization, its chemical structure had not been determined until 1987. Chemical characterization of PTTH should have overcome a multitude of difficulties caused by the low concentration of hormones in insects and the hydrophilic properties of peptides. The keys to eliminating these difficulties could be the collection of large amounts of insects as starting materials for extraction of PTTH and/or application of up-to-date "protein micro-chemistry" as well as the use of new technologies in biosciences, such as monoclonal antibodies, gene cloning and sequencing. Japan has a long history in sericulture (although Japanese sericulture is in decline), and has been able to supply large quantities of silkworms, *Bombyx mori*, for extracting insect hormones, as is well-known by the examples of bombykol and ecdysone.

In 1987, PTTH was first isolated from heads of the adult silkworm, and its amino-terminal sequence was determined (2). Subsequently, the entire amino acid sequence and dimeric structure of PTTH were elucidated by a combination of peptide analysis (3) and molecular cloning of cDNA (4). This article summarizes our studies on *Bombyx* PTTH, and our recent progress towards the identification of PTTH molecules in other lepidopteran insects.

Purification and Primary Structure of *Bombyx* PTTH

The bioassay system we used for PTTH of *B. mori* was developed by Kobayashi and later improved by Ishizaki et al. (5). When the brain is removed from *Bombyx* pupae shortly after pupation, brainless pupae remain as pupae for a while without undergoing adult development. Injection of a solution containing PTTH into debrained dormant pupae brings about resumption of adult development whereas a negative control solution causes no development. The responses are judged by wing apolysis 3-6 days after injection and PTTH activity is quantified in terms of *Bombyx* PTTH unit, which represent a half maximal response (5).

After a long-term effort at preliminary purification to select an efficient procedure, we finally established a scheme for purification of *Bombyx* PTTH (2). The scheme consisted of 16 steps including five HPLC procedures and yielded 5.4 μg pure PTTH from 5×10^5 *Bombyx* heads (3.75 kg). As little as 0.1 ng of pure PTTH induced adult development in a *Bombyx* brainless pupa. The amino acid analysis showed that *Bombyx* PTTH characteristically contained large proportions of Asx, Glx and Pro residues and small proportions of Met and Phe residues. Sequence analysis of pure PTTH revealed that the amino-terminal amino acid sequence of *Bombyx* PTTH is Gly-Asn-Ile-Gln-Val-Glu-Asn-Gln-Ala-Ile-Pro-Asp-Pro (2). Unfortunately, the pure PTTH obtained from 5×10^5 heads was exhausted by these analyses.

To obtain more sequence information, PTTH was purified again from 3×10^6 heads through the same scheme. Following an ion-exchange HPLC, PTTH activity was recovered from four peaks (I, II, III and IV). Fractions II and III were further purified by reversed-phase HPLC and PTTH activity was recovered again in multiple peaks (II-1~5 and III-1~5). These peaks apparently overlapped in profile rather than being sharp and well-separated peaks, giving the impression of an impure state of PTTH. However, the specific activities of these peaks were almost the same among the peak fractions. The overlap of the peaks was assumed to be due to the presence of highly heterogeneous molecular forms of PTTH that were structurally very similar, rather than to contaminating proteins. Amino-terminal sequencing of material from each purified fraction revealed that three

kinds of amino acid sequences were present in all of them. Quantitative analysis of these sequence data established that *Bombyx* PTTH has heterogeneity in the amino-terminal starting position. The amino-terminal amino acid sequence of the most extended form was the same as described above. Moreover, two shortened sequences in which six and seven amino acids were missing from the amino-terminal end of the longest sequence were present, but no other amino acid sequence was detected. However, all fractions possessed similar amino acid composition and showed almost identical peptide elution patterns on HPLC after V8 protease digestion. These data suggest that the heterogeneity of *Bombyx* PTTH is derived at least partly from the amino-terminal length and that the amino acid sequences of PTTH in different fractions are identical or very similar. Based on this assumption, we gathered sequencing data of fragment peptides obtained by enzyme digestion of each fraction and constructed the sequence of *Bombyx* PTTH up to the 104th residue from the amino-terminus (3). The 41st residue was undetermined since no PTH amino acid was detected at this cycle of Edman degradation.

cDNA and Genomic DNA Structures

We tried cloning the gene of PTTH from a cDNA library of *Bombyx* brain using synthetic nucleotide probes designed on the basis of peptide sequences of the amino-terminus and several fragments produced by enzyme digestion of *Bombyx* PTTH, but we could not obtain any clone that had PTTH cDNA. Therefore, we used another cloning strategy using an antibody against a synthetic peptide corresponding to the amino terminal 1-15 amino acid sequence for screening a cDNA expression library.

Two *Bombyx* PTTH cDNAs were obtained: one covered the entire coding region while the other lacked the 5' portion of the coding region. Both contained a putative PTTH domain that codes for 109 amino acids at the 3' end of the coding region, the first 104 of which were identical to the sequence of the PTTH described above. Thus it became clear that peptide analysis of purified natural PTTH had only 5 amino acids left to be determined before reaching the carboxyl-terminus. The 41st residue, which remained unidentified by peptide analysis, has now been shown to be Asn, indicating that a carbohydrate moiety is attached to this residue. As shown schematically in Fig. 1-A, the coding region of the PTTH cDNA encodes a signal peptide (29 amino acids), a 2-kDa peptide (21 amino acids), a 6-kDa peptide (57 amino acids), and PTTH (109 amino acids) in this order from the 5' end. The entire amino acid sequence of *Bombyx* PTTH has thus been elucidated (4).

We cloned two PTTH genes, which were concluded to be allelic, from a *Bombyx* genomic DNA library using the PTTH cDNA as a probe (6). As shown diagrammatically in Fig. 1-B, the genes encoding a precursor protein for PTTH (~3 kb) consist of 5 exons. The second-to-fifth exons contain the regions coding for PTTH. Southern hybridization analysis showed that the *Bombyx* haploid genome contained a single copy of the PTTH gene. In the protein coding region, no homology has yet been found to any other known genes as examined by database searching.

Dimeric Structure of PTTH and Production of *Bombyx* PTTH in *E. coli*

Several purified fractions of *Bombyx* PTTH were subjected to SDS-PAGE under non-reducing or reducing conditions. All fractions under non-reducing conditions showed a broad band corresponding to 30 kDa, whereas under reducing conditions two sharp bands with molecular mass of 17 and 16 kDa were detected, and the

relative density of these two bands varied among the fractions. To characterize the peptides derived from intact PTTH by the cleavage of disulfide bonds, the purified PTTH was reduced, alkylated and fractionated by HPLC. This procedure yielded one or two major peaks together with several minor peaks. Sequence analysis of the major peaks yielded the same single sequence as the amino-terminal sequences of intact PTTH. In addition, the amino acid composition of each peak was almost identical with that of intact PTTH. On the basis of all these data, we concluded the following. *Bombyx* PTTH is a homodimeric peptide, the subunits of which are linked together by one or more disulfide bond(s). The two subunits are nearly identical in amino acid sequence differing by a short sequence deletion only at their chain termini. A carbohydrate moiety, which is presumed to be present in the PTTH molecule, may also be responsible for the molecular variation of the subunits.

To confirm that the peptide, whose sequence determined by sequencing of the purified peptide and molecular cloning of the gene, actually has PTTH activity, we produced recombinant *Bombyx* PTTH using an *E. coli* system. When a portion of the cDNA designed to express the PTTH subunit peptide was introduced into *E. coli*, PTTH activity functionally indistinguishable from that of natural PTTH was detected in cell lysate, showing that the cDNA that we cloned does indeed encode the *Bombyx* PTTH and that glycosylation is not essential for biological activity (4). The recombinant PTTH was purified using the debrained pupal assay. Finally, we obtained 1 mg of biologically active recombinant PTTH from 1 L of culture. The recombinant PTTH possesses a specific activity comparable to that of the natural PTTH. Furthermore, SDS-PAGE analysis showed that recombinant PTTH also has a dimeric structure linked by disulfide bond(s).

Using this recombinant PTTH, we tried to determine the disulfide bond location of *Bombyx* PTTH. First, we partially reduced PTTH using tributylphosphine in the presence of the S-alkylating reagent, 4-vinylpyridine. The partial reduction and S-pyridylethylation broke the interchain disulfide bond but did not affect the intrachain disulfide bonds, generating monomeric PTTH whose intrachain disulfide bonds remained intact. Sequence analysis of the fragments generated by lysyl endopeptidase digestion of this monomeric PTTH after complete reduction and S-carboxymethylation showed that the Cys15 residue was reduced and S-pyridylethylated, indicating that this residue formed the interchain disulfide bonds. The other disulfide bonds, which formed intrachain bridges, were determined by sequence and mass analyses of the fragments generated by two successive enzyme digestions of monomeric PTTH. In conclusion, the disulfide bond location of PTTH was assigned to Cys15-Cys15' as an interchain disulfide linkage and Cys17-Cys54, Cys40-Cys96, and Cys48-Cys98 as intrachain disulfide linkages as shown in Fig. 2 (7). Interestingly, the partially reduced and S-pyridylethylated PTTH, in which all intrachain disulfide bonds remained intact, retained PTTH activity, although the specific activity of the partially reduced and S-pyridylethylated PTTH was about one-half that of intact PTTH. Since the fully reduced and S-alkylated PTTH monomer had no PTTH activity, the intrachain disulfide bonds must be essential for producing a biologically active conformation, but the interchain disulfide bond is not essential for activity. Although any significant sequence homology to other proteins has not been discovered by data base searching, the sequential arrangement of intrachain disulfide bonds indicates a striking similarity to that of several growth factors, such as β-nerve growth factor, transforming growth factor-β and platelet-derived growth factor. All these growth factors are characterized by two β-sheets and three intra-chain disulfide bonds that link the β-strands, forming a cystine knot motif. By sequence profile analysis and modeling of the structure of PTTH based on these growth factors, PTTH was shown to have two β-sheets and a similar hydrophobic cluster forming
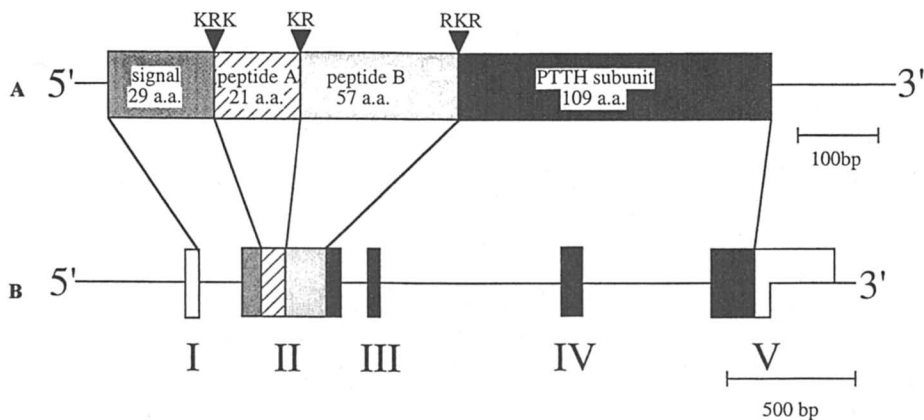
Fig. 1. Schematic model of the cDNA (A) and the genomic DNA (B) of *Bombyx* PTTH.
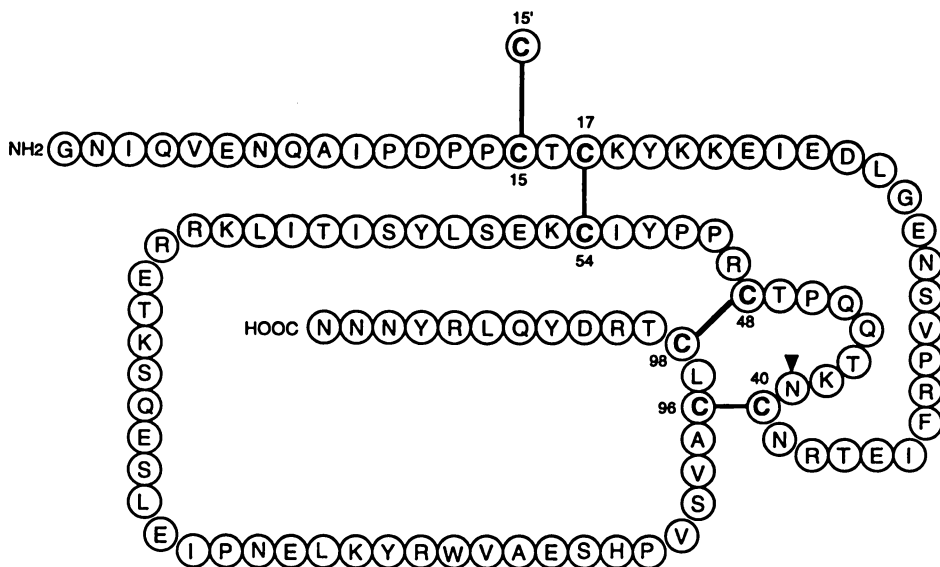
Fig. 2. Amino acid sequece of *Bombyx* PTTH and arrangement of disulfide bonds. N-linked glycosylation site is indicated by a closed triangle.

the cystine knot motif. Thus, PTTH appears to be a member of this growth factor superfamily (*8*).

Structure of the Carbohydrate Chain of *Bombyx* PTTH

The PTTH gene was expressed in a baculovirus system to obtain glycosylated *Bombyx* PTTH. The recombinant plasmid encoding the PTTH gene was cotransfected with a baculovirus in cell culture to yield a recombinant virus. The resulting recombinant virus was then injected into *Bombyx* larvae. After 3 or 4 days, hemolymph from infected larvae was harvested and purified. The yield of recombinant PTTH was about 0.15 mg/larva. Results of HPLC analysis and peptide mapping after lysylendopeptidase digestion showed that the recombinant PTTH was identical with the native hormone, suggesting that the recombinant PTTH expressed in the baculovirus system was glycosylated at the 41st asparagine. Then the structure of the glycosidic side chain on the recombinant PTTH produced by the baculovirus system was determined to be Manα1-6Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc-. Also, the saccharide chain of the native hormone has been determined to be identical to that of the recombinant hormone (*9*). The biological activity in brainless *Bombyx* pupae was carefully compared between the native hormone and the recombinant hormones produced in *E. coli* and in the baculovirus expression system. In conclusion, the native hormone and the recombinant hormone from the baculovirus system showed completely identical dose-responses, whereas the recombinant hormone produced in *E. coli* showed slightly weaker activity, indicating that the glycosidic side chain in prothoracicotropic hormone stabilizes the hormone molecule in the hemolymph to reinforce the hormonal activity.

Biological Characterization of *Bombyx* PTTH

Since we used an *in vivo Bombyx* pupal-adult assay for determination of PTTH activity during purification, it is of interest whether this PTTH has activity *in vitro* and at other stages. Both the purified PTTH and the recombinant PTTH produced in *E. coli* activated day-0 pupal prothoracic glands to secrete ecdysone at a concentration of 10^{-11} M in the culture medium (unpublished data). This result indicates that the purified PTTH and the recombinant PTTH act directly on prothoracic glands to induce adult development in pupae. In addition, when injected into debrained 4th instar larvae, the purified PTTH induced larval molting, suggesting that it functions in the same manner as does the PTTH present at the larval stage. All these data show that the PTTH purified from adult heads is a genuine PTTH in *B. mori*. Moreover, when changes in the amount of PTTH mRNA in the *Bombyx* brain from the first instar larvae to the pupae was examined by means of Northern-blot analysis, the PTTH mRNA amount/total RNA ratio did not change appreciably during the developmental period examined (*6*). This result indicated that the timing of PTTH function is not regulated at the transcriptional level, but might be regulated at the point of release into the hemolymph.

A monoclonal antibody recognizing PTTH was raised against a synthetic peptide corresponding to the amino-terminal 1-15 amino acid sequence of *Bombyx* PTTH (*10*). This antibody immunostained two pairs of dorsolateral neurosecretory cells of *Bombyx* brain. The immunoreactive material was also detected in the axons of these neurosecretory cells which run across the brain midline down to the contralateral corpora allata, suggesting that PTTH might be liberated into the hemolymph from the corpora allata as shown using *in vitro* bioassay by Agui *et al.* (*11*). *In situ* hybridization using the *Bombyx* PTTH cRNA as a probe located the PTTH mRNA in the same two pairs of brain dorsolateral neurosecretory cells (*4*),

```
                                                             20                              40 ▼
Bombyx: G N I Q V E - - N Q A I P D P P Q T Q K Y K K E I E D L G E N S V P R F I E T R N Q N K T Q Q P T Q
Samia:  G D L R R E K H N Q A I Q D P P Q S Q G Y T Q T L L D F G K N A F P R H V V T R N Q S - D Q Q Q S Q
                                               20                                           40 ▲

                                     60                                       80
Bombyx: R P P Y I Q K E S L Y S I T I L K R R E T K S Q E S L E I P N E L K Y R W V A E S H P V S V A Q L Q
Samia:  L F P Y V Q K E T L Y D V N I L K R R E T S T Q I S E E V P R E L K F R W I G E K W Q I S V G Q M Q
                                  60                                       80

           100
Bombyx: T R D Y Q L R Y N N N
Samia:  T R D Y R N S T E D Y Q P R L L T K I I Q Q R D L S
           100                              120
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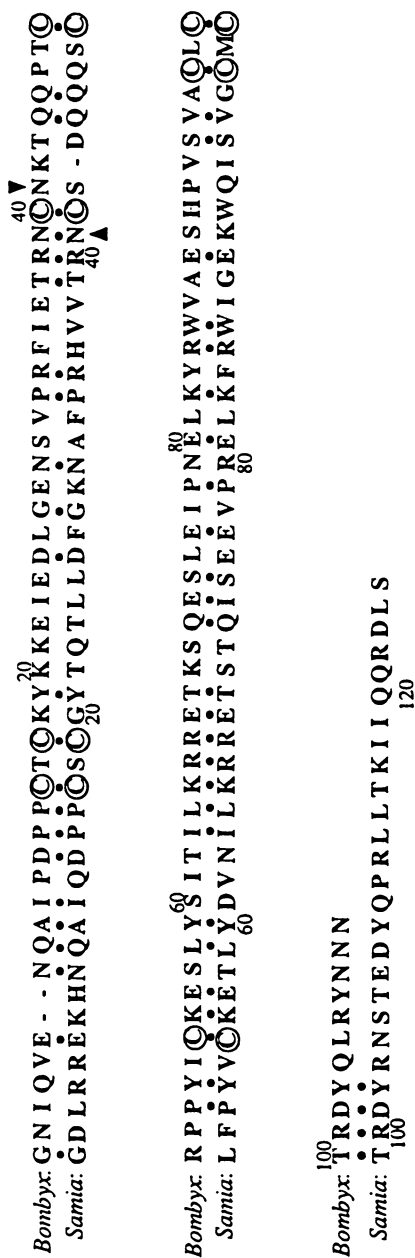
Fig. 3. Amio acid sequences of the Bombyx PTTH and Samia PTTH subunits. Gaps are introduced for maximum alignment. Dots indicate identities. Possible N-linked glycosylation site is indicated by closed triangles.

indicating that PTTH is actually synthesized in these cells. Recently, we made a polyclonal antibody against the recombinant PTTH produced in *E. coli*, that recognized the entire PTTH molecule. So far, the same dorsolateral neurosecretory cells in the brain of *B. mori* and *Agrius concolvoli* were immunostained using this antibody. Now we are trying to detect PTTH-producing cells of other lepidopteran insects using this antibody.

Molecular Cloning of Samia PTTH

Since immunohistochemical studies revealed that a *Bombyx* PTTH homologue exists in other lepidopteran insects, we tried to isolate cDNA of these homologues using *Bombyx* PTTH cDNA as a probe. Quite recently, we succeeded in cloning several cDNAs from the saturniid moth, *Samia cinthia ricini*. The amino acid sequence of the codeing region was predicted as shown in Fig. 3 (*12*). The structure of the precursor molecule is similar to that of *Bombyx* PTTH and a region homologous to *Bombyx* PTTH is located in the carboxyl-terminus of the precursor.*Samia* PTTH has seven cysteine residues and one potential glycosylation site. These features are highly similar to those in *Bombyx* PTTH, although the glicosylation site is slightly different. The homology between *Bombyx* and *Samia* PTTH is relatively low (about 50%) and *Samia* PTTH has an additional 15 residues at the carboxyl-terminus, compared to *Bombyx* PTTH. We have developed a method of *Samia* PTTH expressing in *E. coli* and the PTTH activity of *Samia* was determined by a *Samia* pupal assay. This recombinant *Samia* PTTH was capable of inducing adult development at a dose of 0.3 ng/pupa when injected into brainless *Samia* pupae. This activity is about the same as that of *Bombyx* PTTH (0.3ng/Bombyx brainless pupa).*Samia* PTTH showed PTTH activity in *Samia*, but not in *Bombyx*. Conversely, *Bombyx* PTTH showed PTTH activity in *Bombyx*, but not in *Samia*. From these results, we conclude that a molecule homologous to *Bombyx* PTTH acts as PTTH in insects, at least, in lepidopteran insects, and that PTTH has species specificity due to low amino acid sequence homology between species.

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Chapter 21

Active Conformation and Mimetic Analog Development for the Pyrokinin–PBAN–Diapause–Pupariation and Myosuppressin Insect Neuropeptide Families

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Members of the insect pyrokinin/PBAN and myosuppressin neuropeptide families regulate and/or influence such physiological processes as pheromone production, oviduct contraction, digestive enzyme release and the developmental processes of egg diapause and larval pupariation in a variety of insects (1, 2, 3). However, these peptides hold little promise as pest control agents due to their susceptibility to enzymatic degradation in the target insect, and to an inability to readily penetrate the insect cuticle or gut wall. In this chapter, we discuss active conformations adopted by the peptides at the receptor site and the development of active pseudopeptide and nonpeptide mimetic analogs with enhanced resistance to peptidase attack and/or topical activity in an intact insect. Disruption of peptide-regulated processes or behavior by mimetic agonist and/or antagonist analogs could form the basis for future pest insect management strategies.

The Pyrokinin/PBAN/Diapause/Pupariation Family

The pyrokinin family of insect neuropeptides is widespread in several insect orders and has been associated with a variety of physiological functions (1). Although the first pyrokinin, leucopyrokinin (LPK) was originally identified as a hindgut and oviduct myotropin of the cockroach *Leucophaea maderae* (4), additional members have since been isolated from other insect species on the basis of their ability to stimulate contractions of the oviduct of the locust (5-8), sex pheromone biosynthesis in the corn earworm (9), gypsy moth (10), and silkworm (11,12), reddish coloration and melanization in larvae (13) and diapause induction in eggs of the silkworm (14).

The cockroach peptide LPK has also been found to accelerate pupariation of larvae of the fleshfly *Sarcophaga(Neobellieria) bullata*, mimicking the activity of natural, proteinaceous factors in the hemolymph and extracts of various neural and neurohemal organs (2, 15). Although the members of this class of insect neuropeptides vary from 8 to 33 amino acids in length (1), they share the common C-terminal pentapeptide Phe-X-Pro-Arg-Leu-NH$_2$ (X=Gly, Ser, Thr, Val) (16). The common C-terminal pentapeptide accounts for the considerable cross-activity observed for members of this family of peptides from diverse insect sources in different physiological systems (16-19). PBAN demonstrates significant myostimulatory activity on the isolated cockroach hindgut (17, 20). Myotropic pyrokinins from the locust elicit activity greater than the natural peptide factors in diapause induction and pheromonotropic assays of the silkworm (16, 17) and corn earworm (19), despite containing a fourth of the amino acid constituents. For instance, the dose of the dodecapeptide locust pyrokinin Lom-PK (variable X = Val) required to elicit 50% egg diapause in the silkworm *Bombyx* was 3.4 pmol/pupa, over threefold more potent than the longer Bom-DH-I [19-Cys] (10.5 pmol/pupa) and over sixfold more potent than Bom-DH-I [19-Trp] (21 pmol/pupa) (16). This superagonist activity is all the more exceptional given that Lom-PK is half the size of the hormone Bom-DH. The cross-activity observed for the smaller members of the pyrokinins emphasize the importance of the C-terminal region to activity in the biological systems associated with this family. Indeed, the C-terminal pentapeptide common to all members of the pyrokinin/PBAN family is both essential and sufficient to elicit at least some myotropic, pheromonotropic, diapause induction, and melanization and reddish coloration hormone activity (16-20). The observed potency is, however, only a fraction of that of the respective parent pyrokinin peptide in pheromonotropic (17, 19), melanization (18) and diapause induction assays (16). By contrast, in the cockroach hindgut myotropic assay, the pentapeptides Phe-X-Pro-Arg-Leu-NH$_2$ (X = Gly, Ser, Thr) demonstrate activity at a threshold concentration of about 2 nM, or a considerable 30% of the potency of the parent cockroach leucopyrokinin (21). The C-terminal tetrapeptide Thr-Pro-Arg-Leu-NH$_2$ is equipotent with LPK in eliciting pupariation acceleration in larvae of the fleshfly, whereas the tripeptide Pro-Arg-Leu-NH$_2$ elicits pupariation at 10% of the potency of the parent structure (15).

Data on the "active conformation" adopted by the C-terminal pentapeptide region of the pyrokinins was obtained by analysis of the conformation of an active and rigid, cyclic analog (*cyclo*[Asn-Thr-Ser-Phe-Thr-Pro-Arg-Leu]) by a combination of spectroscopic and computer molecular dynamics techniques (22, 23). Analysis of a rigid, restricted-conformation pyrokinin analog, as opposed to flexible linear forms, is a requirement for a valid assessment of the conformation adopted by this peptide family at the various receptor sites. A computer graphics representation of the cyclic structure revealed a ß-turn encompassing residues Thr-Pro-Arg-Leu in the C-terminal pentapeptide core region (Figure 1). NMR spectroscopic data demonstrated that the cyclic peptide was highly rigid and that the bond between the Thr and Pro in the core region was exclusively *trans* (22). The rigid, cyclic pyrokinin analog has been shown to retain considerable activity in cockroach hindgut (22) and oviduct (24) myotropic bioassays and the pheromonotropic assay in *B. mori* (23, 25). In each case, the activity of the cyclic analogs proved to be considerably greater than that of linear fragments containing an intact active-core sequence that could have arisen from peptidase cleavage during the course of the bioassay. The cyclic analog retained about 10% of

the activity of PBAN in a *B. mori* pheromonotropic assay and 4% of the myotropic activity of the parent peptide [Asn[1]]LPK on the cockroach hindgut assay. The linear, C-terminally "appended" fragment-analogs Thr-Ser-Phe-Thr-Pro-Arg-Leu-Asn-OH, Ser-Phe-Thr-Pro-Arg-Leu-Asn-Thr-OH and Phe-Thr-Pro-Arg-Leu-Asn-Thr-Ser-OH, each containing an intact pentapeptide pyrokinin core sequence, are at least two orders of magnitude less active than the cyclic analog on the cockroach hindgut and demonstrate no detectable pheromonotropic activity at concentrations an order of magnitude greater than the threshold of the rigid, cyclic analog. The results demonstrated that the activity observed for the cyclic pyrokinin analog is not attributable to linear fragments generated *in situ* by enzyme degradation. They also suggest that much of the reduction in potency observed for the cyclic analog can be attributed to modification of the sensitive C-terminus resulting from addition of amino acid residues at this position. In other words, it is valid to compare the activity of the cyclic analog not only to Bom-PBAN-I, PBAN fragments, and LPK but to these C-terminally "appended" fragment-analogs as well. The retention of activity demonstrated by the cyclic analog suggests that the rigidly held ß-turn in the active core represents the conformation necessary for the pyrokinins to interact with the receptor systems that mediate pheromonotropic and hindgut/oviduct myotropic responses in the two insect systems (22, 23).

The cyclic analog also demonstrates considerable activity in the silkworm diapause induction bioassay, at a potency only somewhat less than that of the parent sequence, LPK (ED_{50} = 2.5 nmol/pupa). In addition, the cyclic analog demonstrates significant activity in the fleshfly pupariation acceleration bioassay, eliciting 10% of the behavioral and 1% of the tanning aspects, respectively (15). The isolated C-terminal tetrapeptide, Thr-Pro-Arg-Leu-NH_2 , which is the minimal lenght sequence to retain the β-turn found in the cyclic analog, is equipotent with LPK. This result underscores the importance of this conformational component of the pyrokinins to a fully successful interaction with the pupariation acceleration receptor of the fleshfly. The structure-activity and pyrokinin cross-activity data suggest that the silkworm diapause hormone and fleshfly pupariation acceleration receptors share homologous features with the silkworm pheromonotropic and cockroach hindgut myotropic receptors, particularly at the site of interaction with the pyrokinin C-terminal region.

Strategies for pyrokinin mimetic analog development have focused on the minimum active sequence ("active core"), the C-terminal pentapeptide Phe-X-Pro-Arg-Leu-NH_2 (X = Gly, Ser, Thr, Val) common to all members of the pyrokinin family. Replacement of the three N-terminal residues of the pyrokinin pentapeptide with the straight methylene chain structure 9-phenylnonanoic acid (9Pna) allowed retention of the phenyl ring of the critical phenylalanine residue, but nevertheless led to a large drop in cockroach hindgut myotropic activity. The analog 9Pna-Arg-Leu-NH_2 stimulated contractions of the cockroach hindgut at an extremely weak threshold of 8 μM. The replacement of the three N-terminal residues represented a loss of two peptide bonds and a proline residue. These chemical groups contribute to the conformation (22, 23) adopted by the pyrokinin peptides during interaction with the receptor and their loss diminishes the turn conformational component of the pseudopeptide analog. Knowledge of the conformational preference of the pyrokinins at the receptor site obtained from studies on the aforementioned rigid, cyclic analog was utilized to design a more potent pseudopeptide analog. In order to promote a
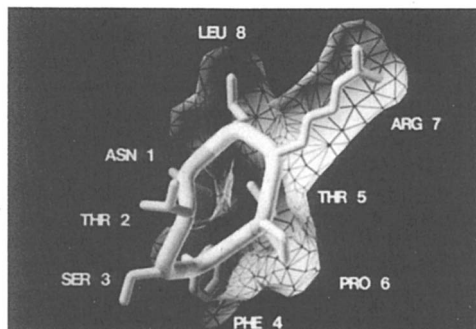
Figure 1. The conformation of cyclo[Asn-Thr-Ser-Phe-Thr-Pro-Arg-Leu], a cyclic pyrokinin analog, includes a β-turn over residues Thr-Pro-Arg-Leu (22). In this conformation, the pentapeptide core residues form a continuous surface that may be presented to the receptor. This figure, as well as Figures 3 and 6, were rendered with the AVS graphics program (Advanced Visual Systems, Inc., Waltham, MA).
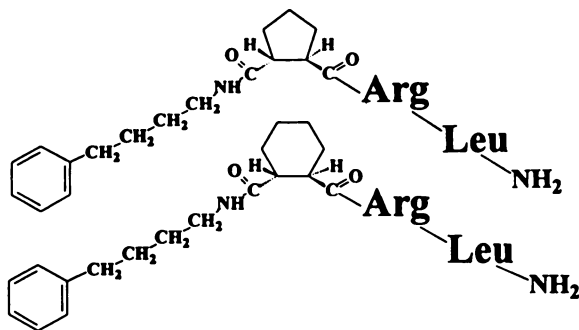
Figure 2. Pyrokinin pseudodipeptide analogs containing 5- (2a; top) and 6-membered (2b; bottom) carbocyclic Pro-mimetic components (Adapted from 26).

conformational preference for a ß-turn-like structure, carbocyclic Pro-mimetic moieties were incorporated into the straight chain of the 9Pna pseudodipeptide analog. Five- and six-membered carbocyclic rings with adjacent carboxyl groups were used as replacements for the Pro residue of the pyrokinin pentapeptide. Specifically, *trans*-DL-1,2-cyclopentanedicarboxyl-(tCpd)- and *trans*-DL-1,2-cyclohexanedicarboxyl-(tChd) moieties were incorporated into the pseudodipeptide structure (26) (Figure 2).

The resulting analogs possess either the five-membered ring or six-membered ring structure of Pro or homoproline (hPro), respectively, but lack the ring nitrogen of the proline residues. Furthermore, the amide bond that links the Pro-mimetic carbocyclic ring with 4-Pbm is reversed compared with a peptide bond, i.e., its order is -HN-C(O)- instead of -C(O)-NH-. Reverse amide or peptide bonds demonstrate marked resistance to peptidase attack (26). Nevertheless, the carbonyl group remains in the same position as in the pyrokinin pentapeptide and is therefore available to participate with the Leu N-H in the hydrogen bond that stabilizes the ß-turn of the X-Pro-Arg-Leu region of the pyrokinins (Figure 3). Evaluation of these carbocyclic pseudodipeptide analogs in the cockroach hindgut bioassay demonstrates that they show improved potency over the straight-chain 9Pna pseudodipeptide. Pseudopeptide 4Pbm-tCpd-Arg-Leu-NH$_2$, with a threshold concentration (TC) of 6 nM and an EC$_{50}$ of 90 nM, is over 1300-fold more potent than 9Pna-Arg-Leu-NH$_2$ and approaches the activity of the parent pyrokinin pentapeptide fragment [TC: 2 nM, EC$_{50}$ = 32 nM]. The pseudodipeptide 4Pbm-tChd-Arg-Leu-NH$_2$ (TC: 64 nM, EC$_{50}$ = 0.9 µM) demonstrated a lower potency by an order of magnitude, indicating that the 5-membered carbocyclic ring of 4Pbm-tCpd-Arg-Leu-NH$_2$ is a better mimic of the 5-sided ring of Pro.

In the typical chair form of the six-membered ring of 4Pbm-tChd-Arg-Leu-NH$_2$, the two adjacent *trans* carboxyl groups can be oriented in a diequatorial or a diaxial conformation. Both the diequatorial and diaxial conformations were superimposed onto the rigid ß-turn involving residues Thr-Pro-Arg-Leu of the cyclic pyrokinin/PBAN analog *cyclo*[Asn-Thr-Ser-Phe-Thr-Pro-Arg-Leu] (26). In the diequatorial orientation, torsional rotation of the cyclohexyl substituents, which have a gauche conformation, provided a hydrogen bond that corresponds to that of the pyrokinin ß-turn (Figure 3a). In this orientation, the carbocyclic ring, the phenyl ring, and the Arg and Leu residues of the tChd pseudodipeptide analog could be readily superimposed onto the proline ring, the phenyl ring, and the Arg and Leu residues of the cyclic pyrokinin/PBAN analog. On the other hand, a corresponding turn conformation could not be formed in the diaxial conformation of the tChd pseudodipeptide analog, which places the cyclohexyl substituents in the anti configuration. Superposition of the main chain of Pro-Arg-Leu in the cyclic pyrokinin/PBAN analog onto the corresponding atoms of the tChd pseudodipeptide analog in the diaxial conformation caused the 4Pbm side chain to extend in the direction of the Pro side chain rather than along the cyclic analog backbone (Figure 3b). This orientation, given the sensitivity of myotropic activity to subtle changes in the group mimicking the Pro side chain (5- versus 6-membered ring, for example), suggests that the diaxial conformation is not the biologically active one. Therefore, a gauche conformation for the adjacent cyclohexyl side chains provides a better mimic of the rigid, cyclic pyrokinin/PBAN analog than the anti conformation, both by allowing hydrogen bond formation and by providing a similar functional group arrangement.
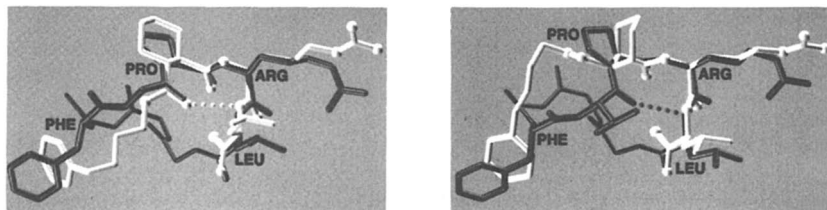
Figure 3. Diequatorial (3a; left) and diaxial (3b; right) conformations of the pseudodipeptide 4Pbm-tChd-Arg-Leu-NH$_2$ (white) (see Figure 2b) superimposed onto the ß-turn conformation present in rigid, cyclic prokinin analog *cyclo*[Asn-Thr-Ser-Phe-Thr-Pro-Arg-Leu] (black) (see Figure 1). The diequatorial but not the diaxial form can mimic the ß-turn of the cyclic analog stabilized by a hydrogen bond (small spheres) (Reproduced with permission from ref. 26. Copyright 1995 Elsevier).
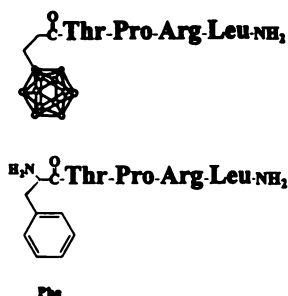
Figure 4. Carboranyl pseudotetrapeptide analog (top) and pentapeptide core (bottom) of the pyrokinins. The cage-like *o*-carborane moiety replaces the phenyl ring of the Phe residue (Reproduced with permission from ref. 27. Copyright 1995 Industrial Publishing and Consulting, Inc.)

In order to impart greater hydrophobic and/or amphiphilic character to the pyrokinin pentapeptide core, the phenyl ring of the Phe residue was replaced with a carborane moiety. The dodecahedral volume created by rotation of the planar phenyl ring is mimicked by the ball shaped carborane structure. It also mimics the aromatic character of the phenyl ring, but is a considerably more hydrophobic structure. Specifically, the Phe residue was replaced by the carborane-ethanoylacyl group (**Cbe** = Cb-CH_2-CH_2-C(O)-[Cb = *o*-carborane]) to yield the pseudotetrapeptide **Cbe**-Thr-Pro-Arg-Leu-NH_2 (27) (Figure 4). On the cockroach hindgut myotropic assay, the carboranyl analog stimulated spontaneous contractions at a TC of 70 pM, over 30x and 10x more potent than the parent pentapeptide core Phe-Thr-Pro-Arg-Leu-NH_2 and LPK, respectively. Remarkably, the carboranyl analog demonstrated a propensity to remain tightly bound to the receptor, as the stimulatory response could not be reversed following repeated rinses with bioassay saline. While the hindgut myostimulatory response of LPK and its pentapeptide core subsided immediately following a saline rinse, the hindgut did not return to a resting state for at least 30 minutes following exposure to **Cbe**-Thr-Pro-Arg-Leu-NH_2 and a saline rinse (27, 28). The greater surface area and hydrophobic character of the carboranyl moiety apparently accounts for the strong receptor-binding characteristics of the pseudotetrapeptide analog.

In contrast with the cockroach hindgut myotropic bioassay, the pyrokinin pentapeptide core demonstrates very little potency in pheromonotropic (17, 19) and diapause induction (16) bioassays. Remarkably, the carboranyl pyrokinin analog demonstrated potent activity in a *Heliothis virescens* pheromonotropic assay with an ED_{50} of 0.1 pmoles/female and elicits a 100% response at 2.5 pmoles/female (27, 28). It is therefore 10x more potent than the 33-membered PBAN molecule. By contrast, at a dose of 70 pmoles/female the pyrokinin pentapeptide core Phe-Thr-Pro-Arg-Leu-NH_2 elicits a truncated maximal response in *H. Zea* of only 30% of the pheromone titre of the carboranyl analog and Hez-PBAN (20). An 1800x greater dose of the pentapeptide core is required to achieve a 30% pheromonotropic titre in *Heliothis* in comparison with the carboranyl pseudotetrapeptide.

While the pyrokinin pentapeptide core Phe-Thr-Pro-Arg-Leu-NH_2 elicits only a 10% egg diapause rate at a dose level of 10 nmoles/pupa in *B. mori* (16), the carboranyl pseudotetrapeptide analog requires only 0.16 nmoles/pupa to achieve a 10% diapause rate and 3 nmole/pupa to reach a 50% egg diapause rate. Therefore, over 60x more pentapeptide core is required than the carboranyl analog to elicit a 10% egg diapause rate. The unmodified pentapeptide core does not appear to be capable of inducing a percentage higher than 10% of the eggs to diapause. Furthermore, the carboranyl analog can induce greater than 40% egg diapause at 1.25 nmoles/pupa and 60% egg diapause at 10 nmoles/pupa. By comparison, the native hormone Bom-DH-I[19-Trp] elicits 40% and 60% egg diapause at 18.5 pmoles and 75 pmoles/pupa, respectively. Carboranyl pseudotetrapeptide analogs containing Val or Gly, instead of Thr, in the variable X position may elicit greater egg diapause activity in the silkworm (27).

The results demonstrate that the C-terminal pyrokinin pentapeptide core and/or analogs contain all of the chemical and conformational information required to interact with both binding and activation regions of the silkworm diapause induction receptor and to <u>fully</u> <u>activate</u> the cockroach hindgut myotropic and tobacco hornworm

pheromonotropic receptor. The relatively high potency observed for the carboranyl pseudotetrapeptide analog is potentially due to its strong receptor binding characteristics. The native peptides form a dynamic equilibrium between a bound state and a solution state in the hemolymph, where they can be inactivated by peptidases. The carboranyl analog is perhaps afforded greater protection from peptidases by virtue of its propensity to remain in a receptor-bound state, in addition to its resistance to aminopeptidases.

The presence of the hydrophobic carborane moiety coupled with the hydrophilic, positively charged Arg residue confers amphiphylic properties to the pseudopeptide analog. Aqueous solutions of the carboranyl peptide readily "wet" the lateral abdominal surface of female *H. virescins*. These topically applied analog solutions also elicit pheromone production after about 15 minutes with an EC_{50} of 25 pmoles/female and a maximal response at about 60 pmoles/female. In contrast, 100 pmole doses of HezPBAN in aqueous solution at 15 min, 1 hr, 2 hr, and 3 hr, following topical application, led to no statistical increase in pheromone production (28). The ability of the analog to penetrate the cuticular surface of the moth means that female moths can be induced to produce pheromone without using invasive procedures, such as injection. Therefore, the carboranyl analog can potentially serve as a useful tool to insect researchers studying aspects of reproduction, endocrinology and behavior of not only *H. virescens* and *zea* but of a wide range of moth species. For example, PBAN was used as a tool in the identification of the pheromone components of *Plodia interpunctella* (29). Injection of PBAN has also been used to study the biosynthetic pathways for pheromones (30). The carboranyl analog could replace PBAN in situations such as these and be delivered in a noninvasive manner. Continuous stimulation of pheromone production by PBAN has been shown to deplete the amount of pheromone produced by *Manduca sexta* by decreasing the triacylglyceride precursors of pheromone components (31). In the future, continuous stimulation of pheromone production may be induced via timed release penetration of the cuticle by topical agents such as the carboranyl pseudopeptide. Should continuous stimulation of pheromone by topically applied agents lead to low levels of pheromone release in moths, the result could be the disruption of species propagation by impairing the ability of males to locate females. Alternatively, should an agent bind irreversibly and desensitize the PBAN receptor, the result could also lead to low levels of pheromone production and, therefore, disruption of reproduction.

Myosuppressin Family

The myosuppressins represent a subfamily of insect FMRFamide-related peptides that share the common C-terminal heptapeptide sequence Asp-His-Val-Phe-Leu-Arg-Phe-NH$_2$ (32, 33), and have been identified in diverse insect species, including the cockroach *Leucophaea maderae* (34), locusts *Schistocerca gregaria* (35) and *Locusta migratoria* (36, 37), the fleshfly *Neobellieria bullata* (38), and the fruitfly *Drosophila melanogaster* (39). The myosuppressins have demonstrated potent inhibition of cardiac and visceral muscle contraction (1, 33, 34, 37, 38, 40). A related peptide, named *Manduca*FLRFamide, was isolated from the hawkmoth *Manduca sexta*

and demonstrates myostimulatory activity on the dorsal longitudinal flight muscles of this moth (41).

The first member of this family to be identified was leucomyosuppressin (LMS: pGlu-Asp-Val-Asp-His-Val-Phe-Leu-Arg-Phe-NH$_2$), isolated from head extracts of the cockroach *L. maderae* on the basis of its ability to inhibit spontaneous contractions of *Leucophaea* proctodeum (hindgut) preparations at a potent threshold concentration of 8.4 x 10^{-11} M (34). The smallest peptide fragment, or "active core", required for cockroach hindgut myoinhibition is the C-terminal pentapeptide fragment VFLRFa, which retains activity at a reduced threshold level of 3.5 x 10^{-8} M (32).

Recently, LMS has been shown to stimulate release of the digestive enzyme α-amylase from digestive tract preparations of two invertebrates: the scallop *Pecten maximus* and the red palm weevil *Rhynchophorus ferrugineus,* the most important pest of the coconut palm throughout South and Southeast Asia (3). LMS elicits over a two-fold increase in α-amylase from a cell suspension of the scallop stomach-digestive gland complex at an EC$_{50}$ of 0.2 nM. In the red palm weevil LMS elicits a two-fold increase of amylase secretion from the isolated midgut when exposed to concentrations between 500 nM and 1 mM (3). LMS mimicked the effect of unidentified factors in extracts of the midgut epithelial tissues of several insects (42). Interestingly, expression of the LMS gene of the cockroach *Diploptera punctata* occurs in cells of the midgut (43), consistent with a possible regulatory role for the myosuppressins in carbohydrase release in insects.

Yamamoto and coworkers (44) have demonstrated that LMS can attenuate evoked transmitter release from the presynaptic membrane of excitatory motor neurons terminating on skeletal muscle of the mealworm, *Tenebrio molitor*. The homologous *Schisto*FLRFamide has been isolated from extracts of the locusts *Schistocerca* (35) and *Locusta* (36, 37) and demonstrates inhibition of proctolin-induced oviduct contractions (37) at a threshold of around 10^{-9} M. The active core for locust oviduct contractile inhibition is the hexapeptide HVFLRFa, which retains potency similar to the parent peptide (37, 45).

Recently, it was observed that the nonpeptide benzethonium chloride (Bztc) shares several chemical features with the sequence VFLRFamide (33, 45). As can be seen in Figure 5, Bztc has zones with branched-chain and basic character and two separate zones with a phenyl ring. It was conjectured that two or more of these structural zones of Bztc might bind with those portions of the LMS receptor that interact with side chains of Leu or Val, Arg, and two Phe residues of the C-terminal pentapeptide and, thus, may be a ligand for the LMS receptor.

Indeed, Bztc mimics the inhibitory activity of the myosuppressins on the isolated cockroach hindgut, mealworm neuromuscular junction, and locust oviduct. On the *Leucophaea* cockroach hindgut, Bztc reversibly inhibits spontaneous contractions at a threshold concentration of 60 nM, close to the 35 nM threshold observed for the myosuppressin C-terminal pentapeptide (45). A dose-response curve for Bztc in the cockroach hindgut assay revealed that myosuppression is observed at an EC$_{50}$ of 6.4 x 10^{-7} M, comparable with the EC$_{50}$ of 4.2 x 10^{-7} M noted for the C-terminal pentapeptide, but less potent than the 2 nM recorded for the full LMS sequence (45).

On a *Tenebrio molitor* mealworm neuromuscular junction preparation, Bztc mimics LMS by attenuating the evoked transmitter release from the presynaptic

membrane of excitatory motor neurons terminating on the skeletal muscle (33, 45). In experiments performed on the ventral longitudinal muscle fibers of the mealworm, Bztc induced reversible suppression of the EPSPs evoked by stimulation of the innervating nerve with a pair of silver electrodes. LMS, when applied to the bathing solution at concentrations higher than 100 nM, produced a reversible reduction of the amplitude of EPSP evoked by nerve stimulation in the muscle fiber. An EC_{50} on the order of 100 µM was observed for Bztc as compared with an EC_{50} of 8 µM for the complete LMS sequence (33, 45) on the mealworm neuromuscular junction preparation. The lipoxygenase inhibitor nordihydroguairetic acid blocks the actions of arachidonic acid and LMS, whereas indomethacin has no effect. Exogenously applied arachidonic acid (75 µM) suppresses the EPSP amplitude just as LMS does. The results suggest that the inhibitory effects of both LMS and Bztc may be mediated by lipoxygenase metabolites of arachidonic acid in these two physiological processes which occur in diverse insects.

Although Bztc mimicked the physiological effects of the myosuppressin LMS in two distinct insect bioassay systems it was unclear whether the effects of Bztc were attributable to direct interaction with the LMS receptor or via another mechanism. An *in vitro* myosuppressin receptor binding assay, which was recently developed to characterize receptors of a related myosuppressin, *Schisto*FLRFamide, on the locust oviduct, allowed us to address this question. The assay employs [^{125}I-Y^1]-SchistoFLRFamide (Y^1 DVDHVFLRFamide) as ligand and has revealed the presence of both high-affinity (Kd of 9.5×10^{-10} M) and low affinity (Kd of 1.9×10^{-7} M) receptors for the natural myosuppressin peptide (46). The essential core for binding in this receptor system was the C-terminal pentapeptide VFLRFamide, while the active core for suppression of proctolin-induced and/or spontaneous oviduct contractions was observed to be HVFLRFamide (37). This is in contrast with the myosuppressin active core for suppression of spontaneous cockroach hindgut contractions which was observed to be the C-terminal pentapeptide VFLRFamide (33). Prior to conducting receptor binding studies, Bztc was shown to mimic the physiological effects of the myosuppressin *Schisto*FLRFamide on the locust oviduct. Specifically, Bztc reversibly inhibits *proctolin-induced* contractions of the locust oviduct at a threshold concentration of 5×10^{-7} M and an EC_{50} of 6×10^{-5} M as compared with a threshold of 10^{-9} M and an EC_{50} of 2×10^{-7} M for *Schisto*FLRFamide. The nonpeptide Bztc also inhibits *spontaneous* contractions of the locust oviduct at an EC_{50} of 10^{-8} M (45).

In receptor-binding studies, Bztc was found to bind to both high and low-affinity myosuppressin receptors of the locust oviduct, but with a lower affinity than the radiolabelled *Schisto*FLRFamide analog. Binding to both high and low affinity receptors is competitively displaced by [^{125}I-Y^1]*Schisto*FLRFamide with a K_i of 6.9×10^{-10} M, respectively. Bztc competitively displaces the radioligand at a less potent K_i of 6.3×10^{-8} M for the high-affinity site and 1.5×10^{-4} M for the low-affinity site. However, because the small size of the nonpeptide suggests it mimics only the myosuppressin C-terminal tetra- or pentapeptide region, a comparison with the binding constant of a smaller myosuppressin fragment would seem more appropriate. For instance, the displacement curve for Bztc is similar to that of FLRFamide, which demonstrates K_i's of 5.1×10^{-8} M and 8.5×10^{-6} M for high- and low-affinity receptor sites (45, 46).

The combination of biological activity and receptor binding data provides evidence that the nonpeptide Bztc mimics the physiological effects of the myosuppressin via direct interaction with myosuppressin binding sites of the receptor. Thus, the nonpeptide appears to interact with both binding and activation regions of the myosuppressin receptor system.

A molecular dynamics/modeling study was initiated to identify plausible structural explanations for the observed ability of Bztc to mimic the biological and receptor binding characteristics of the myosuppressins in several insect bioassay systems (45). To fit Bztc onto the C-terminal myosuppressin pentapeptide VFLFamide, we assumed a functional correspondence between the two aromatic rings of Bztc and the Phe side chains, between the positively-charged quaternary ammonium group of Bztc and the positively-charged guanidinium group of Arg^4, and between the branched-chain hydrophobic tail of Bztc and either the Leu^3 or the Val^1 side chains. Following an extensive conformational search of the myosuppressin C-terminal pentapeptide, two low-energy candidates were chosen for comparison with Bztc because of the proximity of the Arg^4 guanidinium group and a Phe side chain. This proximity is necessary for reasonable superpositions because of the short distance between the ammonium group and the terminal benzyl ring of Bztc. Selected atoms of Bztc were forced onto corresponding positions in the myosuppressin pentapeptide with the myosuppressin structures held fixed and the Bztc structure allowed full flexibility. In the best superposition, the terminal aromatic ring of Bztc was fit onto the Phe^2 side chain and the internal aromatic ring of Bztc was fit onto the Phe^5 side chain (Figure 6). The opposite correspondence of aromatic rings also gave reasonable fits between the two molecules while retaining good internal geometries for Bztc. In addition, the branched-chain hydrophobic tail of Bztc could be successfully fit to either the branched-chain side chain of Leu^3 or to that of Val^1. In conclusion, the molecular dynamics/modeling study demonstrates that chemical groups on Bztc can readily superimpose with analogous sidechains of the myosuppressin pentapeptide active-core regions, and that several superposition scenarios are plausible.

Myosuppressin analogs containing restricted-conformational components, incorporating the α-carbon of the amino acid position occupied by Met into a cyclopropyl ring, have recently been shown to antagonize the myoinhibitory properties of the myosuppressins on the locust oviduct. The cyclopropyl myosuppressin fragment-analogs His-Val-Phe-**Cpa**-Arg-Phe-NH$_2$ [**Cpa** = cyclopropyl-Ala] (Nachman, R., U.S. Department of Agriculture, unpublished results)(Figure 7) and Phe-**Cpm**-Arg-Phe-NH$_2$ [**Cpm** = 2S, 3S-cyclopropyl-Met] completely antagonize the locust oviduct myoinhibitory activity of 100 nM HVFLRFamide at μM concentrations. The pseudotetrapeptide analog binds to both high affinity (K_i = 42 nM) and low affinity (K_i = 23 μM) myosuppressin receptors on the locust oviduct (Nachman, R., Lange, A., Orchard, I., and Burgess, K., U.S. Department of Agriculture, unpublished data), lower than SchistoFLRFamide (high affinity K_i= 0.9 nM; low affinity K_i = 0.5 μM), but comparable to the myosuppressin tetrapeptide fragment FLRFamide (high affinity K_i = 30 nM; low affinity K_i = 13 μM)(46). The myosuppressin antagonism and/or receptor binding characteristics demonstrated by these cyclopropyl, restricted-conformation analogs carry implications for the
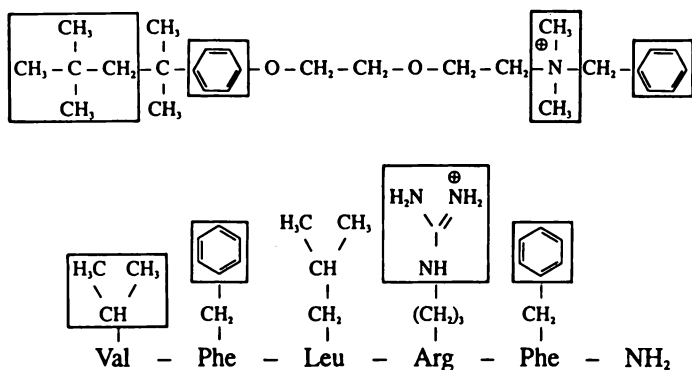
Figure 5. A comparison of similar structural features of the nonpeptide benzethonium chloride (top) with the myosuppressin C-terminal pentapeptide (bottom) (Reproduced with permission from ref. 45. Copyright 1996 Elsevier.)
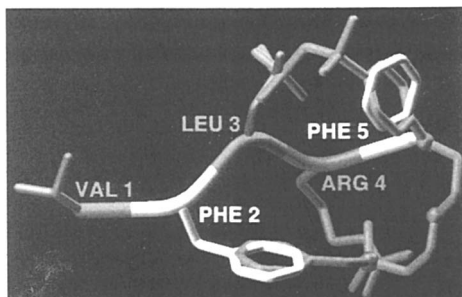
Figure 6. Best fit superposition (RMSD: 0.7 A) of the aromatic rings, positively charged ammonium group and hydrophobic tail of the nonpeptide Bztc with corresponding sidechain groups of a low-energy conformer of the myosuppressin C-terminal pentapeptide (Reproduced with permission from ref. 45. Copyright 1996 Elsevier.)
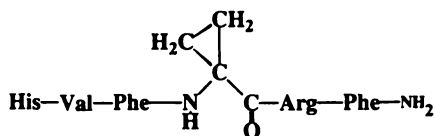
Figure 7. A conformationally-restricted pseudohexapeptide myosuppressin antagonist containing a cyclopropyl-Ala residue (Nachman et al., U.S. Department of Agriculture, unpublished results).

conformational component of the interaction of the myosuppressins with the locust oviduct receptor. NMR studies of the cyclopropyl tetrapeptide analog Phe-**Cpm**-Arg-Phe-NH$_2$ demonstrate a preponderance of a γ-turn conformation over residues Phe-**Cpm**-Arg in solution (47). The data suggest that a tight turn in the core region is adopted by the myosuppressins to facilitate binding with the locust oviduct receptor. Although a thorough investigation of the active conformation of the myosuppressin core region employing conformationally-restricted agonist analogs has not been completed, molecular dynamics calculations on the linear myosuppressin fragment VFLRFamide demonstrate that a low energy conformation can be achieved if the pentapeptide adopts a reverse γ-turn about the Met residue (45). That the myosuppressin fragment containing a cyclopropyl group demonstrates a propensity to form a γ-turn, as opposed to a reverse γ-turn, may explain why this and related cyclopropyl analogs can bind to and yet fail to activate locust oviduct myosuppressin receptors.

Epilog

The chapter describes conformational aspects of the interaction of two insect neuropeptide families with their receptor sites and the development of mimetic analogs with enhanced resistance to peptidase degradation with either partial or, for several examples, complete retention of potency. Remarkably, one amphiphilic analog of the pyrokinin peptide family not only demonstrates superagonist properties, but can induce a physiological response in an intact insect following topical application (28). These and similar enzyme-resistant mimetic analogs of the pyrokinin and myosuppressin neuropeptides can provide useful tools to insect physiologists studying the neuroendocrine control of such processes as pheromone production, oviduct contraction, digestive enzyme release, and the developmental processes of diapause and pupariation. The analogs will also be instrumental in studying the physiological and behavioral consequences of challenging an insect with a neurochemical signal it cannot readily degrade. If such analogs, whether in isolation or in combination with other factors, can disrupt the internal physiological balance of insects, they hold potential utility in future for the control of pest insect populations and the reduction of environmental residues of harmful pesticides.

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Chapter 22

Neuropeptide Biosynthesis: Possible Molecular Targets for the Control of Insect Pests

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The peptide adipokinetic hormones (AKHs) are important regulators of metabolism in many insects. In the locust, *Schistocerca gregaria*, AKHs are produced by glandular cells of major neuroendocrine centers called the *corpora cardiaca* (CC). We have determined the temporal order and the intracellular location of AKH precursor processing events, and have documented the enzymatic steps in precursor to product conversion. In addition, we have used NMR spectroscopy to determine the solution structure of the AKH precursor in hope of identifying features of this protein which govern processing endopeptidase specificity. Our work provides a basis for the design of specific inhibitors of processing enzymes to be used as lead compounds for new insecticides.

Peptides acting as transmitters, modulators and hormones are involved in the regulation of a wide variety of essential physiological processes in insects. While some aspects of peptidergic systems in insects have been examined in detail (e.g. peptide action, release and anatomical localization), relatively few studies have centered on biosynthesis. For this reason some years ago we decided to look for a model preparation in insects in which to study the cellular and molecular mechanisms of the biosynthesis of an important regulatory neuropeptide. Through this work we have now established the major neuroendocrine structure, the *corpora cardiaca* (CC) in the locust (*Schistocerca gregaria*) as an excellent system in which to investigate details of the synthesis of the adipokinetic hormones (AKHs).

Results of pulse-chase experiments (using intact CC maintained *in vitro*), protein chemistry and cDNA cloning (*1-3*) have allowed us to establish a cellular and molecular model for adipokinetic hormone (AKH) biosynthesis (*4*; reviewed in *5*). More recent studies focusing on the structure of an AKH precursor (*6;7*) and on characterization of enzyme activities involved in AKH precursor processing (*8*) have provided further insights into molecular recognition events essential to the production of the AKH peptides and others. This chapter provides an opportunity to update our previous review.

Although our primary interest is in the basic cell biology underlying neuropeptide biosynthesis, our findings may ultimately be of practical significance. Our work suggests novel targets may exist for new agents that disrupt neuropeptide biosynthesis. Provided the peptides in question are essential, such agents are likely to

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be harmful to insects and could therefore lead in the search for new pest control compounds.

Prohormones, Precursors, Products and Packaging

Our work has revealed that in the locust *Schistocerca gregaria* there are three AKH precursors that yield five AKH-related peptide products. These are major stored products of the glandular CC and include the previously identified decapeptide AKH I and octapeptide AKH II (*9;10*) as well as three larger peptides (ca. 6.5 kDa each) named the AKH Precursor-Related Peptides (APRPs; *1;2*). Molecular cloning, peptide purification and protein sequencing revealed that the APRPs are dimers, and furthermore, that the precursors of the AKHs and APRPs are themselves dimers (*1;4*). These dimer precursors (P1, P2 and P3) are formed by oxidation of single Cys residues in the two products of the translation of two mRNAs. These are proAKH I (41 residues) and proAKH II (39 residues). Random association of the monomer prohormones (presumably within the endoplasmic reticulum) yields the two homodimers and one heterodimer. Note that these dimers, not their monomeric prohormones, have been called "precursors" because it is the dimers that are the direct antecedents of the peptide products. In the adult locust there is five fold more proAKH I synthesized than proAKH II resulting in similar ratios for the stored AKH products and predictable binomial ratios of the APRPs. If this all seems a little arcane, Figure 1 should be consulted for clarification.

In vitro pulse-chase studies using intact CC from *S. gregaria* also have indicated the intracellular compartment in which AKH precursor processing takes place. Normally, neuroendocrine cells exhibit orderly trafficking of prohormones through the regulated pathway of secretion (from the endoplasmic reticulum, through the Golgi compartments, and ultimately into secretory granules for temporary storage) and specific processing events have been localized to distinct intracellular compartments (see *11*). The ionophore monensin can be used to disrupt the orderly intracellular trafficking of prohormones and thereby block conversion of precursors to products. In most cases, monensin blocks only processing steps which would ordinarily occur in post-*trans*-Golgi compartments (reviewed in *12*). When *S. gregaria* CC are incubated *in vitro* in the presence of monensin, processing of the AKH precursors is entirely blocked (*13*). We infer from this result that enzymatic cleavage of AKH precursors takes place only upon reaching a post-*trans*-Golgi compartment, most likely in the secretory granules.

Precursor cleavage in the secretory granules implies that the AKHs and APRPs (the products of cleavage) are co-packaged because any opportunity for sorting of the products will have been lost since secretory granules represent an intracellular "dead-end". Immunocytochemical studies support this implication, showing that both AKHs (*14*) are co-localized in CC secretory granules. Moreover, AKHs and APRPs are co-released from CC maintained *in vitro* in ratios reflecting their relative quantities within the CC, as would be expected of co-localized peptide products (*1*).

In summary, these observations together indicate that the three AKH precursors follow a common trafficking pathway so that they are randomly distributed in secretory granules (i.e. are not sorted in the late Golgi compartments) and that AKH biosynthesis results in the co-release of five peptides in ratios reflecting their relative abundance.

When considering the functional consequences of this basic cell biology, the story is further complicated by the fact that ratios of the five peptides vary progressively and systematically during post-embryonic development (*15*). This precise variation in peptide stoichiometry suggests that peptide ratios may have functional significance. A peptidergic message may for example be conveyed in the "cocktail" of co-released peptides, and the message may be altered as peptide ratios change. This possibility suggests that peptides may need to be present at the target or targets in precise ratios in
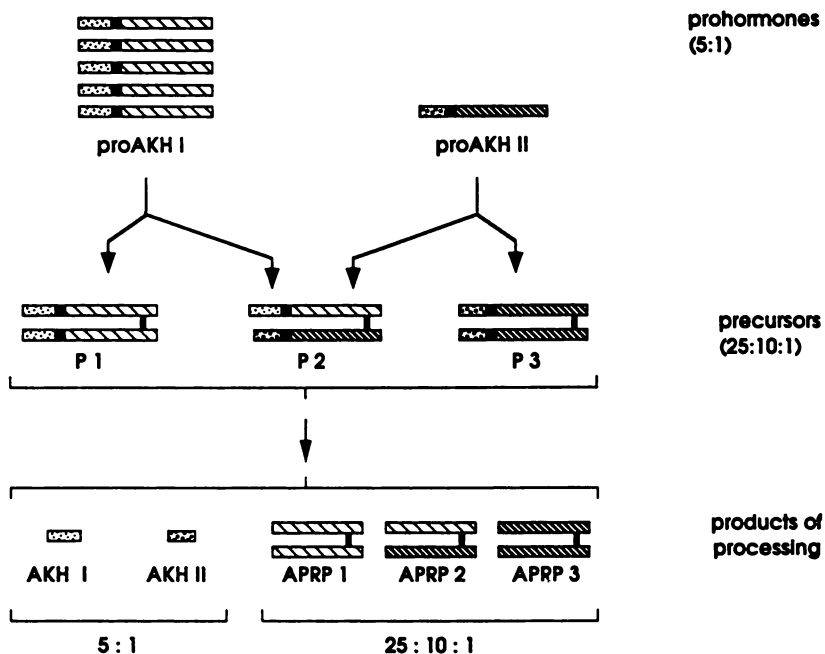
Figure 1. *Schistocerca gregaria* AKH I and II are products of two prohormones which form three disulfide-linked, dimer precursors. The dimers are converted enzymatically to five products of processing: AKH I, AKH II and the APRPs 1, 2 and 3. Molar ratios of prohormones, precursors and products in the adult locust are indicated. The black regions of proAKH I and proAKH II represent the sequences GKR and GRR, respectively, and black vertical bars connecting the prohormones represent disulfide bonds.

order to elicit appropriate physiological responses. Thus it is possible that the APRPs (yet to be ascribed a clear functional role) will reveal their actions only when considered in the context of the total "peptide cocktail" released by the CC. This would require a revision of current understanding of the physiological roles of the AKHs also. Whether the APRPs are an essential component of the APRP/AKH cocktail or merely a garnish remains to be seen!

The Process of Processing

Based on the sequences of proAKH I and proAKH II (see Table 1) it is possible to infer the steps in precursor processing. AKH I and AKH II are at the N-termini of proAKH I and proAKH II respectively and each is followed immediately by a glycine residue. Next come pairs of basic amino acid residues (either Lys-Arg or Arg-Arg) and these are followed in turn by similar (64% identical) 28 residue C-terminal sequences (the component monomers of the APRPs).

Paired basic residues typically precede sites of endopeptidase (prohormone convertase) action and it is therefore logical to assume that processing begins with endoproteolytic cleavage of the dimeric precursors to yield AKH I-Gly-Lys-Arg and AKH II-Gly-Arg-Arg and the respective APRPs. We also may infer that a carboxypeptidase(s) then removes the basic residues from the C-termini leaving glycine extended peptides and that the Gly residues ultimately donate the amides which are known to "block" the C-termini of mature AKHs I and II.

We have gone a step beyond inference and demonstrated this directly for the AKH I precursor (8). Exploiting its small size, we have synthesized a complete proAKH I and produced the homodimer P1 by its oxidation. This synthetic precursor has been used as a substrate for assaying processing enzymes extracted from CC *in vitro*. It is worth pointing out that the proAKHs are by some margin the "world's smallest" known prohormones at just 41 and 39 residues, respectively, and they remain so even though they approximately double in mass upon forming dimeric AKH precursors.

Using the synthetic precursor of AKH I we have shown that a specific endoproteolytic activity (a prohormone convertase or PC activity) in the CC cleaves C-terminal to Arg^{13} at the Gly^{11}-Lys^{12}-Arg^{13} processing sites. Thus the PC activity produces the predicted 13-residue extended AKH I (AKH-Gly^{11}-Lys^{12}-Arg^{13}) and the dimer, APRP 1. Other potential cleavage sites in the prohormone, a dibasic site (Arg^{34}-Lys^{35}) and a monobasic site (Arg^{27}), remain unused *in vivo* and likewise are not cleaved by PC activity *in vitro*. Therefore this endoproteolytic activity is likely to represent the *bona fide* AKH PC. Further processing of AKH-Gly-Lys-Arg in this *in vitro* system includes trimming of the basic residues producing AKH-Gly-Lys, then AKH-Gly by a metal cofactor-dependant carboxypeptidase activity. (Concomitant with our work, Stone *et al.* (16) purified and characterized a carboxypeptidase E-like processing enzyme from the nervous system of *Manduca sexta*). Production of the amidated bioactive product, AKH-NH_2 (AKH I), from the glycine-extended peptide is a two step process requiring the cofactors ascorbate and Cu^{2+}. This enzymatic activity strongly resembles the well-studied PAM (peptidyl-glycine alpha-amidating monooxygenase) enzyme of vertebrates (*c.f. 17*) and a PAM recently described in *Manduca sexta* (18). This sequence of events is summarised in Fig. 1 and more complete details of supporting experiments have appeared in Rayne and O'Shea (13).

Making the Cut

The AKH precursors also represent useful examples for identifying molecular recognition mechanisms by which PCs "select" appropriate processing sites. We know that the mechanism is selective because only one of three potential cleavage sites (basic residue motifs) in each chain of the AKH I precursor or P1 is used by the PC

Table 1. Amino Acid Sequences of AKH Prohormones

Prohormone Name	AKH sequence	Processing Site Sequence	AKH Precursor Related Peptide sequence
Scg AKH I	QLNFTPNWGT	GKR	DAADFGDPYSFLYRLIQAEARKMSGCSN
Scn AKH I	QLNFTPNWGT	GKR	DAGDYGDPYSFLYRLIQAEARKMSGCSN
Lom AKH I	QLNFTPNWGT	GKR	DAADFADPYSFLYRLIQAEARKMSGCSN
Scg AKH II	QLNFSTGW	GRR	YADPNADPMAFLYKLIQIEARKLSGCSN
Scn AKH II	QLNFSTGW	GRR	YADPNADPMAFLYKLIQIEARKLAGCSN
Lom AKH II	QLNFSAGW	GRR	YADPNADPMAFLYRLIQIEARKLAGCSD
Lom AKH III	QLNFTPWW	GKR	ALGAPAAGDCVSASPQALLSILNAAQAEVQKLIDCSRFTSEANS
Mas AKH	QLTFTSSWG	GKR	AMTNSISCRNDEAIAAIYKAIQAEAERFIMCQKN
Drm AKH	QLTFSPDW	GKR	SVGGAGPGTFFETQQGNCKTSNEMLLEIFRFVQSQAQLFLDCKHRE

Single letter amino acid codes are used. Where simple sequence alignments are possible, amino acid substitutions or additions relative to *Scg* proAKH I or proAKH II are indicated by bold lettering. The *Lom* AKH III and *Drm* AKH sequences (octapeptides) have been compared to *Scg* AKH II. *Mas* AKH (a nonapeptide) is compared to *Scg* AKH I (a decapeptide). Citations of publications in which these sequences have appeared are given in the text

(see above). It has been suggested that particular elements of secondary structure (e.g. ß-turns and Ω-loops) provide "molecular signposts", directing a PC to the appropriate domain in the precursor in which basic amino acid residues flank the bond to be cleaved (*19-21*). To test this hypothesis we have monitored the effects of replacing basic residues in the AKH precursor with other amino acids. Replacement *in vitro* of lysine by its analog thialysine at the processing site in the AKH I precursor prevented cleavage of the prohormone (*6*). Control experiments indicated that processing endopeptidases remain competent to cleave AKH prohormones in thialysine-treated *corpora cardiaca*. This indicated that the observed defect in processing was due to incorporation of thialysine into proAKH I and not into the co-synthesized processing enzymes. A molecular model of the AKH I precursor suggests that thialysine substitution may have caused this defect by disrupting a predicted secondary structural motif (an Ω-loop; see *22*) at the processing site (*6*).

We have also employed biophysical methods to learn more about AKH precursor structures. Using circular dichroism (CD) spectroscopy (R.C. Rayne, A. Drake and M. O'Shea, unpublished) and ^{1}H 2D NMR (*7*) we have studied the structure of a complete, synthetic AKH precursor in solution. Two distinct structural domains have been resolved. Each 41-residue chain contains a flexible N-terminal region (residues 1-21) and an alpha helical domain from residues 22 through 37. In this homodimer, the alpha helical domains are parallel and exhibit a slight positive supercoiling (*7*). These studies show that the unused potential processing sites are effectively "buried" within alpha-helical domains. Each used processing site on the other hand lies in a well exposed, flexible domain near the N-terminus of each chain. Unfortunately, structural studies to date have not enabled us to resolve clearly the structure of the AKH precursor in the region of the processing site, leaving us unable to confirm directly the hypothesis that an Ω-loop marks the peptide bond between Arg^{13} and Arg^{14} as the initial cut site.

AKH Biosynthesis in Other Insects

The *S. gregaria* AKHs are members of a family of peptides comprising a structurally related, but functionally diverse group known as the AKH/RPCH (red pigment concentrating hormone) family (See *23* for a review). Members of the family are identified using an acronym produced from the first two letters of the generic name and the first letter of the species name. Hence *Scg* AKH I refers to AKH I in *Schistocerca gregaria*. Other laboratories have studied AKH biosynthesis using species known to contain AKH/RPCH-related peptides. Altogether, molecular studies to identify AKH/RPCH precursors have been performed on five insects (*S. gregaria*, *S. nitans*, *Locusta migratoria*, *Manduca sexa* and *Drosophila melanogaster*) and a crustacean (*Carcinus maenas*). A comprehensive review of the functional implications of these studies is beyond the scope of this chapter, but a few brief points of comparison are appropriate. Table 1 lists the respective AKH prohormone (monomer) sequences.

In *S. nitans*, a species closely related to *S. gregaria*, the structures of the genes encoding AKH I and II have been determined (*24;25*). Comparison of sequences between these species reveals only very slight differences in the prohormone (monomer) amino acid sequences (39 or 41 residues identical in proAKH I and 38 of 39 in proAKH II; see Table 1). Moreover, these differences are found in the C-terminal prohormone domains and therefore occur in the APRPs, not in the AKHs. Although no additional studies of AKH biosynthesis in this species have been published, it is assumed that AKH I and II are produced according to the same biosynthetic model as we have demonstrated for *S. gregaria*.

AKH biosynthesis has also been studied in detail in another locust, *Locusta migratoria*. For AKH I and AKH II, the prohormone sequences are similar to the

corresponding *Schistocerca* prohormones (*26;27;* see Table 1). In fact, *Lom* AKH I is identical to *Scg* AKH I and *Lom* AKH II and *Scg* AKH II differ in only 1 of 8 residues. The additional substitutions in the prohormones (1 in *Lom* proAKH I and 2 in *Lom* proAKH II) occur in the respective C-terminal domains which form the APRPs. *Locusta migratoria* differs markedly from other locusts, however, in having a third AKH (AKH III), the biosynthesis of which occurs via a separate prohormone with little similarity to *Scg* or *Lom* proAKHs (see Table 1). Indeed, proAKH III is regarded as being more closely related to the *Manduca* (*28*), *Drosophila* (*29*) and *Carcinus* (*30*) AKH/RPCH prohormones than to the proAKH I and proAKH II of the locusts (*27*). Studies revealing the biosynthetic pathway of AKH III have yet to appear in the literature.

As indicated above, apart from the conservation of structure between the AKH/RPCH peptide sequences (i.e. within the N-terminal sequences of the respective prohormones), *Manduca* and *Drosophila* AKH prohormones and the *Carcinus* RPCH prohormone exhibit poor homology with locust proAKHs. Consequently, the APRP analogues produced by these species show little homology to locust APRPs. As pointed out by Noyes et al (*29*), the lack of conserved sequences in these precursor-related peptides argues against homologous functions for APRPs in organisms expressing AKH/RPCH family peptides.

Neuropeptide Biosynthesis: New Molecular Targets for Insecticides?

It is clear that a variety of bioactive peptides are involved in regulating a number of essential physiological processes in insects and it is likely that to disrupt peptidergic systems in any of several ways could be insecticidal. The most obvious general targets for interference include neuropeptide receptors, neuropeptide inactivation and neuropeptide biosynthesis. Here, we propose that to design compounds which interfere with neuropeptide biosynthesis may be a particularly effective strategy.

Interference with neuropeptide synthesis could be most readily achieved by the development of specific inhibitors of the enzymes involved in processing of peptide precursor proteins. The design of protease inhibitors has come to the forefront of biotechnology in recent years, driven particularly by the need for anti-viral (especially anti-HIV) compounds. Protease inhibitor design may be rationally accomplished using an "analogue-mechanism based" approach in which the molecular features of the substrate essential for enzyme action are identified and exploited in the design of ideal, mechanism-based inhibitors. Recent advances in structure determination methods and molecular modelling have made possible a more efficient process of rational design. Here, the determination of the structure of the target enzyme is required and once accomplished this allows the examination of interactions between candidate inhibitor and target enzyme to be evaluated at the atomic level. Most recently such a rational drug design approach has been successfully applied to production of anti-HIV compounds (*31*). The target enzyme in this case is the HIV-1 protease, an aspartic protease the action of which is essential for viral replication. Compounds with very high selectivity for the target enzyme have been developed and several products are now in clinical use.

We believe that such an approach is feasible for use in designing selective inhibitors for insect prohormone processing enzymes. Any of the three identified enzymatic steps (endoproteolysis, carboxypeptidase trimming or amidation) could be targeted. Blocking any of these enzymes would prevent the synthesis of fully processed and biologically active AKH. Essential metabolic functions would therefore become unregulated and it seems to us extremely unlikely that this would not either seriously handicap or kill the insect at any stage of development. Conventional screening for lead compounds that affect transmitter-receptor interaction of course has an important role to play, but the alternative approach we suggest here may have advantages. Generally speaking the experience of the pharmaceutical sector suggests

that it may be easier to discover or design effective and selective enzyme inhibitors than to do the equivalent with receptor ligands. By targeting the molecular recognition events essential for hormone synthesis, we may discover compounds against which the insect could not easily develop effective resistance.

If there is to be a serious effort to find inhibitors of insect prohormones convertases, there are two essential prerequisites. Firstly, the target enzyme must be available in sufficient quantity and this would require its cloning, sequencing and expression by suitable recombinant technologies. Secondly, a high throughput assay must be developed to allow for a rational screening program. Neither prerequisite would seem to require the development of novel technologies, nor do they represent significant obstacles to progress. Also there are a number of protocols developed in the pharmaceutical sector that provide encouraging and helpful guidance. Perhaps the time is now right to explore seriously the potential of neuropeptide biosynthesis inhibitors in the continuing search for safer, more selective and effective insecticides.

Acknowledgements

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Chapter 23

Proteolytic Activity in Lepidopterans: Potential for Regulatory Agent Development

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The field of proteolytic enzymes involved with post-embryonic development and metabolism in lepidopterans is discussed. Midgut digestive proteases represent the bulk of the work and information available. Also considered, however, are other key areas of proteolytic enzyme involvement including spermatogenesis, programmed cell death, phenoloxidase cascade, biochemical defenses against pathogens, neuropeptide signal attenuation and specific inhibitors of lepidopteran proteases including the serpins. Speculations on the exploitation of proteases and protease inhibitors in the control of lepidopteran pests are presented.

Proteolytic enzymes are critical to both anabolic and catabolic pathways in development and metabolism. A number of these activities have been described in a variety of insect species. Early reports described rapid degradation of the neurohormone proctolin in hemolymph preparations from locust and cockroach and from *Manduca sexta* larvae (1-3). Carboxypeptidase and aminopeptidase activities were detected. Subsequent work focused on the degradation of neuropeptides. Exo- and endopeptidase activities were described in neural membrane preparations from *Schistocerca gregaria* (4,5), *Drosophila melanogaster* and *Musca domestica* (6,7) and *Lymantria dispar* (8,9). Membrane preparations from *Locusta migratoria* midgut and ovary were found to possess protease activities (10), and neuropeptides in *Schistocerca gregaria* were metabolized in circulating hemolymph (11). Aside from their involvement with neuropeptide metabolism (9), proteases may function in a variety of key metabolic and developmental processes which can be considered as potential targets for which to design control strategies. Given the agricultural importance of lepidopteran species, a survey of proteolytic activities and related factors in this insect family should be useful. This selective review attempts to provide such a survey.

Midgut Proteases

The most extensively studied tissue with regard to protease activity in insects is the midgut. The gut is a rich source of proteases as a result of its digestive function. However, although the list of reports on insect gut digestive proteases is extensive (see 12-14 for reviews) the literature is confusing because of the variety of species involved, variations in diet, and specifics of the assays used to detect and quantify enzyme activities. Attempts are being made to survey selected midgut proteolytic activities using uniform methods (14) and the cataloging of biochemical parameters as reported in the literature (15,16). A number of trends begin to emerge. In a survey of 12 lepidopteran species including *Heliothis armigera, Plodia interpunctella* and *Spodoptera litura*, among others, Christeller et al. (14) observed trypsin, chymotrypsin, elastase, aminopeptidase and carboxypeptidase activities in each species. The relative level of each protease varied depending upon the species. This variation may reflect differences in diet among the species tested. The diet differences may include not only variations in nutrient composition from one plant source to another, but, as the authors suggest, an adaptation on the part of the feeding pest population to differences in specific plant derived protease inhibitors. An array of protease inhibitors from plant and animal sources as well as synthetic chemical inhibitors was tested against crude midgut homogenates from nine species (14). Casein was used as the substrate. Proteolytic activities in extracts from 8 of the 9 species were inhibited a minimum of 80 percent by at least one of the eleven serine protease inhibitors tested. Four of the nine species were inhibited at least 80 percent by each of two serine protease inhibitors. Responses to each of the inhibitors ranged from 0 to 94 percent inhibition depending upon the species tested. None of the non-serine protease inhibitors tested was effective. Phosphoramidon, a specific inhibitor of metalloendopeptidases, and heavy metal cations such as cadmium or copper, inhibitors of aminopeptidase, were ineffective. Thus, only specific inhibitors of serine endopeptidases were effective in reducing the level of casein digestion in the midgut extracts. The pH optimum was alkaline which is characteristic of serine endopeptidases, the only type of endopeptidase detected. The main endopeptidases were trypsin and chymotrypsin. The aminopeptidase and carboxypeptidase activities were probably associated with the gut wall, but this could not be determined from the crude, whole midgut extracts used (14).

Xu and Qin (17) reported serine proteases in midgut preparations from *Heliothis armigera* and *Heliothis assulta* with alkaline pH optima (pH 11) although activity was stable over a wide range of pH (4-11). Lee and Anstee (15) examined the midgut contents from larval *Spodoptera littoralis* and detected both trypsin and chymotrypsin activities. Each had an optimal pH of 10 and molecular weights were 24kDa and 19 kDa, respectively. No metalloendopeptidase activities were detected. The authors noted that endopeptidase activities were profoundly affected by the buffering ions used in the assay buffers, and caution that the effects of gut contents from various species may cause variation in the pH optima of detected proteases. Presumably, this might lead to incorrect classification of enzyme activity, further complicating the cataloging of midgut enzyme activities. In *Heliothis virescens*, trypsin-like activity as well as an enzyme which hydrolyzed synthetic chymotrypsin

substrates were detected in larval midgut contents (18). Together, the two enzymes accounted for over 95% of total protein digestion (casein) by midgut contents. Some elastase activity was detected. The trypsin-like and chymotrypsin-like enzymes were of equal importance, quantitatively, in general protein digestion. The pH optimum for each enzyme was 10-11. In *Helicoverpa armigera*, trypsin-like but not chymotrypsin-like activity was detected in larval midgut contents (19). Molecular weight was estimated to be 24kDa. Plant-derived protease inhibitors such as cowpea, lima bean and soybean trypsin inhibitor were effective against the *H. armigera* midgut trypsin. The vertebrate trypsin inhibitor OMTI (ovomucoid trypsin inhibitor from chicken) was ineffective as an inhibitor of the insect trypsin but very effective against bovine trypsin. Similar observations were made in *Ostrinia nubilalis* (20). Also, calcium ions are not necessary for optimum *H. armigera* midgut trypsin activity whereas such ions are necessary for bovine trypsin activity. Trypsin-like activity and an elastase-like enzyme were found in the midgut contents of *Lymantria dispar* larvae (21). Molecular weights of the serine proteases were 25kDa and 24kDa, respectively. No chymotrypsin or carboxypeptidase activities were detected in any of the midgut preparations. Leucine aminopeptidase (~100kDa) was obtained from brush border membrane extractions. Although no pH optima were reported, assays were conducted at pH 8.0. Leucine aminopeptidase, cystenyl aminopeptidase and dipeptidyl aminopeptidase activities were detected in midgut tissue of larval *Spodoptera littoralis* (16), with pH optima between 7.5-8.5. Leucine and cystenyl aminopeptidases had molecular weights estimated to be near 116kDa. Relative activities and optimal pH were dependent upon the buffer system used in the enzyme assay. Barbitone buffer yields a more alkaline pH optimum than do phosphate or borate buffers. The *Spodoptera* leucine aminopeptidase was unaffected by magnesium cation and inhibited by manganese cation. In contrast, these two ions are required for mammalian aminopeptidase activity.

Midgut digestive proteases present important potential as targets for control agents. Obviously, inhibition of their action would have devastating inpact upon the target pest. Some success has been realized with the incorporation of specific proteinase inhibitors into transgenic plants as protection against *Manduca sexta* (22,23). However, although numerous studies have been reported on lepidopteran midgut proteases, understanding of the interrelationships of these enzymes and the "...control of digestion in lepidopteran larvae..." is poorly understood (14). It appears that lepidopteran larval midgut digestive proteases may be divided into two broad groups. The midgut contents contain primarily serine endoproteases, usually trypsin and chymotrypsin and in some instances elastase, active optimally at alkaline pH with molecular weights near 20-25kDa. They provide the initial digestion steps, reducing the size of food protein molecules (15). Their products then serve as substrates for the membrane-associated exopeptidases. A number of specific inhibitor and cofactor differences between insect enzymes and vertebrate enzymes makes the specific inhibitor approach attractive in light of ecological and food safety considerations. Although prevention of larval growth and development through the inhibition of enzyme activity is conceptually straightforward, and natural plant-derived protease inhibitors are available, selection of the appropriate inhibitor(s) for inclusion in transgenics is complex. Target species, diet, digestive protease array,

biochemical optima, and specific inhibitor response are all essential considerations (14-16, 18, 21). In addition, variations involving the specific assay conditions with which enzyme activity is detected and quantified make correlations among different reporting laboratories difficult. Such logistical problems have to be met to fully exploit this approach to lepidopteran control.

Neural Tissue, Neuropeptides and Hemolymph

Most information on proteolytic activity in neural tissue, specifically neural membrane preparations, comes from work in locusts (4,5,11) and dipterans (6,7). Aminopeptidase and metalloendopeptidase activities were reported. Data for lepidopteran systems has so far been limited to *Lymantria dispar* (8,9). Adult *L. dispar* neural membrane preparations, from brain-subesophageal complexes, contain aminopeptidase and metalloendopeptidase activities similar to those reported in locust preparations. Their pattern of substrate degradation is the same as that reported in the locust (11). Using the N-terminal blocked decapeptide neurohormone locust adipokinetic hormone as substrate, both locust and *L. dispar* neural membrane preparations cleave an internal peptide bond initially, through the action of a phosphoramidon-sensitive metalloendopeptidase. The amastatin-sensitive aminopeptidase then attacks the nascent C-terminal residue of the cleavage product. Observations of the degradation of hemolymph-borne adipokinetic hormone in the *in vivo* locust system suggest that much of this degradation takes place at the surface of hemolymph-bathed membranes (11). Cell surface peptidases are known to be integral to the regulation of peptide messenger molecules in vertebrates (24) and the same paradigm appears to apply to insect systems (7,9,11).

Hemolymph contains proteases capable of the degradation of circulating neuropeptides. *Periplaneta americana* hemolymph degrades proctolin *in vitro* (1,3) and hemolymph from *Musca autumnalis* degrades adipokinetic hormone and hypotrehalosemic hormone (25). Among lepidopterans, *Manduca sexta* larval hemolymph is capable of degrading proctolin (1) and *M. sexta* larval, pupal and adult hemolymph was found to contain an endopeptidase which helped to degrade adipokinetic and related hormones (26). *Bombyx mori* adult hemolymph hydrolyzed the C-terminal hexapeptide of pheromone biosynthesis activating neuropeptide (PBAN) *in vitro* (27). An aminopeptidase was thought to be involved. An aminopeptidase from hemolymph plasma of larval *L. dispar* has recently been isolated (28). The enzyme is distinct from those reported as membrane associated (16) as the plasma enzyme is a hexamer of over 400kDa (28). Similar aminopeptidase activity is present in pupal and adult hemolymph plasma from *L. dispar* (9). Radiolabelled Hez-PBAN was degraded *in vitro* by a hemolymph preparation obtained from *Helicoverpa zea* larvae (29). Specific proteases were not identified, but data indicate the presence of both endo- and exopeptidases which act in concert to inactivate circulating peptide hormone.

Endopeptidases and exopeptidases, in circulating hemolymph and associated with membranes exposed to circulating hemolymph, present an extensive system which is used to regulate the titer and activity of peptide neurohormones and other peptide messengers. Clearly, interference with this regulatory system offers great

potential in affecting insect metabolism, metamorphosis, growth and development. Inhibition of one or more selected peptidases at opportune physiological stages my accomplish this goal. The problems associated with this approach, however, are some of the same encountered in the consideration of midgut proteases. One must identify the "key" enzyme(s) to serve as the target(s), consider the species in question, select appropriate inhibitor(s) and engage a delivery system. Transgenic plants represent one obvious avenue but the inhibitor must be capable of passing through the digestive system and into the hemolymph while retaining activity. Another approach, field spraying, requires an agent which can penetrate cuticle, be highly target-specific, and have a limited half-life so as to present a minimum ecological challenge.

Other Tissues

Molting fluid from pharate pupal *M.sexta* contains numerous proteolytic enzymes (30) including an aminopeptidase of approximately 500kDa. An aminopeptidase purified from pharate adult *M. sexta* molting fluid is characterized as a 240kDa hexamer (31,32). This enzyme, termed moulting fluid protease 2 (MFP-2), is not effective by itself in degrading insect cuticle, but works in concert with moulting fluid protease 1 (MFP-1) for a highly effective degradative system (33). MFP-1 is a trypsin-like endopeptidase from pharate adult *M. sexta* molting fluid which initiates cleavage of cuticular proteins, yielding fragments of from 200-2000 Da which then serve as substrates for MFP-2, and perhaps other proteases (33,34). The levels of activity of MFP-1 and MFP-2 increase late in the molting cycle as adult ecdysis approaches (34). This increase correlates temporally with a decrease in ecdysteroid titer (33), suggesting that the levels of proteolytic activity are under hormonal control. Injection of 20-hydroxyecdysone into pharate adult *M. sexta* prevented the increase in MFP-1 activity but had no effect on MFP-2 activity (33). MFP-1 alone can significantly degrade cuticle, but degradation is more complete when MFP-2 is present (31,32). Hormone responsiveness and degradative dominance suggest that MFP-1 is the initiator of cuticle degradation in the *Manduca* pharate adult system (33). Molting fluid proteinases have also been reported for *Bombyx mori* (35), but have not been isolated. Interference with cuticle degradation through manipulation of protease activities could lead to effective control agents. Clearly, this area of insect enzymology should command attention.

Lepidopteran sperm undergo physiological and morphological changes, during maturation in the spermatophore, which are essential to successful reproduction (36). In *Bombyx mori*, these changes involve mobilization of apyrene (anucleated) sperm and dissociation of eupyrene (nucleated) sperm from sperm bundles. Proteolytic enzymes are implicated in these changes (37). A key protease, initiatorin, has been isolated and characterized (36). Initiatorin is an endopeptidase produced by the prostatic gland and transferred to the spermatophore during mating. It is inhibited by serine protease inhibitors and its N-terminal sequence is homologous with N-terminal sequences reported for serine-type endopeptidases (36). The enzyme has a molecular weight of 29kDa and is thought to act by hydrolyzing protein(s) on the sperm surface or in the extracellular matrix which inhibit motility (36). Mobilized

sperm appear to induce a protein-arginine degradative cascade which liberates arginine, through specific proteolytic action. Liberated arginine is converted to 2-oxoglutarate which serves as an energy substrate for spermatozoa (36). Thus, specific proteases are necessary for sperm release and motility, and consequently, successful fertilization in *B. mori*.

Proteasomes

Programmed cell death, apoptosis, is a feature of most cells, and is exemplified in insects by the neuromuscular cell death in *M. sexta* at eclosion (38). Proteins targeted for degradation during apoptosis are attacked by large, multicatalytic proteases called proteasomes (38,39). Proteasomes isolated from *M. sexta* larval body wall contain five proteases in the 25-36 kDa range and have trypsin-like, chymotrypsin-like and other proteolytic activities (38,39). The highest levels of proteasomes were found in intersegmental muscle and ovary, although lower but measurable levels were also found in other tissues such as Malpighian tubules, ventral nerve cord, flight muscle and fat body (39). In addition to quantitative differences among tissues, differences in subunit composition were observed (39). The precise mechanism of insect proteasome participation in programmed cell death is being investigated. Clearly, an understanding of the components and regulation of this protease-based system will have extraordinary impact upon the design of unique insect control agents.

Endogenous Inhibitors

Proteinaceous protease inhibitors have been characterized from lepidopteran hemolymph and cuticle. There is a fairly extensive literature on their biochemistry and genetics (40,41), but their roles and physiological significance are poorly understood. The inhibitors described thus far are divided into two groups based upon biochemical characteristics (41,42). Activity classification is in large part based upon interaction of inhibitor with vertebrate serine proteases (esp. trypsin and chymotrypsin), although insect proteases are now being characterized (43,44). Low molecular weight (7kDa range), Kunitz-type inhibitors have been isolated from *B. mori* and *M. sexta* hemolymph and are specific for either chymotrypsin or trypsin activities. Large molecular weight (40kDa range) serine protease inhibitors ("serpins") also have been isolated from *B. mori* and *M. sexta* larval hemolymph, inhibiting chymotrypsin and trypsin (40-42). Inhibitors have also been detected in hemolymph of *Antheraea perni* and *Philosamia cynthia ricini* (40).

Serpins are part of a protein superfamily, most of the members of which are serine protease inhibitors (41). Serpins have been extensively studied in mammalian plasma where they are involved in blood clotting, the complement system and with proteases released during inflammation (41). Serpins in insects have been studied primarily from larval hemolymph of *B. mori* and *M. sexta*. Serpin genes representing both chymotrypsin- and trypsin-specific serpins have been cloned in *B. mori* (45-47) and *M. sexta* (48). Serpins are produced by the fat body and to a lesser degree in hemocytes (41,48), and exhibit diversity in selectivity for various serine proteases

(chymotrypsin, elastase, trypsin). In *M. sexta*, there are at least two serpin genes, and one gene (serpin gene-1) has the potential to produce twelve serpin variants, each specific for a different protease (41,49). In fact, a characteristic of insect systems appears to be the presence of numerous protease-specific inhibitors which is in contrast to vertebrates where individual endogenous protease inhibitors have a more broad array of target proteases (40).

Functions for insect protease inhibitors have not been defined. However, the obvious truth that protease inhibitors prevent unwanted proteolytic activity (40) leads to assumptions that such inhibitors have roles in regulating metabolism through the control of endogenous protease activity, and as defensive molecules through inhibition of proteases resulting from pathogen attack or pathogenic conditions. Strong circumstantial data suggest a number of possibilities. Similarity with mammalian serpins suggests that insect serpins may be involved with hemolymph clotting (48). On a wider scale, insect protease inhibitors are thought to be key elements in defense of the insect against pathogen-produced proteases or endogenous proteases released in response to pathogen invasion. Entomopathogenic fungi invade in part by releasing proteases which attack insect cuticle and are also released into the hemolymph. Anti-fungal protease inhibitors have been detected in both insect cuticle and hemolymph (40). Lepidopteran hemolymph protease inhibitors are effective against midgut proteases (40,42) and may function to protect the animal from proteases leaking from the midgut due to injury or infection. Protease inhibitor levels of activity are developmentally related. In *Bombyx* larval hemolymph, highest levels of inhibitor activity were observed in late larvae and pre-pupae (40). In *Manduca*, serpin gene-1 protein in hemolymph is highest in late last instar larvae and pre-pupae, then falls through the pupal stage (41). These developmental correlations suggest that serpins prevent the action of errant proteases released from metamorphosing tissues during the larval-pupal transition. A decline in inhibitor titer during pupal stage tissue reconstruction is logical. In support of an inhibitor role in metamorphic regulation, the levels of serpins appear to be affected by metamorphic hormones. Allatectomy of *B. mori* affected the level of hemolymph inhibitor (40). Fat body is the source of most hemolymph borne protease inhibitors (41). Serpin gene-1 fat body mRNA levels were significantly reduced when fat body was incubated with 20-hydroxyecdysone, resulting in reduced production of serpin (41). Ligation of day-2, fifth instar *M. sexta* larvae prevented the usual decrease in serpin mRNA level associated with larval molting and wandering. Injection of 20-hydroxyecdysone into ligated larvae reversed the effect (41). Another suggested role for inhibitors is in the regulation of the phenoloxidase cascade. Serine proteases typically exist as inactive precursors, zymogens, rendered active through limited proteolysis by activating proteases (44). Such interactions usually take the form of cascades involving several zymogen-active protease transitions, activating proteases and end producer enzymes. In insects, such a cascade has not yet been completely defined, but two enzymes are thought to be part of such mechanisms. Prophenoloxidase activating enzyme and an enzyme which hydrolyzes the synthetic substrate N-benzoyl-L-arginine ethyl ester (BAEEase) exist as zymogens (44). Each active enzyme is produced from the zymogen by the action of a specific proteolytic activating enzyme (44). Recently, the BAEEase activating enzyme in *B. mori* was

shown to be the target of an anti-trypsin serpin (43). Anti-chymotrypsin serpin did not affect the activating enzyme, and no inhibitor for the prophenoloxidase zymogen has been identified. These results illustrate both the specificity and complexity of protease-protease inhibitor interactions in insects. The phenoloxidase system results in the production of reactive intermediates leading to the production of melanin (50). Such activity is necessary to heal wounds and to engulf invading pathogens as part of the insect's defenses. In fact, the presence of bacterial or fungal cell wall components can trigger the phenoloxidase cascade (43). However, unrestrained phenoloxidase activity can lead to pathological melanization (50). Endogenous proteinase inhibitors in *M. sexta* and *B. mori* suppress phenoloxidase activity through inhibition of activating proteases (43,50). Presumably, release of this inhibition is stimulated by injury or infection.

Summary

Proteolytic enzymes are involved in all aspects of lepidopteran growth, development, metabolism and reproduction. There are complex interactions with specific inhibitors, involvement with digestion and nutrient availability, defense against pathogens, participation in metamorphic tissue processing, programmed cell death and sperm activation. Scores of other interactions undoubtedly exist. These interactions do not even take into account such other events as embryogenesis and neuropeptide biogenesis, which have their own sets of enzymatic directives. Clearly, the depth and breadth, the complexity and specificity of protease involvement with lepidopteran physiology, biochemistry and the steady state of normal metabolism suggest an almost endless array of targets at which to aim novel insect control agent design. The task, a daunting one to be sure, will be to assess the possibilities and pursue those with the most promise.

Mention of a proprietary product does not imply endorsement by the USDA.

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Chapter 24

Tyrosinase Inhibitors from Plants

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By bioassay-guided fractionation using mushroom tyrosinase (EC 1.14.18.1), various phenolic compounds were characterized as tyrosinase inhibitors from plants. They inhibited the oxidation of L-3,4-dihydroxyphenylalanine (L-DOPA) by mushroom tyrosinase. The inhibition kinetics analyzed by Lineweaver-Burk plots found them to affect the enzyme in different ways.

   Tyrosinase (EC 1.14.18.1) is a copper containing enzyme that catalyzes the first two distinct reactions of melanogenesis, the hydroxylation of a monophenol (monophenolase activity) and the conversion of an o-diphenol to the corresponding o-quinone (diphenolase activity) (1). As part of our continuing investigation of naturally occurring alternative insect control agents, we searched for tyrosinase inhibitors from plants (2-5). Because tyrosinase is one of the key enzymes in the insect molting process (6), its inhibitors may provide clues in controlling insect pests. Tyrosinase inhibitors have also become increasingly important for cosmetic products in relation to hyperpigmentation (7). Thus, tyrosinase inhibitors may also control production of the dermal melanin pigment since tyrosinase plays an important role in the process of melanin biosynthesis (8,9). In addition, disturbances in the amount and distribution of melanin formation may provide clues to systemic diseases (10-12). Tyrosinase is also known as a polyphenol oxidase (PPO) (13,14) and the browning of some fruits, vegetables, and crustaceans is due to tyrosinase, and subsequently causes a significant decrease in their nutritional and market values. This unfavorable darkening from enzymatic oxidation of phenols has therefore been of

great concern (15), and tyrosinase inhibitors should have
broad applications. These concerns led us to search for
naturally occurring tyrosinase inhibitors.

In our preliminary screening using mushroom
tyrosinase, plant extracts such as three Bolivian
medicinal plants, *Buddleia coriacea* REMY (Loganiaceae),
Gnaphalium cheiranthifolium LAM (Compositae), and
Scheelea princeps (MART.) KARST (Palmae), and a Mexican
medicinal plant *Heterotheca inuloides* CASS (Compositae),
were found to significantly inhibit the oxidation of L-
3,4-dihydroxyphenylalanine (L-DOPA) by mushroom
tyrosinase. In addition, anacardic acids and cardols
isolated from various parts of the cashew *Anacardium
occidentale* L. (Anacardiaceae) fruit were also found to
exhibit tyrosinase inhibitory activity.

RESULTS AND DISCUSSION

Fractionation guided by tyrosinase inhibitory
activity led to the isolation of six phenolic compounds
from *B. coriacea* (2,3), *G. cheiranthifolium* and *S.
princeps* (2), and several flavonoids from *H. inuloides*
(5). The identification of these tyrosinase inhibitors
were characterized mainly by spectroscopic studies.

The MeOH extract of the aerial parts of *B. coriacea*,
a plant locally known as "quishuara", was suspended in
H_2O and extracted with EtOAc, which in subsequent
bioassay was shown to be the active fraction. Two
flavonol glycosides, buddlenoid A and buddlenoid B were
isolated as tyrosinase inhibitors after repeated
chromatographic methods. Their final purification was
achieved by recycle-HPLC (R-HPLC) (16) using an ODS C_{18}
column. These two isolates have been deduced from
spectroscopic evidence to be kaempferol 7-*O*-(6''-*p*-
coumaroyl)glucoside for buddlenoid A (**1**) and isorhamnetin
7-*O*-(6''-*p*-coumaroyl)glucoside for buddlenoid B (**2**),
respectively (3). Bioassays with the purified compounds
indicate that both buddlenoids exhibit potent mushroom
tyrosinase inhibitory activity and showed a dose-
dependent effect on the oxidation of L-DOPA. The ID_{50} were
0.39 mM for buddlenoid A and 0.44 mM for buddlenoid B
(Table 1). Their limited availability prevented further
study to determine their mode of inhibition.

In addition to these two flavonol glycosides, a more
active principle was also purified in large quantities
(0.46%, dry wt) from the same source. This more potent
tyrosinase inhibitor was isolated from the H_2O portion
after repeated chromatography using a variety of methods.
This polar compound has been identified by spectroscopic
studies as the known hydrolyzable tannin, agrimoniin (**3**),
which was previously isolated from two Rosaceae plants,
Agrimonia pilosa and *Potentilla kleiniana* (17). The ID_{50}

of this tannin is 0.06 mM, which is the most potent among the six compounds isolated from the three Bolivian medicinal plants (Table 1). The purified agrimoniin seems to be rather unstable since it gradually changes to a grey color on isolation from the extract. It appears that agrimoniin itself is slowly auto-oxidized. The inhibition kinetics of this tannin were analyzed by a Lineweaver-Burk plot. The three slopes, obtained from the uninhibited enzyme and from the two different concentrations of agrimoniin, were found to be almost parallel, indicating an uncompetitive inhibition. Since tannins are known to react with proteins by cross-links, a process known as tanning, agrimoniin is expected to somehow irreversibly inhibit the tyrosinase (protein). The instability of agrimoniin, coupled with its tanning property, complicated our attempts to characterize the mode of inhibition.

The MeOH extract of the aerial part of *G. cheiranthifolium*, locally known as "huira" in Bolivia, was also suspended in H_2O and extracted with EtOAc, which in subsequent bioassay was shown to be active. Two active principles were isolated after repeated chromatography and identified by means of spectroscopic methods, as gnaphalin (4) (18) and luteolin 4'-*O*-glucoside (5) (19). Their final purification was achieved by R-HPLC (ODS C_{18}). Since luteolin 4'-*O*-glucoside possesses the bulky glucose moiety at the 4'-position but still inhibited the enzyme, it would be interesting to determine its mode of inhibition. However, these two active compounds exhibited significant (almost 100% at 100 μg/ml) inhibition of the L-DOPA oxidation by tyrosinase, but their limited availability prevented further study so neither ID_{50} values nor their mode of inhibition were investigated.

Similar treatment of the MeOH extract of the root of *S. princeps*, known as "mota cú" in Bolivia, yielded a tyrosinase inhibitory EtOAc portion. An active principle was isolated from this fraction after separation by a number of chromatographic methods, and identified as the common *p*-hydroxybenzoic acid (6) by comparison to its spectral data with those of an authentic sample. Interestingly, *p*-hydroxybenzoic acid was the only active compound isolated from this source guided by bioassay at 200 μg/ml. It showed a concentration-dependent effect on the tyrosinase oxidation of L-DOPA. The ID_{50} was determined to be 1.9 mM. Although the ID_{50} is moderate, this tyrosinase inhibitory activity was investigated in more detail because *p*-hydroxybenzoic acid and its derivatives, for example "paraben", have been widely used as preservatives in cosmetic and food products (20).

The inhibition kinetics of *p*-hydroxybenzoic acid were analyzed by a Lineweaver-Burk plot as shown in

Table 1. Tyrosinase Inhibitory Activity of
          Phenolic Compounds

Compounds Tested	ID_{50} (mM)	Mode of Inhibition
1	0.39	-
2	0.44	-
3	0.06	-
6	1.9	Competitive
7	0.06	Competitive
9	0.23	Competitive
12	0.02	Competitive
13	1.1	Competitive
14	3.3	Noncompetitive
15	10.7	-

-, Not tested
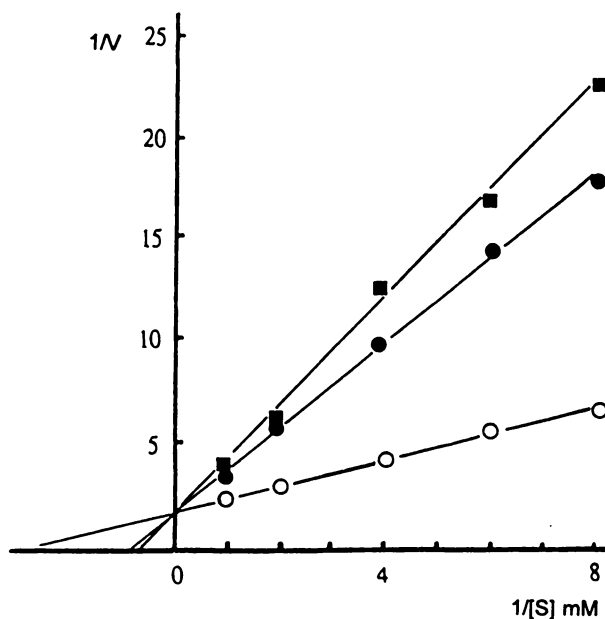
Figure 1. Lineweaver-Burk plots of mushroom tyrosinase and
L-DOPA in the absence (O, control) and presence (●, 133
μg/ml and ■, 233 μg/ml) of p-hydroxybenzoic acid.
1/V:1/Δ475 nm/mim.

Figure 1. The slopes, obtained from the uninhibited enzyme and from the two different concentrations of *p*-hydroxybenzoic acid, intercepted on the vertical axis. The results indicated that *p*-hydroxybenzoic acid was a characteristic competitive inhibitor. However, pre-incubation in the presence of 1.9 mM of *p*-hydroxybenzoic acid and in the absence of the substrate (L-DOPA) indicates that *p*-hydroxybenzoic acid is not a direct inhibitor of the enzyme since it did not significantly decrease enzymatic activity. The inhibition was increased only from 50% to 55%. Thus, *p*-hydroxybenzoic acid appears to competitively displace L-DOPA from the active site. It should be remembered that the pre-incubated enzyme was mostly *met* tyrosinase, known as the resting form of the enzyme. The above pre-incubation data indicate that *p*-hydroxybenzoic acid can react only with the *oxy*-form of tyrosinase.

In addition to the above three Bolivian medicinal plant, flavonoids were characterized as tyrosinase inhibitors from a Mexican medicinal plant *H. inuloides* (21-23). The MeOH extract of the dried flowers of *H. inuloides*, locally known as "arnica", was suspended in H_2O and extracted with *n*-hexane, Et_2O and EtOAc in this order. Subsequent bioassay located the tyrosinase inhibitory activity in the EtOAc fraction. This EtOAc portion by various chromatographic methods led to the isolation of the principal inhibitor which was characterized by spectroscopic method as the common flavonol, quercetin (**7**). Quercetin has previously been isolated from many plants, including the dried flowers of *H. inuloides* (12,13). Four additional flavonols, quercetin 3-*O*-glucoside (**8**) otherwise known as isoquercitrin, kaempferol (**9**), kaempferol 3-*O*-glucoside (**10**), and quercetin 3-*O*-rutinoside (**11**) otherwise known as rutin, were also isolated from the same source (12) and their tyrosinase inhibitory activity were also studied for comparison.

The inhibitory kinetics of quercetin were analyzed by a Lineweaver-Burk plot as shown in Figure 2. The three lines, obtained from the uninhibited enzyme and from the two different concentrations of quercetin, intersected on the vertical axis. It appears that quercetin is a competitive inhibitor for the L-DOPA oxidation by mushroom tyrosinase. Interestingly, similar to *p*-hydroxybenzoic acid, quercetin was not oxidized by the enzyme as a substrate at all. In addition, a pre-incubation experiment of the enzyme in the presence of 0.06 mM of quercetin and in the absence of the substrate increased the inhibition activity from 45% to 77%, indicating that quercetin is a direct inhibitor. This indicates that quercetin can react with the *met*-form of tyrosinase before oxygen binds.
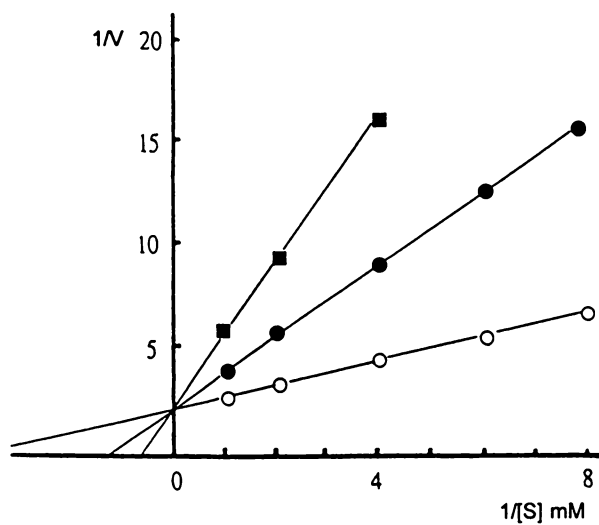
Figure 2. Lineweaver-Burk plots of mushroom tyrosinase and
L-DOPA in the absence (O, control) and presence
(●, 12 μg/ml and ■, 15 μg/ml) of quercetin.
1/V:1/Δ475 nm/mim.

Tyrosinase contains a strongly coupled binuclear copper active site and functions both as a monooxygenase (monophenol + O_2 → o-diphenol + H_2O) and as an o-dihydroxyphenolase (o-diphenol + ½O_2 → o-quinone + H_2O) (24-26). Tyrosinase apparently has been described to have separate catalytic sites for these two oxidations, and yet another independent binding site for L-DOPA as a cofactor (26-31). Based on consideration of these previous reports, we proposed that quercetin, a competitive inhibitor, preferentially displaces L-DOPA from the active site of the cofactor because of its structural resemblance (5). Thus, most competitive inhibitors closely resemble, at least in part if not all, the structure of the substrate. In the case of quercetin, a portion of the structure "**a**" is clearly analogous to L-DOPA as shown in bold line in Figure 3. That is, it may overlap the lock-and-key model. On the other hand, a bulky sugar moiety attached to the 3-hydroxyl group in the above mentioned 3-*O*-glycoside analogues (**8,10,11**) may hinder their approach to the binding site of the cofactor. Most importantly, this hypothesis seemed to explain our finding that quercetin was not oxidized by the enzyme as a substrate at all.

However, the interactions between tyrosinase and substrates have recently been suggested to take place at the binuclear copper site. The two different types of substrates, monophenol and o-diphenol, are found to react with different oxidation states of the coupled binuclear copper sites (*deoxy*, *met*, and *oxy*) (32). This prompted us to reinvestigate the above inhibition mechanisms of quercetin and its derivatives as well as additional related compounds. After characterization of a number of tyrosinase inhibitors, we became aware that a variety of o-diphenols such as catechol, caffeic acid, and chlorogenic acid also serve as cofactors. The fact that cofactors are reductant or proton donors demonstrates that cofactors do not need the specific binding site. This definition of cofactors does not support the above mentioned lock-and-key concept.

A more practical explanation of potent tyrosinase inhibitory activity of quercetin may come from its ability to chelate copper in the enzyme. This is in agreement with the previous report that some flavonoids including quercetin were described to chelate copper (15,33). In the UV spectrum of quercetin, the absorption maximum of 370 nm was shifted to 432 nm by adding excess Cu^{2+}, extending into the visible region. In contrast, this shift was not observed in the UV spectra of the 3-*O*-glycosides (**8,10,11**). It appears that, combining the above pre-incubation data, quercetin may chelate copper in the *met*-form of tyrosinase before oxygen binds and
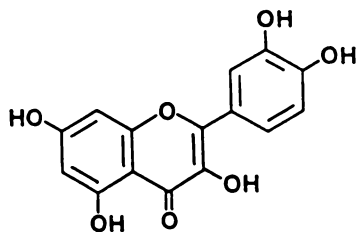
(7)

Shifted from 370 nm to 432 nm by adding Cu^{2+}.

(8)

352 nm → 382 nm

a
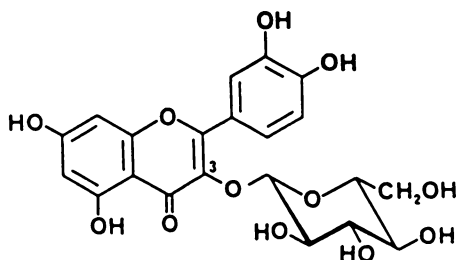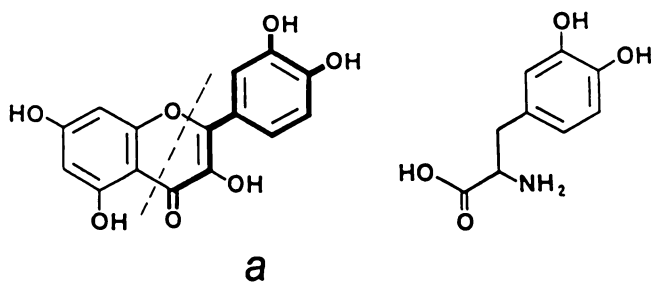
Figure 3. Structural similarity of quercetin and L-DOPA.
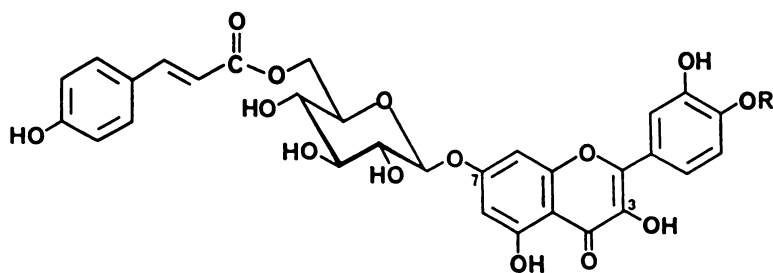
(1) R=H
(2) R=CH₃
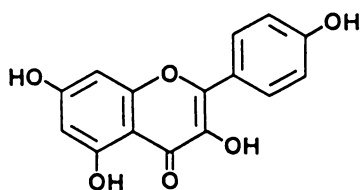
(9)
Shifted from 362 nm to 404 nm by adding Cu²⁺
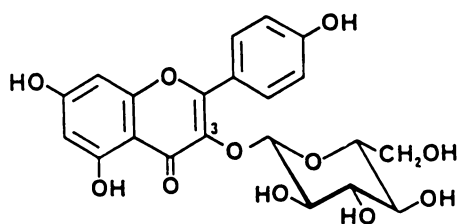
(10)
342 nm → no shift

Figure 3. Continued.

inhibits the enzyme activity. This may explain why
quercetin was not oxidized by the enzyme. As shown in
bold line in Figure 4, a portion of the structure "*b*" (3-
hydroxy-4-keto moiety) in quercetin is clearly analogous
to kojic acid (**12**). The competitive inhibition exerted by
kojic acid is well established to come from its ability
to chelate copper in the enzyme (34,35). Interestingly,
the inhibition exerted by kojic acid was reported to
affect through the *oxy*-form of tyrosinase (36). The
result with kojic acid seems to differ from quercetin
which affects the *met*-form of the enzyme. This difference
may indicate a certain interaction between inhibitors and
the tertiary structure of the enzyme. For example, there
is a positive contribution to the entropy of binding when
the hydrophobic portion of the inhibitor is moved from
the aqueous environment to a hydrophobic pocket in the
active site (37). However, this remains to be solved
since the chemical structure of mushroom tyrosinase has
not yet been established. Needless to add, in contrast to
quercetin, its 3-*O*-glycosides, quercetin 3-*O*-glucoside
(**8**) and quercetin 3-*O*-rutinoside (**11**), did not inhibit
the oxidation of L-DOPA by the enzyme, and they were not
oxidized themselves either (5). Nevertheless, it seems
that the modes of inhibition of *p*-hydroxybenzoic acid and
quercetin are different, even though both were found to
be competitive inhibitors (2,5).

Recently, we reported the tyrosinase inhibitory
activity of 6-[8(Z),11(Z),14-pentadecatrienyl]salicylic
acid otherwise known as anacardic acid (**13**) isolated from
the cashew *A. occidentale* fruit (4). Since it is a
salicylic acid derivative with a $C_{15:3}$-alkyl side chain,
its activity was compared with that of salicylic acid
(**14**). The ID_{50}s were found to be 3.3 mM for salicylic
acid and 1.1 mM for anacardic acid, respectively. More
importantly, the inhibition kinetics analyzed by
Lineweaver-Burk plots found salicylic acid to be a
noncompetitive inhibitor on the L-DOPA oxidation by
mushroom tyrosinase while anacardic acid to be a
competitive inhibitor (4). As far as the ID_{50} is
concerned, salicylic acid showed nearly the same ID_{50}
value compared to that of anacardic acid, indicating that
the addition of a $C_{15:3}$-alkyl side chain does not seem
directly related to tyrosinase inhibitory activity.
However, the inhibition kinetics illustrated that
anacardic acid is a characteristic competitive inhibitor
(4). In addition, pre-incubation of the enzyme in the
presence of anacardic acid and in the absence of the
substrate indicated that anacardic acid was a direct
inhibitor of the enzyme since it significantly increased
the inhibitory activity. The inhibition was increased
from 45% to 75%. On the other hand, pre-incubation of the
enzyme in the presence of salicylic acid and in the

absence of L-DOPA indicated that salicylic acid is not a direct inactivator of the enzyme since it did not increase the enzyme inhibitory activity significantly. Interestingly, the inhibitory activity exerted by salicylic acid was slightly reversed by adding excess Cu^{2+}, although UV absorption of salicylic acid did not shift by adding excess Cu^{2+}. In contrast to salicylic acid, the absorption maximum of 245 nm was shifted to 310 nm by adding excess Cu^{2+} in the UV absorption of anacardic acid. The high affinity of anacardic acid with Cu^{2+} has been reported previously (38). The results obtained with anacardic acid differ from those of salicylic acid, clearly indicating that salicylic acid and anacardic acid affect mushroom tyrosinase in different ways. Thus, the addition of a $C_{15:3}$-alkyl side chain obviously affects the inhibition of the tyrosinase activity. It should be remembered again that the enzyme pre-incubated was mostly *met* tyrosinase. The above results indicate that anacardic acid also may react with the *met*-form of tyrosinase similar to quercetin.

The lag phase is known for the oxidation of monophenolic substrates such as L-tyrosine by tyrosinase. This lag can be shortened or abolished by the presence of reducing agents (hydrogen donors), also known as a cofactor, especially *o*-diphenols such as L-DOPA (32). It appears that quercetin (*o*-diphenol) is an alternative cofactor to initiate this hydroxylase (monophenolase) activity, but kaempferol (monophenol) obviously is not. In the presence of quercetin, L-tyrosine was oxidized by the enzyme without the lag phase as shown in Figure 5. Thus, quercetin completely abolished this lag time. As expected, kaempferol did not suppress this lag time. It appears that quercetin inhibits *o*-diphenolase activity but activates monophenolase activity in the shortening L-tyrosine lag time.

In conclusion, competitive inhibition exerted by the above mentioned flavonols such as quercetin and the buddlenoids should come from their ability to chelate copper in the enzyme. The competitive inhibitor *p*-hydroxybenzoic acid, on the other hand, binds to the binuclear copper center preferentially with the more acidic carboxylic group and competes with L-DOPA (25) as illustrated in Figure 6. This is agreeable with the previous reports that phenolic acids bind to the binuclear copper active center with the carboxylic group and are competitive inhibitors as long as a free carboxyl group exists in the molecule (24,27,39). However, this seems not to be the case for the *ortho*-hydroxybenzoic acid (salicylic acid) and its congeners. Thus, the hydroxyl and carboxyl groups in salicylic acid form a quasi six-membered ring through intramolecular hydrogen bonding and produces a stable chelate structure. Hence,
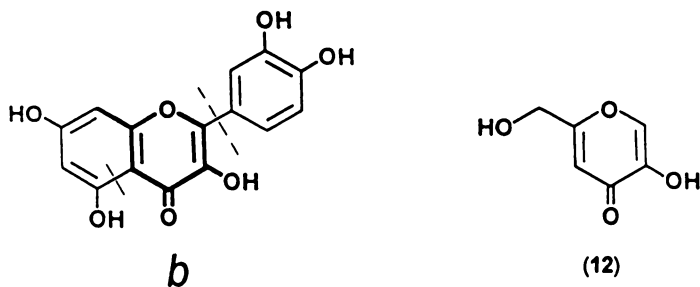
Figure 4. Structural similarity of quercetin and kojic acid.
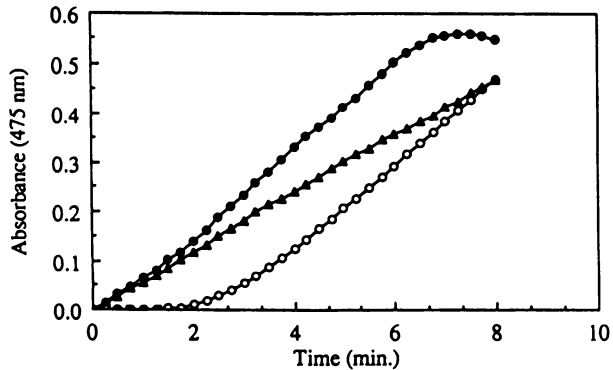
Figure 5. Cofactor activity of quercetin. O, L-Tyrosine (0.85 mM); ●, +L-DOPA (0.85 mM); ▲, Quercetin (0.02 mM).
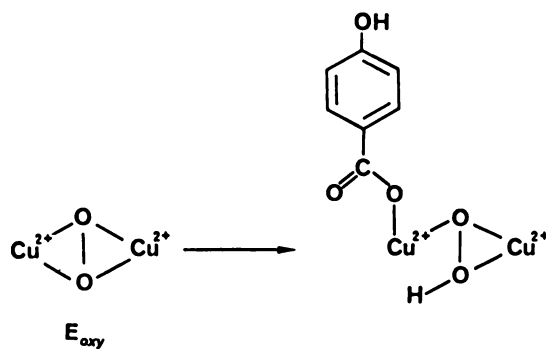
Figure 6. Binding model of p-hydroxybenzoic acid to the binuclear copper active center in the oxy-form of tyrosinase (After Reference 39).

the carboxyl group no longer binds as a HA-type inhibitor like p-hydroxybenzoic acid. In addition, esterification of p-hydroxybenzoic acid, for example with propyl alcohol converts it to propyl paraben (**15**) and decreased the inhibitory activity, but did not destroy it since the affinity of paraben for the enzyme increased (40). The ID_{50} of propyl paraben was lowered 5-fold compared to p-hydroxybenzoic acid. In general, esterification of the carboxylic group decreased the inhibitory strength (41), and more importantly, these esters were oxidized as substrates.

Interestingly, all the tyrosinase inhibitors isolated by bioassay guided fractionation were found to be various common phenolic compounds, but they affect the enzyme in different ways.

ENZYME ASSAY

The mushroom tyrosinase (EC 1.14.18.1) used for the bioassay was purchased from Sigma Chemical Co. (St. Louis, MO). Although mushroom tyrosinase differs somewhat from other sources (1,6,13), this fungal source was used for the present experiment due to its ready availability. Since mode of inhibition depends on the structure of both the substrate and inhibitor, L-DOPA was used as the substrate in this experiment, unless otherwise specified. Therefore, inhibitors discussed in this paper are inhibitors of diphenolase activity of tyrosinase and their effect on the enzyme was determined by spectrophotometry (dopaquinone formation at 475 nm). Since tyrosinase catalyzes a reaction between two substrates, a phenolic compound and oxygen, the assay was carried out in air-saturated solutions. All the samples tested were preliminary assayed at 167 µg/ml. It should be, however, noted that several samples such as kaempferol and anacardic acid are hardly soluble in the water based test solution at this concentration.

The samples were first dissolved in H_2O or DMSO and used in the experiments at 30 times dilution. The assay was performed as previously described (9,42). Thus 1 ml of 2.5 mM L-DOPA was mixed with 1.8 ml of 0.1 M phosphate buffer (pH 6.8), and incubated at 25°C for 10 min. Then 0.1 ml of each sample solution and 0.1 ml of 138 units/ml enzyme in aqueous solution were added to the mixture. This solution was immediately monitored for the formation of dopachrome by measuring the linear increase in optical density at 475 nm.

The pre-incubation mixture consisted of 1.8 ml of 0.1 M phosphate buffer (pH 6.8), 0.6 ml of water, 0.1 ml of the samples solution, and 0.1 ml of the aqueous solution of the mushroom tyrosinase (138 units). The

mixture was pre-incubated at 25° for 5 min. Then, 0.4 ml of 6.3 mM L-DOPA was added and the reaction was monitored at 475 nm for 4 min.

Acknowledgement

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Chapter 25

Comparative Molecular and Pharmacological Properties of Cholinergic Receptors in Insects and Mammals

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The nicotinic acetylcholine receptor (nAChR) is composed of five subunits and its activation produces a conformational change in the protein that results in opening of its central cationic channel. There are numerous subtypes of nAChRs in mammalian brain, ganglia and skeletal muscle, while in insects fewer subtypes were detected only in the central nervous system. The latter receptors are simpler and more similar in subunits and drug-specificities to certain mammalian brain receptors. The muscarinic acetylcholine receptor (mAChR) is formed by a single gene and only five have been identified (m1-m5). Insect mAChRs resemble mostly the m3 receptor in drug specificity, but other subtypes possibly exist. Pharmacological differences between mammalian and insect receptors can be utilized to develop insect-selective insecticides. It is also possible that differences between receptors in insect species can be used for species-selective toxicants.

Neurotransmitter receptors are highly specialized cellular biosensors that regulate cell function upon binding of their chemical signals (i.e. neurotransmitters). They are classified into two major classes based on their transduction mechanism. Cholinergic receptors are cell surface receptors which recognize acetylcholine

(ACh) as their neurotransmitter and respond to its presence by initiating cellular responses in the effector cells that express them. They are divided into the following two major classes: Nicotinic receptors (nAChRs), which belong to the ionotropic class of receptors, that initiate cellular response by changing cell membrane potential due to their function as chemically gated cation channels (1). Muscarinic receptors (mAChRs), which belong to the family of G-protein-coupled metabotropic receptors, that initiate cellular response via activation or inhibition of specific enzymes and increased stimulation or inhibition of synthesis of second messengers (2). ACh, the neurotransmitter for these two classes of cholinergic receptors, is synthesized in cholinergic nerve cells by the enzyme choline acetyltransferase and is released upon nerve stimulation. It's synaptic action is quickly terminated by the highly efficient acetylcholinesterase (AChE), which is abundant in cholinergic synapses (3).

Nicotinic Receptors

The nicotinic receptor was discovered by Langley in 1901 as the receptive substance present in autonomic sympathetic ganglia and skeletal muscle motor endplates that mediate the physiological responses of these tissues to nicotine (4). Years later, after the discovery of ACh and AChE and establishing the chemical nature of transmission at cholinergic synapses in both tissues and their stereospecific responses to nicotine enantiomers, the receptor retained the popular name nAChR (5).

In mammals nAChRs are found in skeletal muscles, autonomic ganglia and in the central nervous system (CNS), i.e. brain and spinal cord. In skeletal muscles it is found in the motor endplates which are specialized synapses between skeletal muscles and motoneurons, that regulate the voluntary movements of skeletal muscles. In sympathetic and parasympathetic ganglia, nAChR regulate the response of ganglionic neurons to presynaptic cholinergic input received from the CNS mostly via reflex neuronal circuits. In insects nAChR are found only in the CNS (i.e. brain and ganglia). They are not found in skeletal muscles. On the other hand, voluntary regulation of insect skeletal muscle is mediated by an excitatory glutamatergic and an inhibitory γ-aminobutyric acid motoneurons (6).

The nAChR of electric organs of electric fish was the first neurotransmitter receptor identified in subcellular preparations, purified, cloned and its subunit composition, amino acid sequence and its membrane topography defined (7-11).

Molecular properties: The nAChRs are fairly large glycoproteins (Mr $\approx$ 250 kDa), consisting of five subunits each having four transmembrane-spanning

domains (TM_1-TM_4) with both the amino and carboxy terminals located extracellularly. The nAChR forms a pentameric structure with a central ion channel lined by TM_2 segments of the five subunits (*9*). The subunit structure of the muscle nAChR is $\alpha_2 \beta \gamma \delta$ (i.e. it is the product of four genes expressing four peptides α, β, γ and δ) (Fig. 1). The ACh binding site is on the α subunit and this is the site that binds other nicotinic agonists (e.g. nicotine) as well as competitive antagonists (e.g. the arrow-head poison d-tubocurarine, the quasi-irreversible binding snake venom α-bungarotoxin (α-BGT) (*12*) and the more reversible marine cone snail α-conotoxin (*13*).

   All neuronal nAChRs are believed to be composed of 5 subunits and have structural and membrane topography similar to that of the skeletal muscle nAChR (Fig. 1). Mammalian and insect neuronal nAChRs are less complex genetically than the skeletal muscle nAChR. Currently, 9 α subunits and 4 β subunits have been shown to exist in mammalian brain nAChRs (Table I). They may be homomeric proteins formed by five identical α subunits. Examples are the α_7, α_8 and α_9 homomeric receptors in mammalian brain (*10, 14*), which when activated influx significant amounts of Ca^{2+} in addition to Na^+ and afflux K^+. There is also the receptor expressed from only a locust α-subunit mRNA in *Xenopus* oocyte (*15*). They are inhibited by α-BGT like the mammalian skeletal muscle nAChR. However, the majority of mammalian nAChRs discovered so far are the products of two genes, are composed of α and β subunits and are insensitive to α -BGT.

Table I. Subtype diversity of nAChRs

	Mammalian		Insects
	Muscle	Neuronal	Neuronal
Subunits	$2 \alpha, 1\beta, 1\gamma, 1\delta$	$2 \alpha, 3\beta$	5α
Genes	4	2	1
Subtypes	1	>10	?
α–bungarotoxin	+	- *	+
k-bungarotoxin	--	+	--
ACh	+++	+++	+
Nicotine	+	+++	+
Cytosine	+	+++	+

* The homomeric α_7, α_8 and α_9 are sensitive to α-bungarotoxin
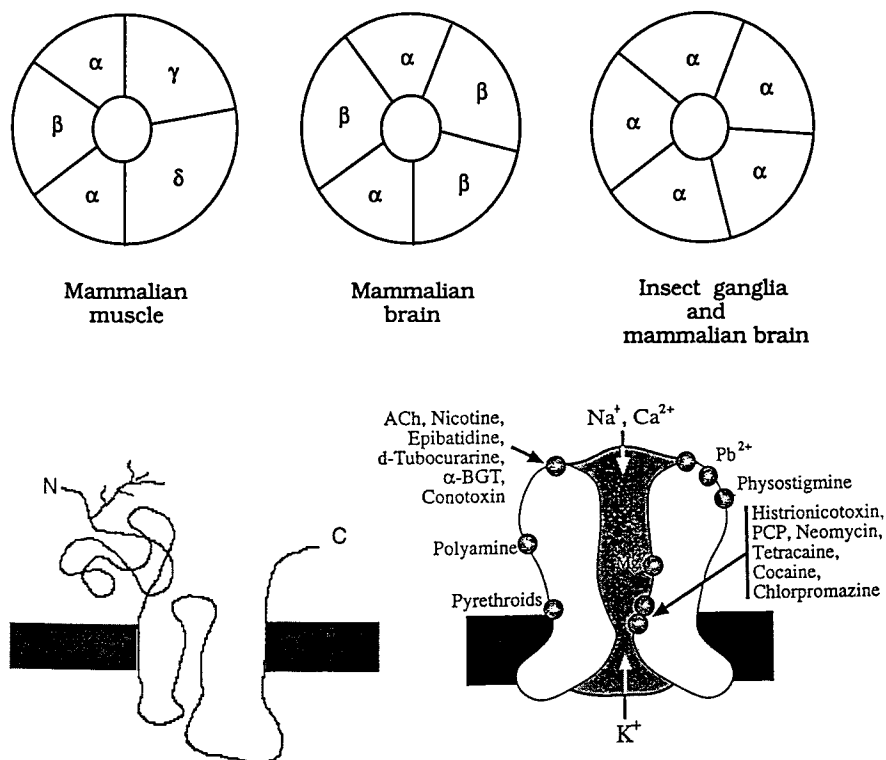
Fig. 1   Structural features of nAChRs. Top: Diagrams of horizontal cross sections of nAChR subtypes showing their five subunit composition surrounding the receptor's central ionic channel. Bottom: (Left) A diagram of the membrane topography of the amino acid sequence of a subunit with both amino (N) and carboxy (C) termini extracellular. (Right) Vertical cross section showing the receptor location in the membrane and its binding sites. (Bottom right reproduced with permission from ref. 36. Copyright 1996, Elsevier Science).

The nAChRs of insect ganglia resemble more the homomeric mammalian nAChRs than the muscle nAChR in molecular weight, subunit structure and sensitivity to α-BGT (*15, 16*) (Fig. 1). Multiple conductances of neuronal nAChRs were detected in house fly brain neurons (*17*). There may be a larger diversity of nAChRs amongst insect species, which is extremely important to know for selective pest management.

Pharmacological properties: The nAChRs are activated by ACh, other choline esters (e.g. carbamylcholine, succinylcholine, suberyldicholine) and several natural products that include the insecticides nicotine, anabasine, lobeline, anatoxin (from the blue green algae *Anabaena flos-aqua*) and cytisine. Activation is very rapid occurring in milliseconds, but fades if the agonist is not removed quickly. This results from receptor desensitization. The synaptic action of ACh is transient because of its quick effective removal by AChE. Agonists of nAChRs, which are resistant to hydrolysis by AChE, produce nAChR desensitization more readily. The biphasic physiological effects of nicotine on autonomic ganglia (stimulation followed by inhibition) is due to activation followed by desensitization of nAChRs. The site where ACh and other agonists bind to initiate receptor activation is on the α subunit. The muscle and electric organ nAChRs have two ACh-binding sites, one on each of its two α subunits; which is reflected in a Hill coefficient of receptor response that is higher than one. Important features of the ACh-binding site include two cysteines forming a disulfide bridge that is highly conserved among all nAChRs, 3 tyrosines and 1 tryptophan.

Antagonists are ligands that bind to the receptor, cause no activation and inhibit its function. There are two groups of nAChR antagonists: Competitive antagonists that bind to the ACh recognition site, such as d-tubocurarine, α-BGT and κ-BGT and the partial agonist but mostly antagonist nereistoxin (*18*). They also include the therapeutic peripheral muscle relaxants atracurium and vecuronium (*19*). A large number of non-competitive antagonists inhibit nAChR function by binding to allosteric sites within or close to the receptor's ion channel. Best known amongst them is the frog skin neurotoxin perhydrohistrionicotoxin (*20*) and the general anesthetic drug of abuse phencyclidine (PCP) (*21*). Numerous other chemicals bind to another set of allosteric sites and enhance receptor desensitization, such as the anti-AChE diisopropylfluorophosphate (*22*).

There is a variety of mammalian neuronal nAChRs that vary in their pharmacology. Generally, all α β neuronal nAChRs are insensitive to α-BGT and have higher affinity for nicotine (100-1000 folds) than does the skeletal muscle

nAChR. Furthermore, agonist selectivity varies according to the α and ß subunits that make the receptor (*10, 13*). There are pharmacological differences such as the lower affinity of the insect nAChR for α-BGT than the mammalian skeletal and neuronal nAChRs. The nAChRs of insects have lower affinity for nicotine and other natural nicotinoids than the mammalian nAChRs (*23*). In this regard, they resemble more the mammalian muscle than the neuronal nAChRs. However, different insect species may have varied sensitivities to nicotinoids because of the diversity of their nAChR subtypes. Also PCP, which is strictly a noncompetitive antagonist of mammalian nAChRs, is also a competitive antagonist of insect nAChR (*24*). There are nAChRs in insect ganglia that are even less sensitive to α-BGT, which may be due to an αß oligomeric structure similar to that of most mammalian neuronal nAChR.

Muscarinic Receptors

The mAChRs are the cholinergic receptors present in mammalian smooth muscles (e.g. intestines and blood vessels), cardiac muscles, glands and other tissues (e.g. blood, liver and pancreas). These receptors were shown to mediate the actions of the mushroom poison muscarine which were recognized as parasympathetic activators and inhibitors long before the discovery that ACh is a neurotransmitter. Activation of mAChRs by a stable agonist such as muscarine, or an anticholinesterase that protects ACh against hydrolysis by the AChE, gives a generalized over-stimulation of parasympathetic function. The mAChRs are also present in autonomic ganglia on the postsynaptic ganglionic neurons and in brain. mAChRs are widely but unevenly distributed in the brain and spinal cord. They mediate numerous brain functions including learning and memory, locomotion and aggression. The mAChRs in insects are detected in the brain and ganglia (*25*), but little information is available on their presence elsewhere.

Molecular properties: Five mAChR genes (m1-m5) have been cloned and the five receptor subtypes (M_1-M_5) are well conserved across various mammalian species (with > 90% amino acid homology) (*26*). The mAChR is a much smaller glycoprotein than the nAChR and is made of a single subunit (Mr 50-70 kDa), that has seven transmembrane segments (TM1-7) (Fig. 2). Mammalian mAChR subtypes differ in their distribution, drug specificities, transduction mechanisms and functions (Table II). The M_1,M_3 and M_5 subtypes are generally excitatory and their activation stimulates phosphoinositol hydrolysis by phospholipases A_2, C and D and tyrosine kinase, while activation of the M_2 and M_4 mAChRs inhibits synthesis of cAMP thereby producing inhibitory cellular responses. Expression models have revealed that a single mAChR subtype can activate multiple signaling
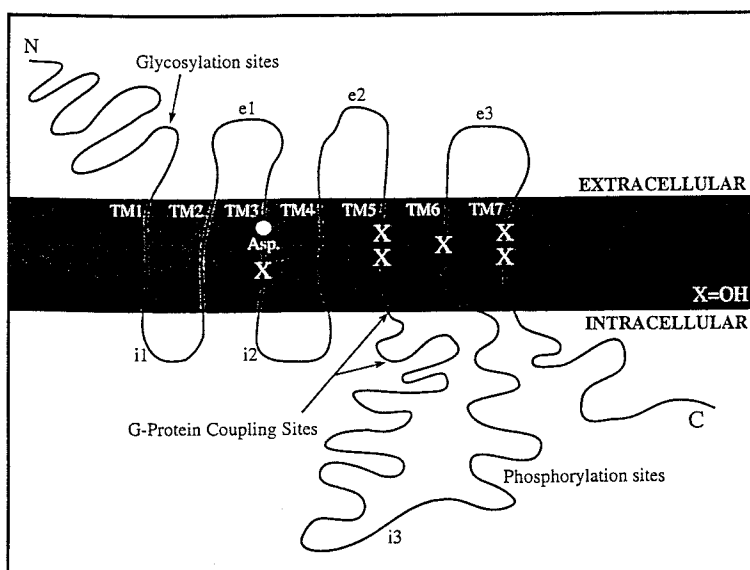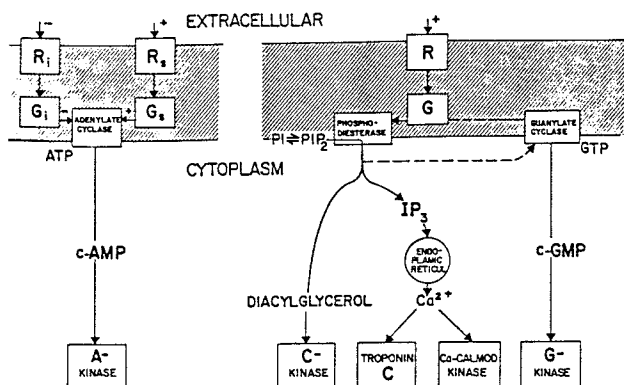
Fig. 2   Muscarinic receptors. Top: A schematic depiction of coupling of mAChRs to second messengers, either via inhibition of adenylate cyclase or activation of phosphodiesterase. Bottom: Model of a mAChR shown as the aminoacid sequence with the amino (N) and carboxy (C) termini outside and inside the cell membrane, respectively, 7 transmembrane (TM) regions, 3 extracellular (e) and 3 intracellular (i) regions. The agonist binding site is depicted as consisting of an aspartate carboxyl group and several hydroxyl groups (X) in different TM regions. (Top reproduced with permission from ref. 6; Copyright 1990, Marcel Dekker. Bottom reproduced with permission from ref. 36. Copyright 1996, Elsevier Science)

effectors (27). The 3^{rd} cytoplasmic loop of the receptor peptide is quite variable among the five subtypes and carries the G-protein recognition domain. It is much larger in insect than mammalian mAChRs.

It is believed that several transmembrane regions contribute to the ACh-binding site, which is located in a "pocket" within the membrane. The positively charged ammonium group of muscarinic ligands bind to the carboxylate of Asp 147 residue and a series of OH-containing Thr and Tyr residues in TM regions 3, 5, 6 and 7 contribute to the high affinity agonist binding.

The mAChRs on presynaptic nerve terminals are mostly M_2 subtype and act as autoreceptors to regulate ACh release in mammals and insects. Distribution of mAChRs in different mammalian brain regions and organs is uneven (Table II). The M_1 is more dominant in brain cortex, intestinal and smooth muscles, the M_2 is dominant in presynaptic membranes of heart muscle and brain and the M_3 is in glands (Table II). Insect mAChRs seem to be mostly of the M_3 subtype (25, 28). However some studies suggest the presence of M_1 mAChRs as well (28).

Table II - Mammalian muscarinic receptor subtypes:
locations, antagonist selectivities and functions

Receptor properties	M_1	M_2	M_3	M_4	M_5
Gene	m1	m2	m3	m4	m5
Location	Neuronal, brain, ganglia	Heart, smooth muscle, brain, presynaptic	Glands, smooth muscle, brain	Brain	Brain
Affinity for :					
pirenzepine	High	Low	Intermediate	Low	
himbacine	Low	High	Intermediate	Intermediate	
4-DAMP	Intermediate	Low	High	Low	
PI hydrolysis	Stimulates		Stimulates		Stimulates
cAMP synthesis		Inhibits		Inhibits	

Pharmacological properties: mAChRs are activated by natural agonists (e.g. muscarine and pilocarpine) and also inhibited by a number of natural competitive antagonists (e.g. atropine and scopolamine). Numerous synthetic agonists and antagonists of mAChRs are used therapeutically. Radioactively-labeled mAChR agonists and antagonists have been used in receptor binding assays to identify and

study mAChR in mammalian and insect preparations. Agonists and antagonists bind to a hydrophobic pocket buried in the transmembrane core of the receptor. All subtypes have similar affinities for ACh and agonists as well as atropine and quinuclidinyl benzylate (QNB), but different affinities for specific antagonists, though not more than 2-fold for a single subtype over all others. This selectivity depends on several regions on different TM domains, which differ from tricyclic antagonists (e.g. pirenzepine and himbacine, which are more selective for M_1 and M_2 respectively) to 4-diphenylacetoxy-N-methylpiperidine (4-DAMP, M_3 selective). There is also an allosteric site on the M_2 receptor which inhibits receptor response upon binding of the drug gallamine. However, most known mAChR antagonists are competitive, are potentially toxic to mammals and some that gain access easily to the brain (e.g. QNB) have serious effects on brain function.

Few pharmacological studies of insect mAChRs exist. These receptors seem to have similar pharmacology to their mammalian counterparts. However, their neurological functions may be quite different. The mAChRs of insects mediate tonic activation of interneurons and motoneurons resulting in long term potentiation. They seem to be mostly of the M_3 subtype, which are coupled to phosphoinositide metabolism (*28*). This long term potentiation could lead to excessive increase of cytosolic Ca^{2+}, which can cause cell death.

Action of Anticholinesterase Insecticides on Cholinergic Receptors

Anticholinesterases (antiChEs) fall into three classes: Reversible (e.g. the therapeutic drug edrophonium), slowly reversible (e.g. the therapeutic and insecticidal carbamates (CB)) and irreversible (e.g. the nerve agents and insecticidal organophosphates (OP)). Anti-AChEs are considered indirect cholinergic agonists because they protect ACh from hydrolysis by AChE; and ACh activates the cholinergic receptors. They also have direct effects on both nAChRs and mAChRs. Some organophosphate anticholinesterases have been shown to have very high affinity for the heart M_2 receptor (*29*). Others cause a dose and time-dependent-desensitization of nAChRs (*22*), are direct agonists of M_4 mAChR in rat brain striatum (*30*) and possibly bind to both M_3 and the Gi-protein-adenylyl cyclase system (*31*). CB anti-AChEs have also been shown to bind directly to the ACh-binding sites of nAChRs acting mainly as partial agonists (e.g. neostigmine and pyridostigmine) or in the case of physostigmine by binding mainly to channel sites and inhibiting receptor function (*32*). It also binds to a specific allosteric agonist-like site (*33*).

Insecticidal potencies of CB and OP antiChEs are explained mostly by their potencies as anti-AChEs. In the mammal mild poisoning with an OP or a CB antiChE is expressed as symptoms of overstimulation of muscarinic function (e.g.

miosis and abdominal cramps from activation of M_1 mAChRs of iris and intestinal smooth muscle, lacrimation and salivation from stimulation of glandular M_3 mAChRs, low blood pressure and low heart rate from stimulation of the M_2 inhibitory cardiac and vascular receptors. These symptoms are not life threatening except in cases of severe poisoning due to heavy exposure and lack of medical attention. The fatal lesion is respiratory failure resulting from both inhibition of cholinergic receptors that regulate the respiratory centers of the brain and more importantly paralysis of the intercostal and diaphragm muscle nAChRs due to their desensitization. The high insecticidal activity of antiChE insecticides may be due to special physiological roles that mAChRs of insects play in causing long term potentiation and the associated events (e.g. increased intracellular Ca^{2+} that may result in nerve cell death). In other words, the fatal lesion in insects may be associated more with interference with mAChRs than with nAChR functions.

Concluding Remarks

Cholinergic receptors are vital regulatory proteins in the nervous system of mammals and insects. The nAChR carries a variety of ligand-binding sites. It undergoes conformational changes upon binding of agonists that result in opening of its ionic channel, which has additional binding sites. The affinities of these sites for ligands vary in many cases depending on the receptor conformation (resting, active or desensitized). The nAChR is a three-fold larger protein than the mAChR. Because of the variety of nAChR subunits and their combinations, compared to only a few mAChR subtypes, it is expected that differences between mammalian and insect receptors will be much more in nAChRs than mAChRs. Despite the overall similarity in the molecular and pharmacological properties of insect and mammalian nAChRs, there are some distinct differences that may be exploited for discovery of new selective pest control agents. Differences in amino acid sequences are reflected in changes in pharmacological specificities of the nAChR's binding sites. For example, while phencyclidine and the nicotinoids anabasine and nitenpyram bind to both the ACh- and channel-binding sites of insect nAChRs, the former bind only to the ACh site while the latter bind only to the channel site of the *Torpedo* and vertebrate nAChRs (*23*). On the other hand, the insecticide 2(nitromethylene)tetrahydro-1,3-thiazine acts as an agonist on cockroach nerve cord and *Torpedo* nAChR; binding only to the ACh-binding site (*34*). Another binding site that is found on nAChRs is for polyamines. Philanthotoxin, which was isolated from the venom of the solitary digger wasp *Philanthus triangulum* bound at low concentrations to a site in the receptor's channel and at high doses to its ACh-binding sites (*35*). Differences in AChEs between insects and mammals should receive attention as well, since the indirect actions of OP and CB insecticides on receptors play major roles in their insecticidal potencies.

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Chapter 26

Philanthotoxins and the Nicotinic Acetylcholine Receptor

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The venom of the Egyptian wasp *Philanthus triangulum* is philanthotoxin-433 (PhTX-433) is a polyamine amide with butyryl / tyrosyl / spermine moieties. It is a noncompetitive inhibitor of the nicotinic acetylcholine receptors (nAChR) and various glutamate receptors (GluR). Over 100 analogs have been synthesized for structure / activity relations (SAR) and various other purposes. Preliminary photo-crosslinking results, coupled with SAR, have led to a putative model representing the binding of philanthotoxins in channels gated by nAChR. Exciton coupled circular dichroic studies with amphiphilic analogs designed to elucidate their mode of entry into the receptor show that the philanthotoxins are capable of forming micelles in aqueous solutions. It has also been found that PhTX can apparently form sodium transporting transmembrane channels when added to phosphatidylcholine vesicles. The sodium transporting capability of the PhTX vesicles are comparable to those of other efficient channel forming compounds such as gramicidin and amphotericin B; furthermore, the PhTX molecules in the vesicle can undergo intervesicular transfer. These findings suggest a possible mode of action of the PhTXs. Namely, PhTX may first enter the lipid bilayer and then enter the nAChR from the cytoplasmic side rather than the extracellular side. Preliminary results on solid state ^{19}F-NMR of fluorinated PhTXs and nAChR complexes are also mentioned briefly.

The venoms of funnel-web spiders and the parasitic wasp produce various neurotoxins, including a group of compounds called polyamine amides (1). They secrete toxins of similar structure and biological function, and employ identical molecular strategies to paralyze their victims. The same toxins are relevant to human neuropharmacology as well because the receptors present in the nervous systems of mammals, including Homo sapiens, share many structural properties with the target receptors of these toxins found in the excitable systems of arthropods. These low molecular weight molecules exhibit complex pharmacologies in which a hydrophilic polyamine and aromatic and/or other hydrophobic moieties play key roles. In 1986 the first two polyamine amides were isolated from spider venoms: argiopine (or argiotoxin 636) by Grishin et al. (2) and JSTX-3 by Aramaki et al. (3), while in 1988 the wasp neurotoxin philanthotoxin-433 was isolated by Eldefrawi et al. (4) and Piek

et al. (5) (Figure 1). The wasp produces only a few variants, but ca. 100 variants have been isolated from various spiders and the number continues to grow. These discoveries have converged into an area of intense research which embraces basic investigations of learning and memory and the potential for the developments of new classes of pharmaceutical agents for preventing or limiting brain cell damage following a stroke.
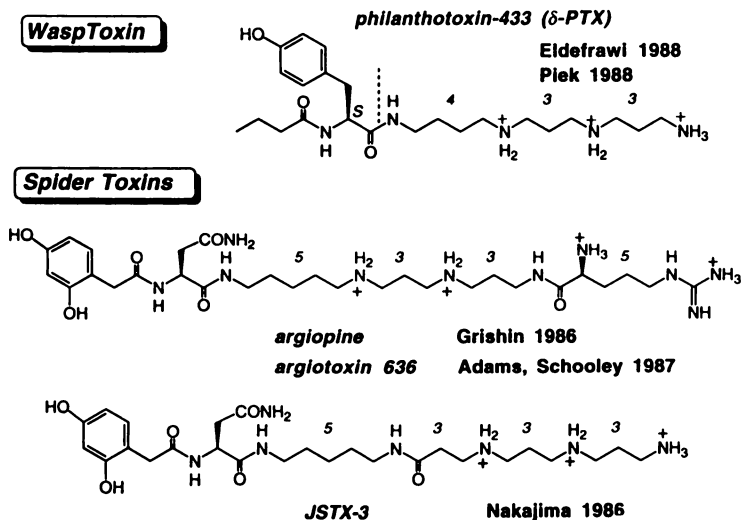
Figure 1. Some early polyamine amide toxins.

The spider and wasp toxins are non-competitive antagonists of the nAChR and GluRs. The former receptor, sequenced in 1986 (6) is the best characterized type of ligand-gated channel protein. Electron microscopic, biochemical, mutagenetic and other studies performed on the receptor isolated from the electric organs of Torpedo rays and other sources have led to a gross three dimensional structure (7-10). Recently, several genes encoding the important GluRs have been cloned and sequenced (11) but since none of them have been overexpressed in quantities sufficient for biochemical structural studies, our efforts have been focused on the readily accessible nACh-R isolated from the electric organs of the ray *Torpedo californica* and the Skate (Naja). Using nACh-R as a probe, we are aiming to develop new general approaches towards facilitating tertiary structural studies (at a chemical level) of the ligand/receptor interactions. The focus of the approach is to use synthetic philanthotoxin analogs which are ideally suited for use as templates, streamline and drastically scale down the amount of receptor required, and to eventually apply the experience gained through nAChR studies to the much more challenging and important glutamate receptors (GluRs), even if they have not yet been overexpressed.

Extensive structure activity relationship (SAR) studies have been performed to determine the channel blocking potencies of PhTX analogs. Assays were performed with nAChR, GluR and quisqualate type GluR (qGluR) using tritiated perhydro histrionicotoxin displacement and/or neurally evoked twitch contraction of locust metathoracic retractor hind muscle (for qGluR). Over 100 analogs have been synthesized by systematically modifying the molecule selectively or simultaneously in the four regions I - IV, shown in Figure 2. SAR results are summarized in the Figure (12); the IC_{50} for PhTX-433 in two assays are also shown. In most cases, analogs exhibited higher potency than the native 433 toxin, the more potent analogs exhibiting ca. 30-fold activity.

philanthotoxin-433 IC$_{50}$ $-\begin{cases} \text{nAChR } [^3\text{H}] \text{ perH-histrionicotoxin} : 1.1 \times 10^{-6} \text{ M} \\ \text{qGluR locust muscle contraction} : 1.8 \times 10^{-5} \text{ M} \end{cases}$

hydrophobic anchoring groups
para-OH has little effect

$\begin{cases} \text{nAChR : activity increases with iodination;} \\ \text{Tyr, Leu, Ala, Phe, Gly, Trp all active} \\ \text{qGluR} : I_2 > Br_2 > Cl_2 > F\text{-Tyr} \end{cases}$

longer chain with
N$^+$ increases activity

S, R configurations:
comparable activity

Philanthotoxin-433

IV

HO

III

n-Bu here increases activity

polyamine chain essential
N-permethylation reduces activity

hydrophobicity and/or aromaticity enhance activity
hydrophilic groups strongly reduce activity (nAChR)

Figure 2. Summary of SAR

The nAChR is a membrane-bound 270 kDa glycoprotein consisting of five subunits, α, α, β, γ and δ, which encircle an internal ion channel; two external agonist binding sites are located on the extracellular surface of the two alpha subunits (Figure 3A, view into receptor from extracellular side). Each subunit consists of four membrane-spanning regions, M1-M4, with the M2 segments, believed to be α-helical, lining the channel. The receptor has a large hydrophilic extracellular region which connects via the transmembrane region to a hydrophobic cytoplasmic moiety (Figure 3C). Binding of the neurotransmitter acetylcholine to its two receptor sites on the extracellular surface of two α subunits leads to opening of the channel lined by the M2 segments. These segments form several well-defined rings (8) which directly affect the functional properties of the channel (Figure 3C): there is negatively charged ring (1) at the top, then a large hydrophobic region (2), a leucine ring (3) which forms a constriction in the gate, two hydrophilic rings (4), and two anionic rings (5), and finally a hydrophobic domain (6) in the cytoplasmic interior; close to the receptor in the cytoplasmic interior, there exists a 43 kDa protein (Figure 3B).

Preliminary photoaffinity labeling together with SAR have led to a working stereo model for the ligand/receptor binding, where the polyamine ammonium groups (tail) of PhTX are lined against the hydrophilic circles consisting of five α-helical subunits of the open channel of the receptor in a tail-up configuration. However, in the photoaffinity studies, the channel blocker was applied in a manner that it had access to both the extracellular and cytoplasmic sides of the receptor. As a result, the model may not represent the native mode of binding; however, subsequent studies have indicated that this could well be the major binding mode (unpublished, see below).

The radioactive and photolabile PhTX analog, N$_3$-Ph-^{125}I$_2$-PhTX-343-Lys (see Figure 3), specific activity 25 Ci/mmole, was synthesized, and photoaffinity labeling studies with pure nACh-R of the electric organ of the fish *Torpedo marmorata* as well as the membrane-bound nACh-R from this source were performed (13.). The receptor was incubated with N$_3$Ph-^{125}I$_2$PhTX-343-Lys and
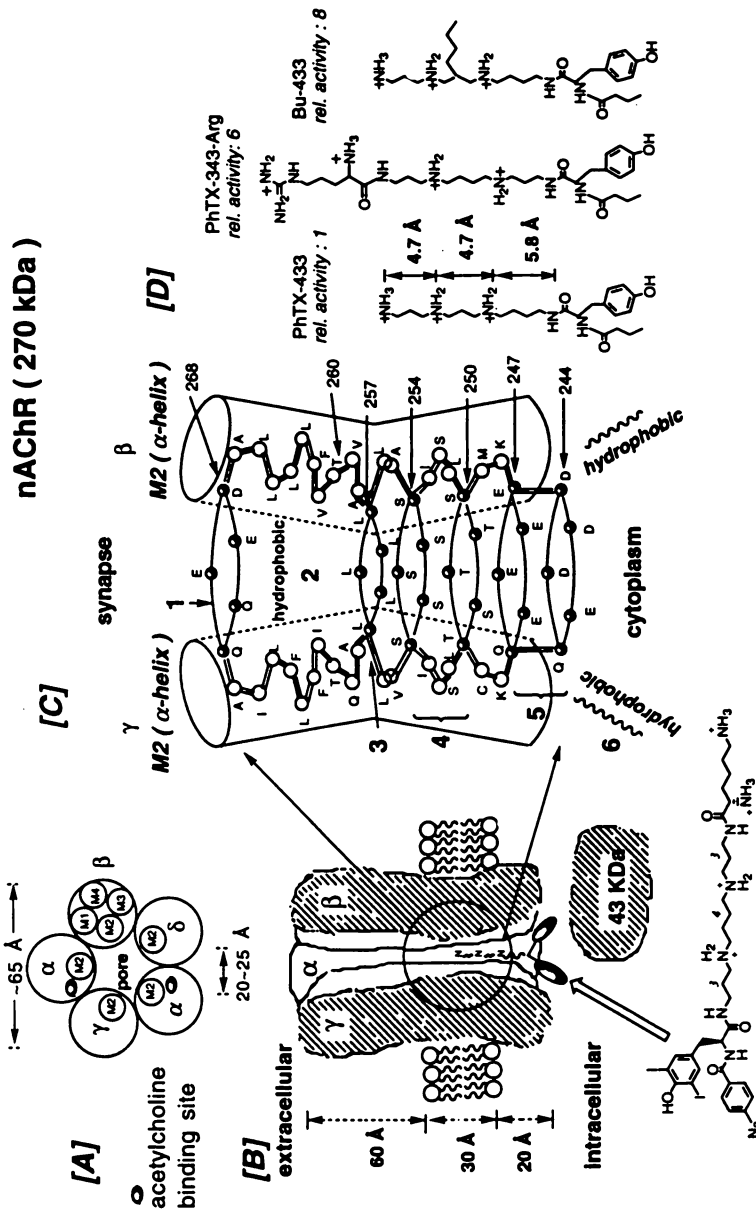
Figure 3. The nAChR and binding of some PhTX analogs (Adapted from ref. *13*).

the agonist (channel opener) carbamylcholine, photoactivated, the $\alpha/\beta/\gamma/\delta$ subunits were separated by SDS-PAGE, and the eluted α-band was proteolysed. The main radioactivity was associated with the 20 kDa Ser[173]-Glu[338] segment encompassing M1, M2, M3 segments (Figure 4). In a separate run, the α-subunit was digested with
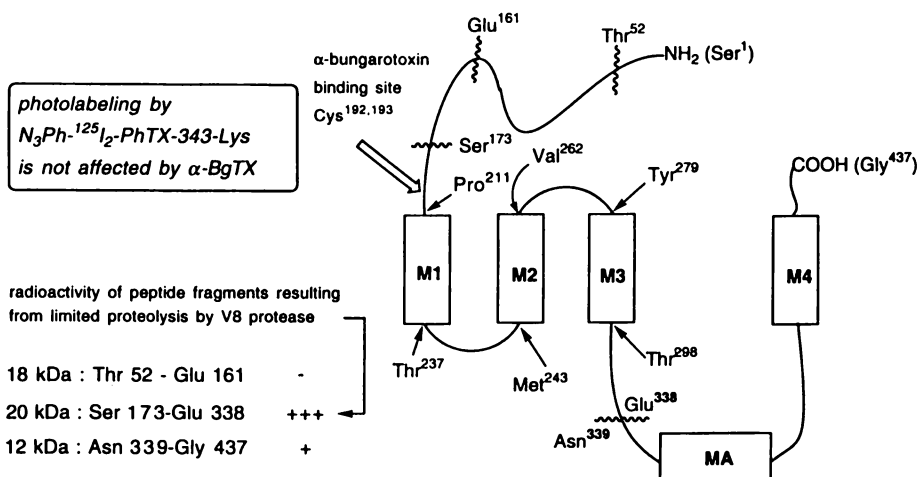
Figure 4. Photoaffinity of nAChR with PhTX analog (see Figure 3).

trypsin and submitted to HPLC to locate the radioactivity; a parallel experiment was performed with cold ligand. Mass spectrometric measurements of all bands (cold) corresponding to the radioactive bands indicated that the mass ranges were less than 2,000. The cross-linking site of the azido nitrogen is presumably on the intracellular loop bridging M1 and M2 and/or the intracellular segment extending from M3 up to Glu[338]. The short extracellular segment leading into M1 from Ser[173] can be excluded because the same results were obtained when experiments were performed in the presence of α-bungarotoxin that binds to this short segment; α-bungarotoxin is an antagonist which competes with acetylcholine for its binding sites on nACh-R.

Almost identical results were obtained from similar experiments performed on the δ-subunit. Since all of the membrane-spanning peptides expected from tyrosine cleavage of nAChR have molecular weights much larger than 2.5 kDa, it follows that the azidophenyl moiety was bound to a cytoplasmic (intracellular) domain of this receptor protein (Figure 3B, unfilled arrow). However, this finding is not so surprising as it might first appear. It was shown earlier that when PhTX-343 is injected *into* locust muscle fibers rather than being applied by topical application, the GluR present in the surface membrane of these fibers are antagonized, a result which suggests that there is a binding site for polyamine amides on the cytoplasmic domain of these receptors (*14*).

The polyamine ammonium tail of PhTX-433 is lined against the hydrophilic circles /5, and the ydrophobic regions III and IV in the hydrophobic domain 6 (Figs. 3); if the orientation were opposite, the hydrophobic head would be in hydrophobic domain 2 of the receptor. The distances between the ammonium groups 4.7 - 5.8 Å   cl se to the 5.4 Å pitch of α helices. In Figure 3 the PhTX molecules are depicted with the "head" group lying in the cytoplasmic hydrophobic region 6. Although S R (*12*) and affinity labeling studies (*13*) lead to this orientation, it is not known whether this is actu ly the case under all conditions.

Some comments on the model depicted in Figure 3 follows. Past assay results have shown that the following comments commonly apply to GluR (of insect muscle and mammalian brain) as well as nAChR (similar comments are applicable to the reverse orientation of PhTX where the head, Regions III and IV, is in hydrophobic space 2).

(i) Region I. The long polyamine chain is essential. Small differences have been observed in activity against insect Glu-R of PhTX-343 (relative activity 1.0), PhTX-433 (natural, 1.3) and PhTX-334 (1.5, not listed); this could arise from subtle differences in the alignment of the hydrophilic Ser, Thr, Glu and Gln groups in the three rings and the distances between the ammonium groups in the polyamine (Figure 3D). The 8-fold increased potency relative to PhTX-433 exhibited by Bu-433 (Figure 3D) which has a Bu side-chain extending from the hydrophilic polyamine chain, should be noted. This could be accounted for by hydrophobic binding of the Bu group to Leu-251 (?), one of the questions which will be addressed by photoaffinity labeling. Quaternization of amino groups drastically reduces activity; it is likely that the bulk of the hydrophobic methyl groups hinders the formation of hydrogen-bond stabilized links between, for example serine and the polyamine chain.

(ii) Region II. Extension of the polyamine chain enhances activity. For example, the 6-fold enhanced activity of PhTX-343-Arg (Figure 3D) may be due to the generation of a H-bond between the terminal guanidinium group and Thr-260 (?).

(iii) Region III. Activity is enhanced by hydrophobic groups but is drastically reduced by hydrophilic groups, i.e., substitution of the butyl group with an Asp moiety reduces affinity to nACh-R to 10 %. Regions III and IV probably interact with the hydrophobic amino acid moieties present in the nAChR space 2 or 6. The high potencies of the azidophenyl analog (6~12-fold) and the cinnamate analog (12~15-fold) are encouraging since these aromatic residues can be utilized in photolabeling studies.

(iv) Region IV. Here some differences were noticed between insect muscle Glu-R and *Torpedo* nAChR. In the former case, a bulky anchoring group with moderate hydrophobicity appears to be necessary. The hydroxyl group on Tyr is not required; rather activity is increased in the Phe analog. The systematic activity enhancement accompanying halogenation of Tyr ($I_2 > Br_2 > Cl_2 > F$) is noteworthy. It is conceivable that this trend is related to the operation of a polarizability effect during binding. The enhanced activity in the iodinated analog is important since this not only leads to analogs with 4 to 9-fold enhanced affinity through iodination that can be performed at the last stage, but also to radiolabeled ^{125}I analogs (*15*). Activity with nAChR is not affected by removal of the bulky aromatic moiety.

Finally, GluR affinity is not dependent on the configuration of the Tyr group, the D-isomer being as active as the native L-isomer. This is not surprising in consideration of the pseudo-centrosymmetric structure of the channel of these receptors.

Elucidation of the *in vivo* mode of entry of the toxin into the open gate as well as its mode of attachment to the receptor is a focal point in understanding the mode of action PhTX on nAChR and eventually the GluRs. Regarding the mode of entry of PhTX into the receptor, five possibilities are conceivable (Figure 5).
(i) PhTX enters R from the synapse and settles in head-up direction.
(ii) PhTX enters R from the synapse, flips over, and settles head-down.
(iii) PhTX with BIG head cannot pass constriction and settles head-up.
(iv) PhTX enters R from the synapse, the positively charged polyamine complexes with Cl$^-$ anion, or the neutral polyamine chelates with Na$^+$ or other cations and settles head-up.
(v) PhTX penetrates the lipid bilayer into the cytoplasm and settles head-down. The mechanism of penetration is not clear (see below).
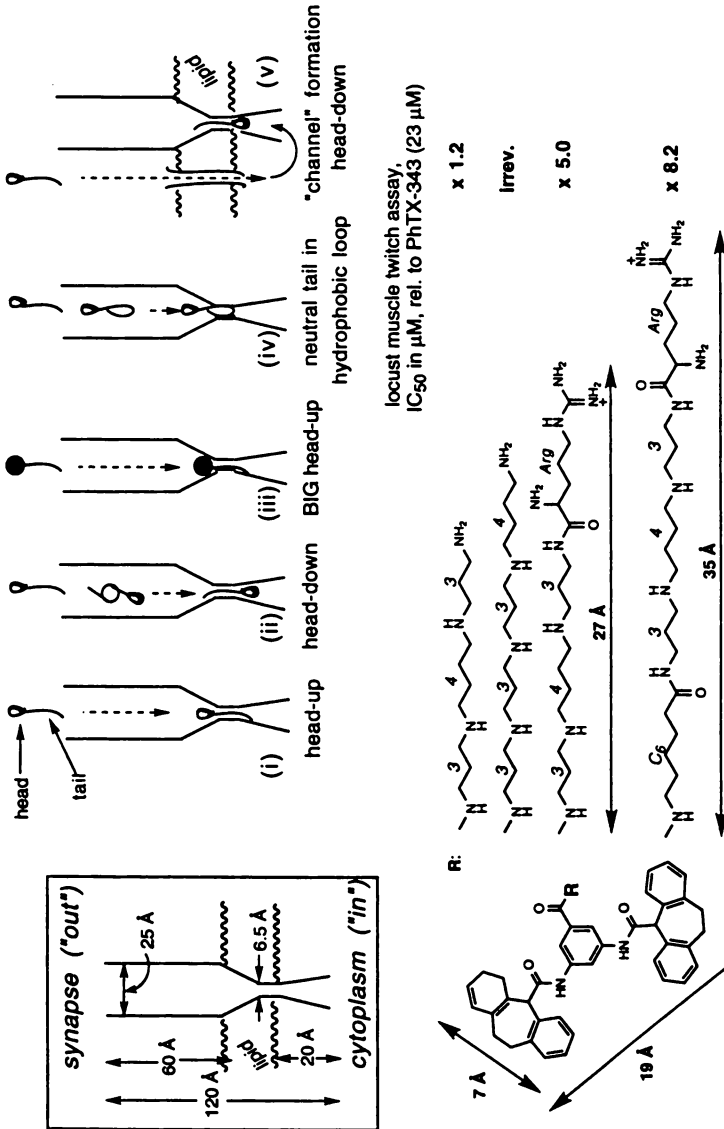
Figure 5. Modes of entry of PhTX into nAChR and BIG PhTX derivatives.

Although (iv) may occur in the case of analogs with specific structures, e.g., PhTX with a macrocyclic polyamine (aza-crown-PhTX)(*16*), it cannot be the general entry mechanism for several reasons. The 8-fold enhanced activity of the analog Bu-433 (Figure 3D) is specific only for this position isomer; when the alkyl branch is moved to the terminal or to the first methylene chain flanked by the two nitrogens, the activity drops drastically to 0.5 (unpublished). A cyclic polyamine structure as in (iv) would not account for this drop because of the quasi-centrosymmetric structure of the rings surrounding the channel.

In order to check mode (iii), analogs with BIG head groups shown in Figure 5 were synthesized (*17*) and bioassayed against qGluR (muscle twitch assay) (unpublished). The iminodibenzyl-5-carbonyl benzoyl moiety cannot hardly change its dimension by rotating around single bonds, and therefore would not be possible to pass the 6.5 Å constriction of the receptor around the leucine ring (Figure 3C, ring 3). The assay showed that the longer the tail, the higher the activity, thus implying a tighter fit into the receptor. Thus the activities of these BIG head analogs can be accounted for by mode (iii) in which the BIG head is directed toward the synapse. This is opposite to the configuration resulting from photo-cross linking (Figure 3B)(*13*).

The following unexpected results, transmembrane sodium transport by PhTX, are in agreement with mode of entry (v) and also supports configuration (ii), i.e., results of the photoaffinity labeling (*13*) (unpublished). Egg phosphatidylcholine vesicles were prepared in 100 mM LiCl, and to this was added 100 mM NaCl solution containing 0.3 mol % of PhTX-343 (Figure 6). Transport rate was estimated by ^{23}Na/^{7}Li NMR upon which an NMR shift reagent (*18*) which cannot penetrate the vesicle is added; measurements of the Na and Li signals, inside and outside the vesicle, respectively, gives the cation transport rate if it occurs; this protocol follows that of Lehn et al. (*19*). The observed rapid transport rate, plotted in Figure 6, was comparable to those of brevetoxin B (Matile, S.; Nakanishi, K. *Angew. Chem.*, in press), amphotericin B (*20* and gramicidin (*21*). It was further demonstrated that PhTX molecules undergo intervesicular transfer (bottom plot of Figure 6). Thus upon treatment of the PhTX-containing vesicles with the same amount of empty phosphatidyl choline vesicles, after 24 hours or when the Na/Li exchange had attained equilibrium, the height of the Na "in" peaks, after immediately dropping to 50 %, again attained equilibrium after 24 hours. PhTX-343 is ca. 25 Å long, and shorter than the ca. 40 Å thickness of average lipid bilayers. The mechanism of Na/Li transport across the membrane is thus not clear. But the rapid transport rate implies ion transport via a "channel-like" mechanism and not via a carrier mechanism. If PhTX can undergo intervesicular transfer, PhTX molecules can also enter a vesicle, namely PhTX can penetrate a membrane. *This makes mode of entry (v) depicted in Figure 5 feasible, which in turn is in agreement with affinity cross-linking results* (Figure 3)(*13*).

Porphyrin derivatives of PhTX were made (*17*) in order to utilize their size as well as their intense and sharp Soret band at 415 nm (ε 350,000); these spectral properties make porphyrins one of the most powerful chromophores for studies of exciton coupled CD (*22*). They were used successfully for investigating the transmembrane ion pore properties formed by brevetoxin B (submitted). It was hoped that PhTX porphyrin analog (I)(Figure 7) might enter the receptor according to mode (iii) of Figure 5, similar to the case of BIG analogs. However, when dissolved in buffer B, an exciton coupled CD with Cotton effects of equal intensities of opposite signs (conservative CD) were observed at 439 nm (Δε -3.2) and 415 nm (Δε +2.9)(Figure 7) (*17*). This conservative shape of the negatively split CD demonstrates that the split CD is due to intermolecular helical stacking with negative chirality because of

Transmembrane sodium transport of PhTX is comparable to brevetoxin B, gramicidin and amphotericin B

- Egg PC vesicles prepared in 100 mM LiCl
- PhTX (0.3 mol %) in 100 mM NaCl added

   i) PhTX enters membranes
   ii) PhTX crosses membranes
   iii) PhTX transports sodium
       through membranes
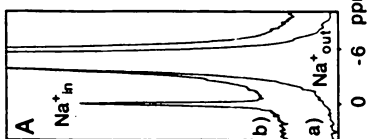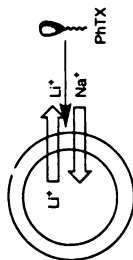
PhTX molecules undergo intervesicular transfer

1) *PhTX vesicles* equilibrated for 24 h
2) *Empty vesicles* equilibrated for 24 h

- Egg PC vesicles prepared in 100 mM LiCl
- 100 mM NaCl added

3) PhTX vesicles and empty vesicles are mixed.
   Na^+_{in}/Na^+_{out} ratio drops to 50%.

4) Na^+_{in}/Na^+_{out} ratio reaches 100 % in ca. 24 h.
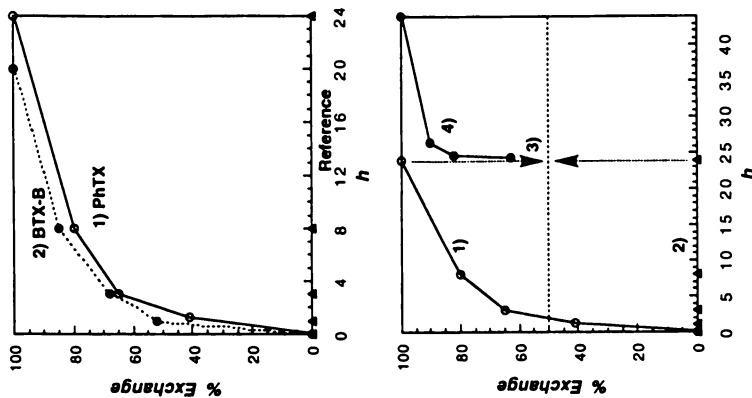
Figure 6. Sodium movement across lipid bilayers containing PhTX-433.
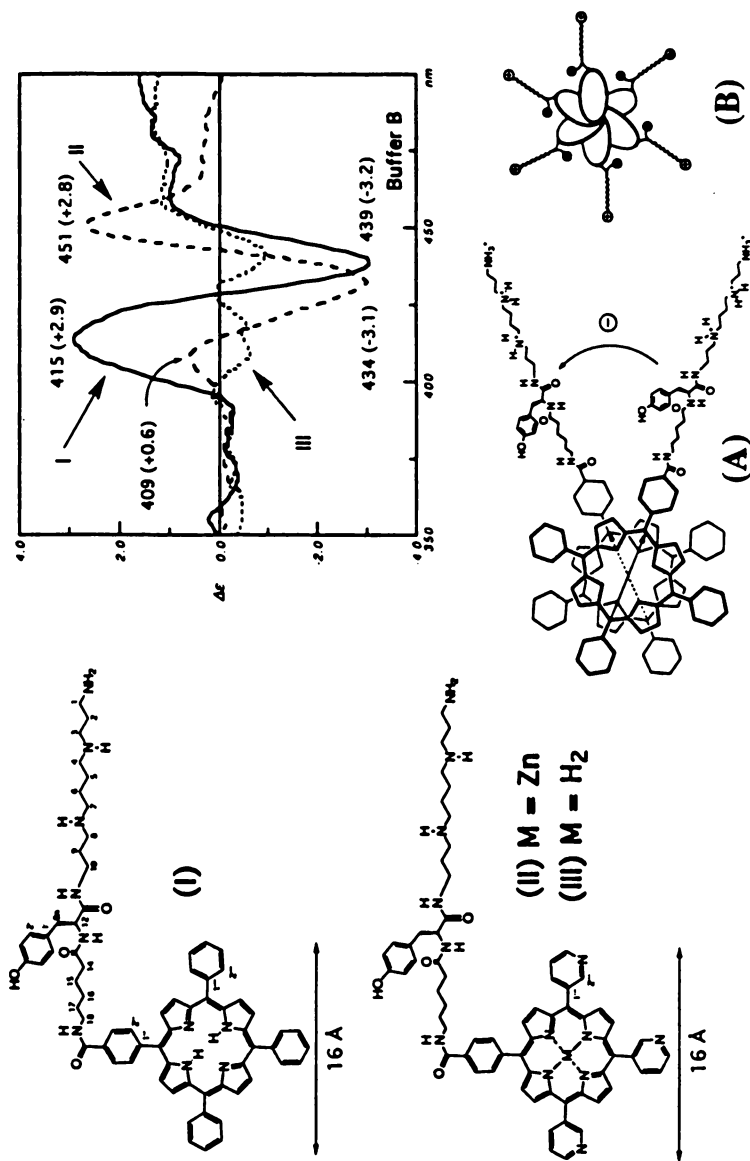
Figure 7. CD of porphyrin analogs of PhTX in aqueous buffer.

the insolubility of analog (**I**). The more soluble trispyridine porphyrin analog of PhTX was prepared as its zinc complex (**II**); this again exhibited a bisignate CD; the positive sign of the split CD (Figure 7) showed that coordination of zinc had induced intermolecular stacking with opposite chirality of that of (**I**). However, removal of zinc now yielded the PhTX analog (**III**) which was soluble in buffer B as seen by the weak Cotton effects due to the (remote) chiral center of the PhTX moiety (*17*). This water-soluble analog and other similar analogs will now be used for further studies of the mode of interaction between the PhTX analog and the nAChR.

[19]F-solid state (SS)-NMR should become an extremely tool to explore various aspects regarding ligand/receptor binding. The advantages of FNMR (*23*) include its high sensitivity comparable to [1]H, no background signals and stronger homo-nuclear dipolar couplings. Since no F exists in native proteins or membranes, subtraction is unnecessary. If decoupling from neighboring protons is sufficient, the line shape would be dominated by chemical shift anisotropy, which typically is averaged by magic angle spinning (MAS). This gives rise to sharp F peaks spread over a wide chemical shift range. The following unpublished experiments have been performed in collaboration with the group of Professor Ann McDermott of this department and Dr. Takashi Iwashita of the Suntory Institute for Bioorganic Research, superb NMR spectroscopists . To insure authenticity of observed peak, a highly purified nACh-R enriched membrane (1.3 mg) was incubated with 5-10 μg of PhTX derivatives **I**- **III** and 1 mM of the agonist carbamylcholine to open the receptor gate. After

(**I**)   PhTX-343-Gly(N-CH$_2$CF$_3$)

(**II**)   R = H, C$_T$-F-PhTX-343-Lys-NHCOCF$_3$

(**III**)   R = F, C$_T$-F-PhTX-343-Lys-NHCOCF$_3$
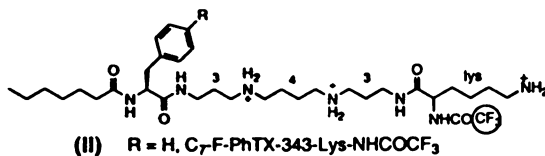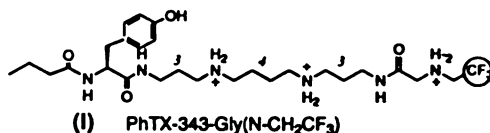
centrifugation, MAS-NMR of the centrifuged sample-containing pellets gave sharp diagnostic [19]F signals spread over the range of -70 to -120 ppm. This is probably the first SSNMR of any protein/ligand complex of comparable size; the receptor nAChR is 270 kDa. The high sensitivity of the [19]F nucleus, coupled with its sensitivity to environmental changes shows that F-SSNMR will provide a unique and powerful tool for mode of action and/or tertiary structural studies of ligand/receptor complexes.

The development of novel techniques by McDermott and coworkers has also led to successful F-F distance measurements in SSNMR. Simple phenyl or linear polyphenyl derivatives have been selected due to ease of estimation of the CF$_3$/CF$_3$ distance (Figure *8*). The F chemical shift of each group is -43 ppm (SCF$_3$) and -64 ppm, respectively, which is in the convenient range for distance measurements by

Table 1. Activities on qGluR of photosensitive PhTX analogs (adapted from ref. 24)

Neurally-evoked twitch contraction of locust metathoracid retractor unguis muscle.
Numerals denote IC_{50} in μM; IC_{50} for the reference PhTX-343 (A) is 23 μM.

IC_{50} for the the native toxin PhTX-43 (B) is 17.7 μM.

Experiments performed in subdued light.

All compounds irreversibly inhibit contraction if applied during UV irradiation.

A) PhTX-343 : 23

B) PhTX-433 (natiove 17.7

(1) N3-benzoyl-PhTX-343 : 4.2

(2) N3-benzoyl-PhTX-343-Lys : 3.3

(3) N3-benzoyl-I2-PhTX-343-Lys : 1.6

(4) N3-Cin-PhTX-343 : 38

(11) Diene-N3Phe-PhTX-343 : 16

(12) Diene-N3Phe-PhTX-343-Lys : 3

(13) Diene-N3Phe-PhTX-343-Arg : 2.6

(14) diene-N3Phe-Asn-533-Arg : 2.9

(5) N₃-Cin-I-PhTX-343 : 6.2

(6) N₃-Cin-I₂PhTX-343 : 4.7

(7) N₃-Cin-PhTX-343-Arg : 3.3

(8) N₃-benzyloxy-diene-PhTX-343 : 23

(9) N₃-benzyloxy-diene-PhTX-343-Arg : 1.5

(10) N₃-phenacetyl-Asn-533-Arg : 3.8

(15) C₁₀-N₃Phe-Asn-533-Arg : 4.1

(16) PhTX-343-benzoyl-N₃ : 30

(17) PhTX-343-Cin-N₃ : 42

(18) C10-PhTX-343-Phe-N₃ : 58

(19) C10-I₂-PhTX-343-Phe-N₃ : 3.3

Figure 8. Reference F compounds for F-F distance calibration

SSNMR. In order to keep the fluorine containing samples isolated in the solid state, the samples have to be diluted with the dilutants or deuterated samples. After repeated attempts, the dilutants shown in Figure 8 were employed and calibration curves for various CF_3-CF_3 distances up to ca. 20 Å were obtained. The triphenyl derivative with a distance of 16 Å gave an almost horizontal calibration curve showing that this distance is the limit of fluorine distance measurements by this method (submitted). The calibration curves will enable us to incorporate bisfluoro-PhTX derivatives such as **III** shown above and others into the receptor and measure the F-F distances of ligands within the receptor. Furthermore, photolabile analogs containing two fluorine groups will be used to measure the F-F distances in the ligand/receptor complex, which in turn should give the distances between the cross-linked amino residues.

Many photolabile analogs of PhTX have been prepared in order to secure information which would assist in crosslinking studies for mode of action or tertiary structural studies of the complex. Some of the results are summarized in Table 1 (*24*). The lower the numeral, the more potent. The presence of the azido group on either Region III (Figure 2) or the phenyl group as in compounds **11-15** increases potency, except for **4** and **8**. As noted earlier (*15*) iodination also increases activity; compare **2/3**, **4/5/6**. Potency is reduced when the azidophenyl is on the tail end, i.e., **16-18**; however, **19** is exceptional for which there is no rationalization at this stage (*24*). Several photolabile analogs have been made based on the SAR data of Table 1.

As mentioned at the outset, we plan to employ these and other methods to other ligand/receptor complexes as well as to understand the mode of binding of the polyamine amides using the versatile philanthotoxin molecules as rulers.

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