

BIOLOGICALLY ACTIVE NATURAL PRODUCTS: Agrochemicals



edited by
Horace G. Cutler
Stephen J. Cutler

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Preface

Forty-five years ago, agricultural and pharmaceutical chemistry appeared to be following divergent paths. On the agricultural scene industrial companies were concentrating on the synthesis of various classes of compounds and when a successful chemical candidate was discovered, there was a good deal of joy among the synthetic chemists. We were told that as a result of chemistry life would be better and, indeed, it was. Armed with synthetic agrochemicals, the American farmer became the envy of the world. Essentially, with a vast series of chemical permutations, the synthetic chemist had tamed nature and the biblical admonition to subdue the natural world was well underway. One large agricultural chemical company, now out of the business, had in its arsenal plans to pursue “cyclohexene” chemistry among its many portfolios. Plans were already in motion to produce the series and on the drawing board was the synthesis of abscisic acid, later discovered in both cotton bolls and dormant buds of *Acer pseudoplatanus* as a biologically active natural product. The chemical elucidation led, in part, to the winning of the Nobel Prize by Dr. John Cornforth. How different the history might have been if the chemical company in question had synthesized the molecule quite by accident. In the field of pharmacy, natural product therapy was, at one time, the mainstay. With the rapid development of synthetic chemistry in the mid to late 1900s, those agents soon began to replace natural remedies. Even so, several natural products are still used today with examples that include morphine, codeine, lovastatin, penicillin, and digoxin, to name but a few. Incidentally, griseofulvin was first reported in 1939 as an antibiotic obtained from *Penicillium griseofulvum*. However, its use in the treatment of fungal infections in man was not demonstrated until almost 1960. During the 20 years following its discovery, griseofulvin was used primarily as an agrochemical fungicide for a short period. Interestingly, it is a prescription systemic fungicide that is still used in medicine today.

Certainly, the thought that natural products would be successfully used in agriculture was a foreign concept at the beginning of the 1950s. True, the Japanese had been working assiduously on the isolation, identification, and practical use of gibberellic acid (GA) since the late 1920s. And later, in the early 1950s, both British and American plant scientists were busy isolating GA₃ and noting its remarkable effects on plant growth and development. But, during the same period, some of the major chemical companies had floated in and out of the GA picture in a rather muddled fashion, and more than one company dropped the project as being rather impractical. To date, 116 gibberellins have been isolated and characterized.

There was no doubt that ethylene, the natural product given off by maturing fruit, notably bananas (and, of course, smoking in the hold of banana ships was strictly forbidden because of the explosive properties of the gas) had potential, but how was one to use it in unenclosed systems? That, of itself, is an interesting story and involves Russian research on phosphate esters in 1945. Suffice to say the problem was finally resolved on the practical level with the synthesis of the phosphate ester of 2-chloroethanol in the early 1970s. The chlorinated compound was environmentally benign and it is widely employed today as a ripening agent. Indole-3-acetic acid, another natural product which is ubiquitous in plants and controls growth and development, has been used as a chemical template, but has not found much use *per se* in agriculture. Indole-3-butyric acid, a purely synthetic compound, has large-scale use as a root stimulant for plant cuttings. The cytokinins, also natural product plant growth regulators, have found limited use since their discovery in stale fish

sperm, in 1950, mainly in tissue culture. Brassinolide, isolated from canola pollen, has taken almost 35 years to come to market in the form of 24-*epi*brassinolide and promises to be a highly utilitarian yield enhancer. However, there is no doubt that synthetic agrochemicals have taken the lion's share of the market.

In the 1980s something went wrong with the use of "hard" pesticides. Problems with contaminated groundwater surfaced. Methyl bromide, one of the most effective soil sterilants and all purpose fumigants, was found in well water in southwest Georgia. There was concern that the product caused sterility in male workers and, worse, the material was contributing to the ozone hole above the polar caps. Chlorinated hydrocarbons, such as DDT (1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane), were causing problems in the food chain and thin egg shells in wild birds was leading to declining avian populations. Never mind that following World War II, DDT was used at European checkpoints to delice and deflea refugees. The former ensured that the Black Plague, which is still with us in certain locations in the U.S., was scotched by killing the carrier, the flea. The elimination of yellow fever and malaria, endemic in Georgia in the early 1940s, also was one of the beneficial results of DDT. To date it is difficult to envisage that two thirds of the population of Savannah, GA was wiped out by yellow fever 2 years before the Civil War.

During the late 1980s and 1990s, a movement to use natural products in agriculture became more apparent. Insecticides, like the pyrethroids which are based on the natural product template pyrethrin, came to the marketplace. Furthermore, natural products had certain inherent desirable features. They tended to be target specific, had high specific activity, and, most important, they were biodegradable. The last point should be emphasized because, while some biologically active organic natural products can be quite toxic, they are, nevertheless, very biodegradable. Another feature that became obvious was the unique structures of natural products. Even the most imaginative and technically capable synthetic chemist did not have the structural visions that these molecules possessed. Indeed, nature seems to make with great facility those compounds that the chemist makes, with great difficulty, if at all. This is especially true when it comes to fermentation products. It is almost a point of irony that agrochemistry is now at the same place, in terms of the development of new products, as that of pharmaceutical chemistry 50 years ago, as we shall see.

A major turning point in the pharmaceutical industry came with the isolation and discovery of the β -lactam, penicillin by Drs. Howard W. Florey and Ernst B. Chain who, after being extracted from wartime England because of the threat of the Nazi invasion, found their way to the USDA laboratories in Peoria, IL, with the Agricultural Research Service. The latter, in those days, was preeminent in fermentation technology and, as luck would have it, two singular pieces of serendipity came together. First, Mary Hunt ("Moldy Mary" as she was called by her colleagues) had scared up a cantaloupe which happened to be wearing a green fur coat; in fact, *Penicillium chrysogenum*, a high producer of penicillin. Second, there was a byproduct of maize, corn steep liquor, which seemed to be a useless commodity. However, it caused *P. chrysogenum* to produce penicillin in large quantities, unlike those experiments in Oxford where Drs. Florey and Chain were able to produce only very small quantities of "the yellow liquid".

This discovery gave the pharmaceutical industry, after a great many delays and back-room maneuvering, a viable, marketable medicine. Furthermore, it gave a valuable natural product template with which synthetic chemists could practice their art without deleting the inherent biological properties. History records that many congeners followed including penicillin G, N, S, O, and V, to name but a few. But, more importantly, the die was cast in terms of the search for natural product antibiotics and other compounds from fermentation and plants. That does not mean that synthetic programs for "irrational" medicinals had stopped but, rather, that the realization that nature could yield novel templates to conquer

various ills was a reality rather than a pipe dream. To use an old cliché, no stone would go unturned; no traveler would return home from an overseas trip without some soil sticking to the soles of his shoes.

The common denominator in both agrochemical and pharmaceutical pursuits is, obviously, chemistry. Because of the sheer numbers of natural products that have been discovered, and their synthetic offspring, it was inevitable that the two disciplines would eventually meld. Examples began to emerge wherein certain agrochemicals either had medicinal properties, or *vice versa*. The chlorinated hydrocarbons which are synthetic agrochemicals evolved into useful lipid reducing compounds. Other compounds, such as the benzodiazepine, cyclophenol from the fungus *Penicillium cyclopium*, were active against *Phytophthora infestans*, the causal organism of potato late blight that brought Irish immigrants in droves to the New World in search of freedom, the pursuit of happiness, and, as history records, the presidency of the U.S. for their future sons; and, one hopes in the future, their daughters. While not commercially developed as a fungicide, the cyclophenol chemical template has certain obvious other uses for the pharmacist. And, conversely, it is possible that certain synthesized medicinal benzodiazepines, experimental or otherwise, have antifungal properties yet to be determined. It also is of interest to note that the β -lactone antibiotic 1233A/F, [244/L; 659, 699], which is a 3 hydroxy-3-methyl glutaryl CoA reductase inhibitor, has herbicidal activity. Interweaving examples of agrochemicals that possess medicinal characteristics and, conversely, medicinals that have agrochemical properties occur with increasing regularity.

In producing a book, there are a number of elements involved, each very much dependent on the other. If one of the elements is missing, the project is doomed to failure.

First, we sincerely thank the authors who burned the midnight oil toiling over their research and book chapters. Writing book chapters is seldom an easy task, however much one is in love with the discipline, and one often has the mental feeling of the action of hydrochloric acid on zinc until the job is completed. We thank, too, those reviewers whose job is generally a thankless one at best.

Second, we thank the Agrochemical Division of the American Chemical Society for their encouragement and financial support, and especially for the symposium held at the 214th American Chemical Society National Meeting, Las Vegas, NV, 1997, that was constructed under their aegis. As a result, two books evolved: *Biologically Active Natural Products: Agrochemicals* and *Biologically Active Natural Products: Pharmaceuticals*.

Third, the School of Pharmacy at Mercer University has been most generous with infrastructural support. The Dean, Dr. Hewitt Matthews, and Department Chair, Dr. Fred Farris, have supported the project from inception. We also thank Vivienne Oder for her editorial assistance.

Finally, we owe a debt of gratitude to the editors of CRC Press LLC who patiently guided us through the reefs and shoals of publication.

**Horace G. Cutler
Stephen J. Cutler**

Editors

Horace G. (Hank) Cutler, Ph.D., began research in agricultural chemicals in February 1954, during the era of, “we can synthesize anything you need,” and reasonable applications of pesticides were 75 to 150 lbs/acre. His first job, a Union Carbide Fellowship at the Boyce Thompson Institute for Plant Research (BTI), encompassed herbicides, defoliants, and plant growth regulators (PGRs); greenhouse evaluations, field trials, formulations; and basic research. He quickly found PGRs enticing and fell madly in love with them because of their properties. That is, they were, for the most part, natural products and had characteristic features (high specific activity, biodegradable, and target specific). After over 5 years at BTI, he went to Trinidad, West Indies, to research natural PGRs in the sugarcane, a monoculture.

It quickly became evident that monocultures used inordinate quantities of pesticides and, subsequently, he returned to the U.S. after 3 years to enter the University of Maryland. There, he took his degrees in isolating and identifying natural products in nematodes (along with classical nematology, plant pathology, and biochemistry). Following that, he worked for the USDA, Agricultural Research Service (ARS) for almost 30 years, retired, and then was appointed Senior Research Professor and Director of the Natural Products Discovery Group, Southern School of Pharmacy, Mercer University, Atlanta. He has published over 200 papers and received patents on the discovery and application of natural products as agrochemicals (the gory details are available at ACS online). Hank’s purloined, modified motto is: “Better ecological living through natural product chemistry!”

Stephen J. Cutler, Ph.D., has spent much of his life in a laboratory being introduced to this environment at an early age by his father, “Hank” Cutler. His formal education was at the University of Georgia where he earned a B.S. in chemistry while working for Richard K. Hill and George F. Majetich. He furthered his education by taking a Ph.D. in organic medicinal chemistry under the direction of Dr. C. DeWitt Blanton, Jr. at the University of Georgia College of Pharmacy in 1989. His area of research included the synthesis of potential drugs based on biologically active natural products such as flavones, benzodiazepines, and aryl acetic acids. After graduate school, he spent two years as a postdoctoral fellow using microorganisms to induce metabolic changes in agents which were both naturally occurring as well as those he had synthesized.

The latter brought his research experience full circle. That is, he was able to use his formal educational training to work in an area of natural products chemistry to which he had been introduced at an earlier age. He now had the tools to work closely with his father in the development of natural products as potential pharmaceuticals and/or agrochemicals either through fermentation, semi-synthesis, or total synthesis. From 1991 to 1993, the younger Cutler served as an Assistant Professor of Medicinal Chemistry and Biochemistry at Ohio Northern University College of Pharmacy and, in 1993, accepted a position as an Assistant Professor at Mercer University School of Pharmacy. He teaches undergraduate and graduate pharmacy courses on the medicinal chemistry and pharmacology of pharmaceutical agents.

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Agrochemicals and Pharmaceuticals: The Connection

Horace G. Cutler and Stephen J. Cutler

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ABSTRACT Three categories of agrochemicals (or potential agrochemicals) and pharmaceuticals are discussed both in the context of historical discovery and utilitarian development. The benzodiazepines include the natural products cyclopenin and cyclophenol, their potential as plant growth regulators, fungicides, and tranquilizers vs. the synthetics lorazepam, oxazepam, clorazepate dipotassium, temazepam, prazepam, flurazepam dihydrochloride, triazolam, and alprazolam. All exhibited activity in the etiolated wheat coleoptile bioassay. The phenoxy compounds include 2,4-dichlorophenoxyacetic acid, and its 2,4,5-trichloro congener, 2-(3-chlorophenoxy)-propanoic acid, and *p*-chlorophenoxyacetic acid, all of which are plant growth regulators. Juxtaposed to these is the development of clofibric acid, clofibrate, and their derivatives which reduce blood serum cholesterol. Finally, the organophosphates metrifonate, initially used to control schistosomiasis, and dichlorvos are examined. Dichlorvos, a catabolite of metrifonate, was an important insecticide but now finds use in controlling Alzheimer's disease.

1.1 Introduction

There is a certain pleasure that comes from migrating across scientific disciplines. At first, there is suspicion that the translation will dilute the fund of knowledge rather than construct a highly viable hybrid and, furthermore, it is difficult to keep abreast of the discoveries in one discipline let alone taking on a new vocabulary and findings of another. Surprisingly, in this transition it happens that chemical structures and their congeners,

which one has known for many years, turn up like old friends. To put it tritely, different play, different characters, same actors. Perhaps another metaphor for the relationship between agrochemicals and pharmaceuticals also exists. The common thread between the two disciplines is chemistry, both synthetic and natural product chemistry. And, while the thread may be singularly strong, it is only when it is woven into cloth that it becomes exceptionally durable and has major utilitarian value. Paradoxically, the threads in a piece of cloth consist of a warp and a weft, and they are diametrically opposed. It is the precise opposition that maximizes the strength.

Both agrochemicals and pharmaceuticals are two of the pillars upon which modern civilization stands. And *mens sana in corpore sano*, a sound mind in a sound body, is certainly the ideal product of that interdisciplinary marriage. The first set gives rise to an abundant supply of food, the second set keeps the body and mind in a healthy, functioning state. From the economic point of view, pharmaceuticals are lucrative value-added products. Agrochemicals, on the other hand, while they may yield a solid return on investment, do not generate as much income on a “weight to sale” proportion. That is, a few milligrams of a medicinal may sell for several dollars, while several pounds of an agrochemical may sell for the same price. However, between the two disciplines there is an apparently vast no-man’s land. As we shall see, there are a number of chemical templates that have found use in both disciplines. But surely, in the multitude of pharmaceuticals that have been discovered as natural products, their synthetic derivatives, and the logical sequence by which one arrives at an active product that no longer resembles its progenitor, there must be compounds that have alternate uses. Of special interest in agriculture are those natural products that do not adversely affect the environment because they are target specific, have high specific activity, and are biodegradable — all attributes of natural products and, to a certain extent, their synthetically modified products.

It may be argued by the reader that some of the examples presented herein are not all natural products and that the symposium, after all, involved natural product agrochemicals and pharmaceuticals. Like all true penitents we have to say *mea culpa* to the charge, and while we have not kept to the strict letter of the law we have certainly kept to the spirit. And most certainly the phenoxy compounds, for example, had their origin in a natural product. Nature absolutely preceded the synthetic chemist in the biosynthesis of the benzodiazepines. But above all, the intention is to weave the disciplines of agrochemicals and pharmaceuticals to the benefit of all.

1.2 Benzodiazepines

It is erroneously believed that the discovery of the benzodiazepine structure was first revealed in the second half of 1955.¹ In fact, the events leading up to the use of this family of compounds as anxiolytic agents was completely separate to the elucidation and first disclosure of the structure. In retrospect, it is difficult to understand how much literature was ignored by those involved in agrochemistry and pharmaceuticals, and it can only be assumed that the two were operating in isolation. From the pharmaceutical perspective the history is charged with the elements of chance, genius, and geography. In the 1930s, Leo Sternbach had been working in Cracow, Poland as a post-doctoral assistant. At that time, he was looking at the properties of the benzheptoxdiazines for use as dyestuffs, in a very unsettled world. Hitler was already rattling the sabres of war, Poland was becoming a bone of contention in the gathering political storm, and the fortuitous opportunity arose for Sternbach to make his way to America to work at the Roche laboratories.

During the 1950s, the first tranquilizers were made available to the public. They were, in chronological order of the patents that were granted in the U.S., the following: chlorpromazine, 2-chloro-10-[3-(dimethyl amino) propyl] phenothiazine, commonly known as thiorazine (U.S. Patent 2,645,640 to Rhône-Poulenc, 1953) and marketed as the hydrochloride. In 1951, a minor tranquilizer, meprobamate, 2-methyl-2-propyl-1,3-propanediol dicarbamate, had been synthesized by Ludwig and Piech² and this was later patented by Carter Products (U.S. Patent 2,724,720) in 1955. There followed the root of the Indian plant *Rauwolfia serpentina* L. (= *Ophioxylon serpentium* L.) which had been used in the East for generations as an antihypertensive and as a “mood altering” agent with tranquilizing properties. The chemical composition of the root extract was shown to be reserpine, reserpinine, yohimbine (a purported aphrodisiac), ajmaline, serpentine, and serpentinine.³⁻⁵ Of these, reserpine, 11,17 α -dimethoxy-18 β -(3,4,5-trimethoxybenzoyl) oxy]-3 β , 20 α -yohimban-16- β -carboxylic acid methyl ester, was the constituent which possessed antihypertensive and tranquilizing properties.⁵ Most importantly, it contained the indole structure, a key molecule in both psychomimetic and plant growth regulating substances. The material was patented and marketed (U.S. Patent 2,833,771 to Ciba, in 1958).

Ironically, the soma described by Aldous Huxley in 1932 as the opiate of the select masses in his book, *Brave New World*, came in a multitude of guises in the early 1950s with the exception that these were highly useful medicinals. The pharmaceutical industry was quick to realize the potential of these materials and rightly so. One should bear in mind that in 1950 World War II had only been over for 5 years. Horrific events still haunt some of us who lived in European cities a half century later, and the reasons for developing those types of drugs were critical to post-war stabilization. The game was afoot.

So it was that Leo Sternbach found himself placed on a project at Roche to find new tranquilizers. His analysis of the research task went through some dichotomous thinking. None of the available tranquilizers, or sedatives, or hypnotics were structurally similar and, subsequently, there was no common chemical denominator. That left him with the option of either using those compounds that existed and synthetically modifying them or trusting to luck and empirically producing biochemicals on the off chance that they might have the desired activity. On the positive side of the equation, all the necessary bioassays were in place. Fortuitously, he decided that it would be far more profitable if he pursued some chemistry that he knew, namely the benzoheptoxdiazines, the compounds that he had worked on in Cracow which, since that time, had remained in relative obscurity. First synthesized by Auwers and von Meyenburg, and reported in 1891,⁶ the ground had been well cultivated for the prepared mind of Sternbach and, furthermore, for the element of serendipity to come into play.

Oddly, the progression of the research took an odd turn. The chemistry of certain benzoheptoxdiazines was such that hydrogenation produced quinazolines and, more specifically, quinazoline-3-oxides. Once again, fate intervened. First, the pharmacological properties of these were, to overstate the case, uninteresting. Second, in the latter part of 1955 the project was halted because other unrelated, more important research avenues were opening up for the chemical company. Then, as Sternbach so eloquently relates, the laboratory had reached a critical stage by April 1957. The clutter was such that there was no space on which to place anything. And for those who have used coffee cups to hold test tubes, when all the racks have been expended, it is a familiar story. But during the laboratory cleanup two crystalline products, a base and a hydrochloride salt, were found in separate containers and the water soluble material was tendered for bioassay. That product was patented in July, 1959 (U.S. Patent 2,893,992 to Hoffman-LaRoche) and given the trademark Librium®.⁶ The entire process from discovery to application had taken only 2 years which is something of a miracle by present day standards. From that point on, many thousand benzodiazepines were synthesized.

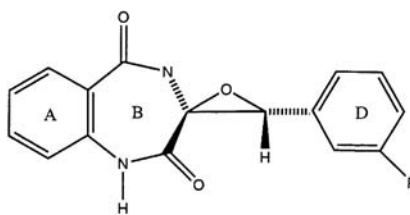


FIGURE 1.1

Structures for cyclophenin and cyclophenol.

R = H Cyclophenin

R = OH Cyclophenol

However, in 1954 a paper was published which, at the time, was of little interest to those working with tranquilizers. The title of the paper, "Studies in the biochemistry of microorganisms. 93. Cyclophenin, a nitrogen-containing metabolic product of *Penicillium cyclopium* Westling," was largely ignored with the exception that the metabolite had demonstrated slight antibiotic activity against *Micrococcus pyogenes* var. *aureus*, and *Escherichia coli*. At best, it could be hoped that synthetic modification would produce a practical antibiotic. But, what was important was the disclosure of the structure,⁷ a benzodiazepine (Figure 1.1). In addition, a second benzodiazepine, cyclophenol, was discovered in 1963^{8,9} and it had its genesis in *Penicillium viridicatum*. Again, the significance of the benzodiazepine structure appears to have fallen on barren ground. It is a point of irony that the original sample of cyclophenin, extracted from *P. cyclopium*, was later shown to be composed of cyclophenin and cyclophenol. It was not until another 20 years had elapsed following the initial discovery that the extent of the biological properties of both cyclophenin and cyclophenol were published.¹⁰ First, it was shown that crude extracts from an aberrant strain of *Penicillium cyclopium* (NRRL 6233) contained cyclophenin and cyclophenol in a ratio of 1:4 and that the crude extract inhibited the growth of etiolated wheat coleoptiles. Upon purification and identification, cyclophenin was shown to inhibit coleoptiles at 10^{-3} and 10^{-4} M, 100 and 33%, while cyclophenol inhibited only 20% at 10^{-3} M, relative to controls. In other intact plant bioassays, cyclophenin induced malformations in the first set of trifoliate leaves of 9-day-old bean (*Phaseolus vulgaris* L. cv. Black Valentine) at treatments of 10^{-2} M and stunting and necrosis in 1-week-old corn (*Zea mays* L. cv. Norfolk Market White). But, there were no visible effects on 6-week-old tobacco plants (*Nicotiana tabacum* L. cv. Hick's). On the other hand, cyclophenol had no apparent effect on bean, corn, or tobacco plants.¹⁰ Conversely, cyclophenol was highly active at rates of 0.025%, while cyclophenin was moderately active at the same concentration, against *Phytophthora infestans*, late blight of potato (*Solanum tuberosum* L.).¹¹ Significantly, this fungus was responsible for the Great Famine in Ireland during the years 1846 and 1847 and led to a massive emigration of those surviving starvation and typhus.¹² Fully one quarter of the population left home aboard the coffin ships, so-called because many souls were lost at sea, the most notable being, quite by chance, the Titanic upon which many emigrants were sailing to the New World in 1912. Oddly, the organism had only been recorded in the U.S. and Europe in 1840,¹³ but it is now a perennial problem in the potato-growing areas of the world and the applications of fungicides required to control the organism ranges from 8 to 18 per season in the U.S.

Pharmaceutically, cyclophenin induced drowsiness in day-old chicks within 2 h when dosed in corn oil *via* crop intubation, at doses of 250 mg/kg. At rates of 500 mg/kg chicks exhibited intoxication, ataxia, prostration, but were not paralyzed, within 1 h. Doses of 25 and 125 mg/kg failed to produce any external symptoms. In all cases, the chicks had fully recovered within 18 h, although those treated at the highest rate remained slightly drowsy. However, cyclophenol induced no response.¹⁰ This poses the question as to the utility

of the hydroxyl on the D ring of the latter. And does a substitution, either electron withdrawing or donating, appreciably affect the performance of the molecule as a plant growth regulator, fungicide, or anxiolytic agent? A further proposition arises in that the possibility of other benzodiazepines being present in other microorganisms also exists. Also intriguing is the observation that benzodiazepines without a C3-OH generally have long half-lives and are converted to this species by hepatic oxidation, while compounds with C3-OH possess short half-lives because they conjugate with glucuronide and are then excreted in the urine.¹⁴ So, does the epoxide form the C3-OH, and if it does in both cyclopenin and cyclophenol, why then is the latter inactive as a tranquilizer? It is possible that the polar nature of the hydroxyl group on the D ring prevents cyclophenol from entering the central nervous system in therapeutic levels. Obviously, the nature of the substituent at C15 is critical and, perhaps, other sites on the D ring would play an important role.

1.2.1 Bioassay

It must be emphasized that the discovery of the plant growth regulating properties of cyclophenin on intact plants and the tranquilizing properties were predicated on the growth inhibiting effects induced in the coleoptile bioassay, a plant bioassay. Briefly, the assay consists in sowing wheat (*Triticum aestivum* L. cv. Wakeland) on moist vermiculite in plastic dishpans, sealing them with aluminum foil and placing them in the dark at $22 \pm 1^\circ\text{C}$.¹⁵ When the seedlings are 4 days old, they are harvested, the caryopses and roots cut from the shoots, discarded, and the shoots are fed tip first into a Van der Weij guillotine. The apical 2 mm are cut away and the next 4 mm are retained for bioassay. Only one 4 mm section is excised from each shoot. Ten sections are then placed in a test tube, with the crude extract or compound to be tested, in 2 mL of phosphate-citrate buffer, pH 5.6, supplemented with 2% sucrose as a carbon source.¹⁶ Test tubes are placed in a roller-tube apparatus and rotated at 0.25 rpm. Following incubation at 22°C the sections are removed, blotted, placed on a glass sheet that is introduced into a photographic enlarger, and the images ($\times 3$) are recorded and the data statistically analyzed. All manipulations are carried out at 540 nm.¹⁷

1.2.2 Synthetic Benzodiazepines

Because cyclophenin was active in the assay, the synthetic benzodiazepines became an obvious area to explore. Diazepam was the first tested and it inhibited coleoptiles at 10^{-3}M , 100% relative to controls.¹¹ When week-old corn plants (*Z. mays* L. cv. Norfolk Market White) were treated with 10^{-2}M solutions of diazepam, the treated portions of the plants became bleached after 4 days, but a week later the plants had recovered and were green.¹¹ Beans (*P. vulgaris* L. Black Valentine) were not affected by diazepam at concentrations that ranged from 10^{-2} to 10^{-4}M . Other benzodiazepines tested in the coleoptile bioassay included lorazepam, oxazepam, clorazepate dipotassium, temazepam, prazepam, flurazepam dihydrochloride, triazolam (Halcion®), alprazolam (Xanax®), and chlordiazepoxide (Figure 1.2). Of these, diazepam, lorazepam, clorazepate dipotassium, temazepam, prazepam, and flurazepam dihydrochloride significantly inhibited ($P < 0.01$) coleoptiles 100% at 10^{-3}M . Triazolam and alprazolam, which are structurally very similar and differ only by the substitution of a fluorine for a chlorine in the latter, significantly inhibited ($P < 0.01$) 42% at 10^{-3}M , while chlordiazepoxide inhibited 40% at 10^{-3}M , and oxazepam inhibited 34% at 10^{-3}M , relative to controls. Of all the compounds tested, lorazepam also was significantly active at 10^{-4}M . However, when applied to week-old corn plants (*Z. mays* L. cv. Norfolk Market White) only flurazepam dihydrochloride and chlordiazepoxide

Effects of Benzodiazepines on Wheat Coleoptile Growth

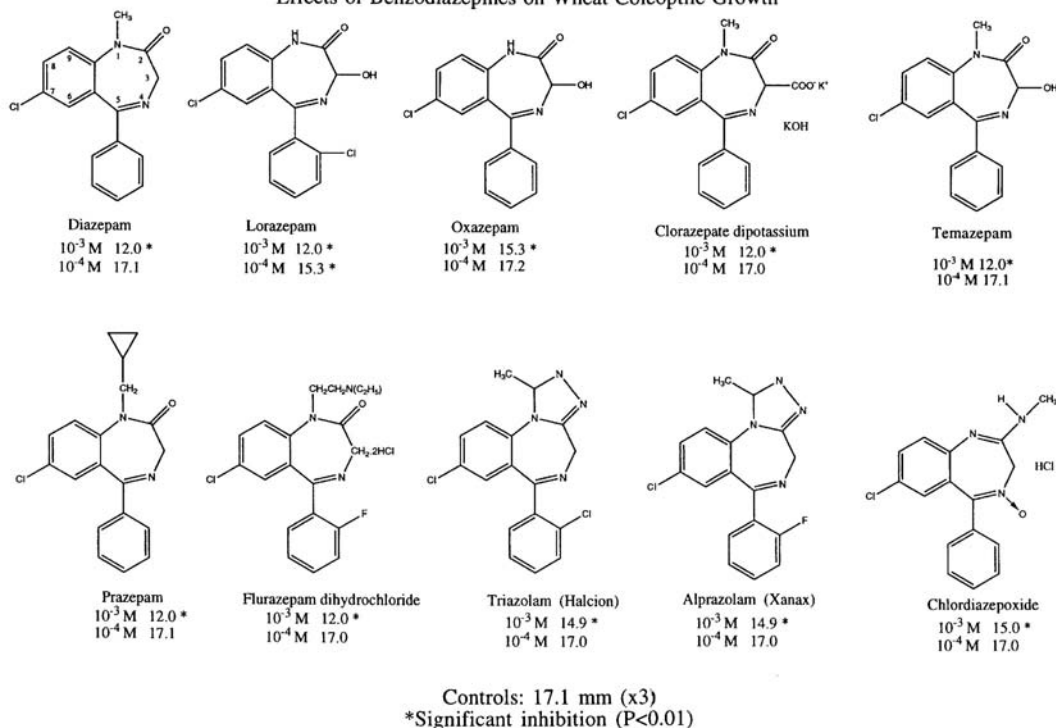


FIGURE 1.2

Structures for assorted benzodiazepines.

induced necrosis within 48 h when applied at 10⁻²M, but the plants resumed normal growth a week later. None of the compounds affected week-old bean plants (*Phaseolus vulgaris* L. cv. Black Valentine).¹⁸

It is of interest that the etiolated wheat coleoptile bioassay detected biological activity with the benzodiazepines which, for the most part, are medicinals. It is also capable of detecting antimicrobials, immunosuppressants, anti-amoebic, and “other” biologically active materials.¹⁷ But, it does not detect neurotoxins. Furthermore, an amplification effect has been observed in that if biological activity is noted in the bioassay then when the “correct” niche for the active metabolite is found, the specific activity of that substance increases by a factor of several hundred times.¹⁸ Hence, while the assay was originally designed to detect plant growth promoting and inhibiting compounds, it also is a very powerful tool for detecting pharmaceutical and other compounds. The mechanism of action by which the bioassay detects structurally diverse, biologically active materials is not known, but it is highly probable that a quick detection *in vitro* system can be developed, thus reducing the assay time from 18 h to less than 1 h.

1.2.3 Phenoxy Compounds

The phenoxy compounds have a long and illustrious history in the field of agrochemicals and much has been written concerning their application. The most famous are 2,4-dichlorophenoxyacetic acid (2,4-D) and its congener 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). 2,4-D had its genesis in troubled times which accelerated its development. In World War II (1939 to 1942), the Allies found themselves fighting a jungle war in Asia. While the jungle

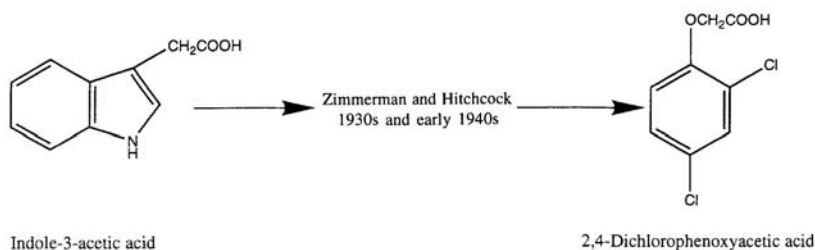


FIGURE 1.3

Schema for transition of indole-3-acetic acid to 2,4-D.

is an excellent environment to fight a guerilla war and hide equipment, it becomes difficult terrain for the hunter to find his quarry and to take a straight shot at an enemy through undergrowth and foliage. 2,4-D was extremely active at low concentrations in killing dicotyledonous plants and its derivative, 2,4,5-T, was most effective against shrubs and defoliating trees. These were still the chemicals of choice during the Vietnam conflict and were used under the name Agent Orange.¹⁹ Unfortunately, the demand exceeded the available supply during that conflict and in order to meet production demands the reaction temperature was increased to raise the Q_{10} of the synthesis rate with disastrous results because a side product, the potent teratogen dioxin (2,3,7,8-tetrachlorodibenzo [*b,e*] [1,4] dioxin), was produced.²⁰ One of the major industrial accidents with this material occurred in Seveso, Italy on July 10, 1976, with disastrous result to the inhabitants.

The history of the development of 2,4-D and 2,4,5-T is somewhat murky. Discussions with Zimmerman and Hitchcock indicate that they realized that the structure of indole-3-acetic acid (IAA) might be a good template for synthesizing agriculturally useful compounds. IAA was a phenyl pyrrole and making substitutions on the phenyl or pyrrole ring might give active compounds (Figure 1.3). Also, naphthalene acetic acid had been shown to have high specific activity as a plant growth regulator. As an interjection, but cogent point, Lawrence J. King who worked at the Boyce Thompson Institute (the same location as Zimmerman and Hitchcock) proposed that a carbamate derivative of naphthalene acetic acid would possibly be a herbicide, or plant growth regulator, and asked his colleague Joseph Lambrecht to make..." six novelty carbamates".²¹ One of these turned out to be not a herbicide but, rather, an insecticide marketed as Sevin®. So the indole ring, the acetic acid group, and probably some other features combined in the minds of Zimmerman and Hitchcock to produce 2,4-D. Their work was carried out in the late 1930s and early 1940s. At about the same time, work was progressing in England on 2,4-D but it was cloaked in the Official Secrets Act, because of the war. The first disclosure of the synthesis of 2,4-D and 2,4,5-T was in 1941²² by Pokorny in an exceptionally brief, 27-line, one quarter page report from the C. B. Dolge Company and the patents issued were: British Patent 573,476 in 1945, and U.S. Patent 2,471,575 to U.S. Rubber in 1949. Obviously the pursuit of patents was rather loose in the early years of agrochemical discovery!

Apart from being used as a broadleaved postemergence herbicide, 2,4-D also has been used to control grasses in sugarcane at very high application rates²³ even though it is not considered to be a grass herbicide. It is also used as a yield enhancer at rates of 4 to 200 mg/L in grapefruit, lemons, and oranges when the fruit is approaching one in. in diameter, which causes the fruit to increase in size. Additionally, it is used to control fruit drop in citrus trees that are over 6 years old. The storage life of lemons may be increased and ripening inhibited by treating fruit with solutions containing 500 mg/L.²⁴

Because of their high specific activities, 2,4-D and 2,4,5-T became model templates for the production of a number of plant growth regulators. In 1945, 2-(3-chlorophenoxy)-propanoic

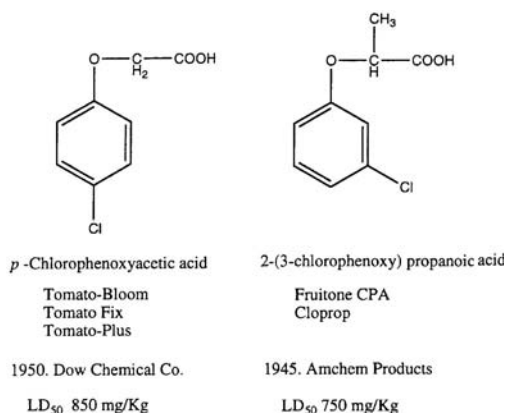


FIGURE 1.4

Structures of chlorophenoxy plant growth regulators.

acid (Figure 1.4) appeared on the market under the trade name Fruitone® and it was used in pineapple to reduce crown growth, increase fruit size and weight, and to lower shipping costs because of injury reduction in the shipping process.²⁴ A congener of Fruitone®, called Tomato Bloom®, was *p*-chlorophenoxy acetic acid and it found use as a compound to increase fruit set in tomatoes.²⁴ The material is applied at 500 mg/L to tomato flowers as they open although it is not necessary to spray all the flowers to induce fruit set. By way of an oddity, it also is used as a soak for mung beans (*Phaseolus aureus*) to inhibit root growth, but the seed has to be washed with water following the 5 to 8 h treatment.²⁴

One of the first pharmaceutical phenoxy compounds was clofibrate, ethyl 2-(*p*-chlorophenoxy)-2-methylpropionate (Figure 1.5). It is administered to control type III hyperlipoproteinemias, where the triglycerides and total cholesterol are elevated, although it is moderately active against type IIb and type IV cases. In the former, triglycerides are slightly elevated and cholesterol levels are high while, in the latter, triglycerides are moderately-to-highly elevated and cholesterol is normal-to-elevated. Upon ingestion, esterases act upon clofibrate to yield the free acid which then binds to serum albumin in the blood. Clofibric acid (Figure 1.5) also can be given, but it absorbs more slowly into the bloodstream relative to the ethyl ester. It has been reported that the most efficient form of the medicinal is the aluminum salt.²⁵ The mode of action of clofibrate is inhibition of *sn*-glyceryl-3-phosphate acyltransferase, the enzyme necessary for the conversion of acetate to mevalonate. It also acts in the liver where it inhibits 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase and subsequently controls cholesterol biosynthesis.¹⁴

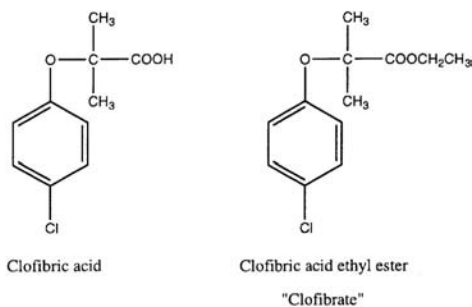


FIGURE 1.5

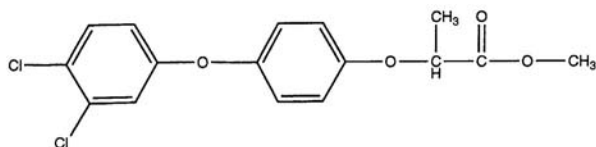
Structures of clofibric acid and clofibrate.

For those involved with agricultural chemicals, the structure of clofibrate and clofibric acid is an intellectual magnet because of the similarities to those compounds already discussed and, therefore, it was surmised that both had plant growth regulatory properties. On examining them in the etiolated wheat coleoptile bioassay it was determined that clofibrate significantly inhibited ($P < 0.01$) 100% at both 10^{-3} and 10^{-4} M. On the other hand, clofibric acid inhibited 100 and 60% at 10^{-3} and 10^{-4} M, relative to controls. This was interesting in that wheat is a monocotyledonous plant, and 2,4-D mainly inhibits the growth of dicotyledonous plants. 2,4-D inhibits the growth of wheat coleoptiles at 10^{-3} and 10^{-4} M, 100 and 50%, respectively, and significantly promotes growth at 10^{-5} and 10^{-6} M, 37 and 34% respectively. At 10^{-2} M, clofibrate induced the production of leathery, malformed leaves in week-old bean plants (*P. vulgaris* L. cv. Black Valentine) 10 days following treatment and they were inhibited approximately 50 and 10%, at 10^{-2} and 10^{-3} M, respectively, compared with controls. With clofibric acid, bean plant first trifoliates were even more leathery and malformed than those treated with clofibrate at 10^{-2} M; intact plants were inhibited 50% relative to controls at the same concentration, 1 week following treatment.¹⁸ Clofibrate, at 10^{-2} M, induced chlorotic streaks on the leaves of week-old corn plants (*Z. mays* L. cv. Norfolk Market White) within 48 h and, after 10 days, plants were inhibited approximately 50% at 10^{-2} M, but the new leaves exhibited no necrosis. With clofibric acid, at 10^{-2} M, week-old corn plants were inhibited and necrotic at 72 h following treatment. Jones et al. were awarded the patent that included both clofibrate and clofibric acid (British Patent 860,303 which corresponds to U.S. Patent 3,262,850 to ICI in 1961 and 1966). But whether the chemistry of these products for use as pharmaceuticals was based on the earlier herbicide discovery impetus with phenoxy compounds remains quite unclear. One point is certain and that is that the aryloxyphenoxypropionate herbicides inhibit the formation of fatty acids thereby interrupting lipid biosynthesis which is essential for the growth of the plant. The susceptible enzyme is acetyl-CoA carboxylase. However, only perennial and annual grasses are affected, while broadleaved plants are not.

The most recent phenoxy herbicides to reach the market are: 2-(4-dichlorophenoxy) phenoxy-methyl propanoate, Hoelon®, 1974; Butyl 2-[4-(5-trifluoro-2-pyridyloxy)phenoxy] propionate, Fusilade®, 1980; Methyl 2-(4-((3-chloro-5-trifluoromethyl)-2-pyridinyloxy)-phenoxy) propanoate, Verdict/Gallant®, 1981; and Ethyl (*R*)-2-[4-[(6-chloro-2-benzoxazolyl)-oxy]-phenoxy] propanoate, Whip®, 1982 (Figure 1.6). All are grass herbicides, in contrast to their progenitor 2,4-D, and it remains to be determined as to whether their structures will influence pharmaceutical applications.

1.2.4 Organophosphates

An odd chemical marriage has taken place between agrochemistry, pharmaceuticals, and the modern disease Alzheimer's. And, like some unions, it has come as something of a surprise to the parties concerned, although the sequence of events is quite logical. Alzheimer's is one of the major diseases affecting the elderly and it is manifest by the loss of short-term memory, cognitive skills, and a general dementia to a greater or lesser degree. The emotional stress on families is incalculable and the institutional costs are exorbitant. While the emotional and social consequences are extremely complex, the chemistry by which the disease progresses is, in comparison, relatively simple. In the aging process, neurons in the brain degenerate, especially in the hippocampus where they play a special role in the accession of new memory. Chemically, the neurons are acetylcholine receptors (cholinergic), but the enzyme acetylcholinesterase also is present in the chemical equation and it plays a role in keeping the acetylcholine in balance under normal conditions. As a protein, it has certain properties and its activity is governed by specific attributes. Among these are the anionic



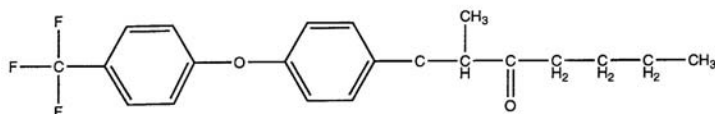
2-(4-(2,4-dichlorophenoxy)phenoxy)-methyl propanoate

Hoelon

1974 Hoechst AG

Preemergence and postemergence grass herbicide

LD₅₀ 2140 mg/kg



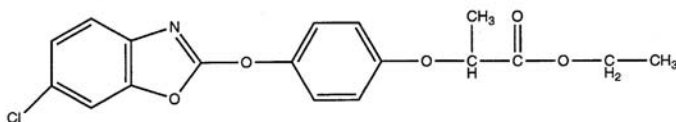
Butyl 2-[4-(5-trifluoro-2-pyridyloxy)phenoxy] propionate

Fusilade

1980 Ishihara Sangyo Kaisha Ltd of Japan

Control grass weeds

LD₅₀ 3328 mg/kg



Ethyl (R)-2-[4-[(6-chloro-2-benzoxazolyl)oxy]phenoxy] propanoate

Whip

1982 Hoechst AG

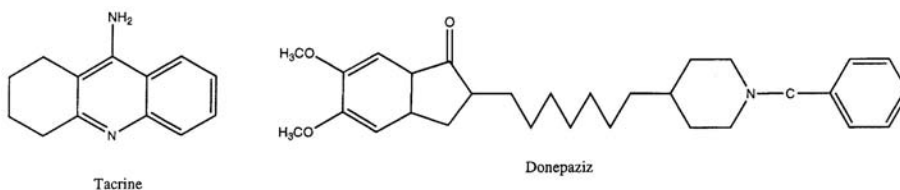
Grass herbicide

LD₅₀ 2357 mg/kg

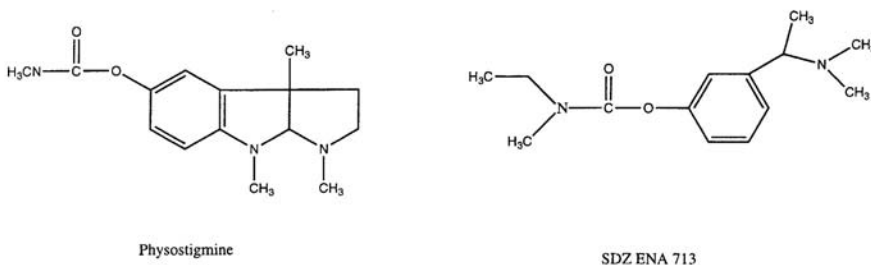
FIGURE 1.6

Structures for Hoelon, Fusilade, and Whip.

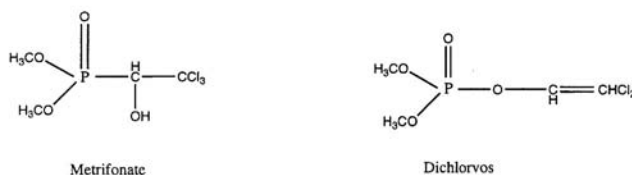
and esteratic sites. Glutamic acid, with its γ -carboxylic group, has a free COO^- function which constitutes the anionic segment, while the esteratic segment is constituted from a tyrosine residue, two imidazole groups from histidine residues, and a serine residue.¹⁴ Because of the dynamics involved which include pH and, therefore, pKas, the molecular formation and conformation of interacting compounds fall into three categories. First are the reversible inhibitors of acetylcholinesterase and include tachrine and donepezil (Figure 1.7), while the second group includes the pseudo- or semireversible compounds such as physostigmine from the dried seeds of *Physostigma venenosum* and SDZ ENA 713 (Figure 1.7). The third group are irreversible and include sarin, the nerve agent released by terrorists in a Tokyo subway in 1995, and dichlorvos. The reason that the latter are considered to be irreversible is because certain organophosphates esterify the serine residue on acetylcholinesterase, but hydrolysis of the phosphorylated serine species to phosphoric acid and the free acid is extremely slow. Enzymes that have been exposed to organophosphates undergo aging, that is, the phosphorylated moiety becomes permanently affixed to the enzyme generating a fully inactivated species so that the original enzyme cannot be



Reversible Acetylcholinesterase Inhibitors



Pseudo-/Semireversible Acetylcholinesterase Inhibitors



Irreversible Acetylcholinesterase Inhibitors

FIGURE 1.7

Classes of cholinesterase inhibitors.

recovered. Conversely, physostigmine is a competitive inhibitor with acetylcholine for acetylcholinesterase and is, thus, a reversible inhibitor.

If there is reduced production of acetylcholine in Alzheimer-compromised patients then selective inhibition of acetylcholinesterase would seem to be an appropriate control measure. In fact, one of the first drugs used to treat patients was metrifonate, *O,O*-dimethyl 2,2,2-trichloro-1-hydroxyethylphosphonate (Figure 1.7) an organophosphate that was originally prescribed for controlling schistosomiasis. Its mode of action consisted in paralyzing the organism rendering it susceptible to phagocytosis.¹⁴ In addition, the compound was an acetylcholinesterase inhibitor and it produced parasympathetic nerve stimulation, all of which were considered side effects relative to its main purpose. Following oral administration, the major metabolic product was dichlorvos (Figure 1.7). The disclosure that dichlorvos, phosphoric acid 2,2 dichloroethenyldimethyl ester, has pharmaceutical properties comes as a surprise to agricultural chemists because of its extensive use as an insecticidal fumigant whose LD₅₀ in rats is 70 mg/kg. It was sold under various trade names including dichlorvos, DDVP, dichlorophos, and vapona. The U.S. Patent 2,956,073 was granted to Shell in 1960.

Recent experiments have been conducted in rats and rabbits. With respect to the former, it has been shown, *in vitro*, that metrifonate induces acetylcholinesterase inhibition through the slow release of dichlorvos in rat brain and blood serum.²⁶ Proof of this biotransformation

lies in the fact that the metrifonate-induced acetylcholinesterase inhibition had the same pH dependence as its dehydrochlorination to dichlorvos, while the cholinesterase inhibition induced by dichlorvos was not pH dependent. Dichlorvos cholinesterase inhibition also was governed by a competitive interaction with the catalytic site of the enzyme and this resulted in irreversible inhibition within minutes; that is, addition of more substrate did not improve drug dissociation or enzyme activity. While the same effects were noted with physostigmine and tetrahydroaminoacridine, their effects were reversible although the means by which this was achieved was different in each case. *In vivo* experiments also have been carried out on 3- and 19-month-old rats, with metrifonate and dichlorvos, measuring cholinesterase in forebrains, erythrocytes, and blood plasma. A single dose of either compound induced cholinesterase inhibition in both brain and blood in conscious rats, and the effects were dose dependent and completely reversible. At the same time, there was good correlation between brain and blood cholinesterase activity. Oral doses of 10 mg/kg of dichlorvos in 3-month-old rats induced maximum inhibition within 15 to 45 min in the brain, and 10 to 30 min in erythrocytes and plasma, with recovery of cholinesterase activity in the plasma within 12 h. However, brain and erythrocyte cholinesterase did not reach control levels after 24 h. Metrifonate had similar, but delayed activity, so that peaks were reached in 45 to 60 min in the brain, and 20 to 45 min in the blood, after dosing, followed by complete recovery in 24 h. Notably, 19-month-old rats were more sensitive to both compounds.²⁷

Rabbit assays also have been useful in evaluating metrifonate as a cholinesterase inhibitor.^{28,29} The compound improved eyeblinking in aging rabbits without inducing the undesirable side effects observed with other acetylcholinesterase inhibitors and, additionally, the dose-dependent results were predictable. Associative learning also was improved from 30 to 80%.²⁸

While rat and rabbit bioassays have given promising results, experiments also have been conducted with Alzheimer's patients using both oral and transdermal delivery systems. Both the pharmacokinetics and pharmacodynamics have been evaluated in oral treatments with metrifonate and dichlorvos, the net results being that metrifonate and, subsequently, dichlorvos because it is the catabolic product, is potentially useful as a treatment.³⁰ Transdermal patches also show promise for metrifonate, dichlorvos, and other cholinesterase inhibitors such as physostigmine, eptastigmine, and tacrine.^{31,32}

1.3 Epilogue

While the connection between certain agrochemicals and pharmaceuticals has been presented, there is another area of chemistry that has not been covered. Briefly, the indole nucleus appears in both plant and animal products, although it may be argued that the latter are the result of the ingestion of plants. Research with indole-3-acetic acid (IAA), sometimes referred to as auxin, has generated hundreds of research articles over the past 50 years and the compound is considered to play a major role in plant growth and development. The indole nucleus also appears in tryptophane, a precursor for IAA by way of deamination, which of itself is an essential amino acid for animals. Coincidentally, one of the original isolations of IAA was from human urine when Fritz Kögl (1897–1959) was working with schizophrenic patients who appeared to metabolize indole in an atypical fashion. Serotonin, 5-hydroxytryptamine, also is synthesized from tryptophane in the brain where it plays a part in regulating central and peripheral nervous systems.³³

Another natural product that has fascinated plant scientists since its discovery in the 1950s is abscisic acid. First found in the buds of *Acer pseudoplatanus*^{34,35} and cotton bolls, *Gossypium hirsutum* L.,³⁶ it plays a significant role in keeping buds dormant, controlling abscission of cotton bolls, opening and closing of stomata in leaf surfaces to control gas exchange, and it protects plants from drought.³⁶ It also mediates other plant functions. It is ubiquitous and it has also been found in various fungi including *Cercospora rosicola*,³⁷ *Cercospora cruenta*,³⁸ and *Botrytis cinerea*,³⁹ although its function is not known. Oddly, it has been found in animal brains including pigs, rats,⁴⁰ rodents, and ruminants,⁴¹ where it may be an integral part of the chemistry, but no role has been ascribed to it.

In the final analysis the relationship between agrochemistry and pharmaceuticals will prove to be an interesting association, not only from an academic perspective, but also a financial one.

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*Terpenoids with Potential Use as Natural Herbicide Templates*¹

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KEY WORDS: *allelopathy, terpenoids, monoterpenes, sesquiterpenes, diterpenes, steroids, commercial herbicides, bioassay, phytotoxicity, standard target species (STS), Lactuca sativa L., Lycopersicon esculentum L., Daucus carota L., Lepidium sativum L., Allium cepa L., Triticum aestivum L., Hordeum vulgare L., Zea mays L., Helianthus annuus L., Melilotus messarensis L., coefficient of variation (CV).*

2.1 Introduction

Between 60 and 70% of the pesticides used in agriculture in developed countries are herbicides.² In the U.S. where herbicides dominate pesticide sales, sales of \$4 billion are expected by the year 2000.³ Herbicides have helped farmers to increase yields while reducing labor. Indeed, without herbicides, labor would be a major cost of crop production in developed countries.

Nevertheless, the indiscriminate use of herbicides has provoked an increasing incidence of resistance in weeds to some herbicides, changes in weed population to species more related to the crop, environmental pollution, and potential health hazards. New, more efficient and target-specific herbicides are needed. One of the following strategies may be used.

1. Biochemically directed synthesis — strategies supported by the knowledge of biochemistry and biotechnology.
2. New structural types — synthesis of new compounds for broad biological screening.
3. New ideas in known areas — add new values to existing classes of chemicals.
4. Natural products as a source of new structural types of compounds.

Plants have their own defense mechanism and allelochemicals are, in fact, natural herbicides. Allelopathy is officially defined by the International Allelopathy Society⁴ as “The science that studies any process involving, mainly, secondary metabolites produced by plants, algae, bacteria, and fungi that influence the growth and development of agricultural and biological systems, including positive and negative effects.” Allelochemicals isolated from plants or microorganisms have ecological implication as biocommunicators in nature^{5a-f} and they are, indeed, a potential source for models of new structural types of herbicides. These natural herbicides should be more specific with new modes of action and less harmful than those actually in use in agriculture.^{6a-f} Allelopathy may help us in providing new concepts on integrated weed control management, crop varieties, and new generations of natural phytotoxins as herbicides. Some new techniques involving allelopathy have been suggested for weed suppression.

- The use of natural or modified allelochemicals as herbicides
- The transfer of allelopathic traits into commercial crop cultivars
- The use of allelopathic plants in crop rotation, companion planting, and smother crops
- The use of phytotoxics mulches and cover crop management, especially in no-tillage systems

With these concepts in mind and with the notion that allelopathic compounds have a wide diversity of chemical skeletons, we have initiated two different research projects: “Natural Product Models as Allelochemicals” and “Allelopathic Studies on Cultivar Species.” Both natural and agronomic ecosystems require previous field observation and preliminary bioassays of crude extracts. Indeed, bioassays are necessary during the complete research process. It is very important to establish a standard bioassay for allelopathic studies of phytotoxicity.

For this purpose, 22 commercial varieties of 8 species (lettuce, carrot, cress, tomato, onion, barley, wheat, and corn) were selected. These were grown at different pH and volumes of test solution per seed. Species with the lowest coefficient of variation (CV) in growth and the highest mean value of two growth parameters (root and shoot length) were selected for further study. Nine commercial varieties that represent the most common weeds families⁷ *Compositae*, *Umbelliferae*, *Cruciferae*, *Solanaceae*, *Liliaceae*, and *Gramineae* (Table 2.1), were selected as standard target species (STS).⁸ Based on these studies we recommend testing compounds in the following order depending on the availability: lettuce, onion, cress, tomato, barley, carrot, wheat, and corn.

In order to evaluate the potential of allelopathic agents for new herbicides, a number of bioassays have been undertaken with these agents and then compared with commercial herbicides which were used as internal standards. Several herbicides provided by Novartis, (simazine, terbutryn + triasulfuron, terbutryn + triasulfuron + chlorotoluron, terbutryn + chlorotoluron, terbutryn, terbumeton + terbuthylazine, terbuthylazine + glyphosate,

TABLE 2.1

Selected Species as STS

Class	Family	Species
Dicotyledoneous	Compositae	<i>Lactuca sativa</i> L. (lettuce)
	Solanaceae	<i>Lycopersicon esculentum</i> L. (tomato)
	Umbeliferae	<i>Daucus carota</i> L. (carrot)
	Cruciferae	<i>Lepidium sativum</i> L. (cress)
Monocotyledoneous	Liliaceae	<i>Allium cepa</i> L. (onion)
	Gramineae	<i>Triticum aestivum</i> L. (wheat)
		<i>Hordeum vulgare</i> L. (barley)
		<i>Zea mays</i> L. (maize)

simazine + amitrole, and terbumeton + terbutylazine + amitrole) were tested.⁹ Test concentrations were 10^{-2} to 10^{-9} M, based on the usual concentration applied in the field ($\sim 10^{-2}$ M).

In this standard phytotoxic allelopathic bioassay, herbicides show strong inhibitory activities only at concentration between 10^{-2} to 10^{-3} M and at a lower concentration this activity disappears or is stimulatory. Based on the most consistent profile of activity of the nine tested herbicides, the mixture terbutryn + triasulfuron (commercialized as Logran Extra) was selected to be used as an internal standard to validate the phytotoxic responses of the chemicals tested.

We are developing a systematic allelopathic study on natural and agroecosystems as well as with synthetics based on bioactive natural product models in order to evaluate their potential as allelopathic agents. The selection of plant material is based on field observations and on preliminary bioassays of the crude water extract. After the first chromatographic separation a second bioassay is performed and the fractions are selected on the basis of their activity. Each pure compound resulting from the separation process is tested using a series of aqueous solutions and an internal standard herbicide in order to establish the structure–activity relationship.

Rice¹⁰ classified allelopathic compounds into 13 types. They involve almost every class of secondary metabolites, thus one may find allelochemicals that vary from simple esters to polyacetylenes, monoterpenes, and alkaloids. From the observation of the range of activity^{6b} of these compounds, we can conclude that terpenoids represent a group of potential natural herbicides.

In this chapter we present a selection of plant terpenoids belonging to natural and agroecosystems, from monoterpenes to triterpenes and steroids, with potential use as natural herbicide models. Activity results are presented in figures where germination and growth of STS are expressed in percentages from control; zero values mean equal to control, positive values mean stimulation, and negative values mean inhibition of the measured parameter.

2.2 Monoterpenes

Monoterpenes exist as hydrocarbons or as oxygenated moieties with aldehyde, alcohol, ketone, ester, and ether functionalities. Moreover, they may be acyclic, monocyclic, bicyclic, or tricyclic in structure.¹¹ Owing to their low molecular weight and nonpolar character, the group as a whole has been classified as volatile. Nevertheless, they operate as chemical defenses against herbivores¹² and diseases,^{13a,b} as fragrances attractive to pollinators¹⁴ and also phytotoxins to other plants.^{15a-c}

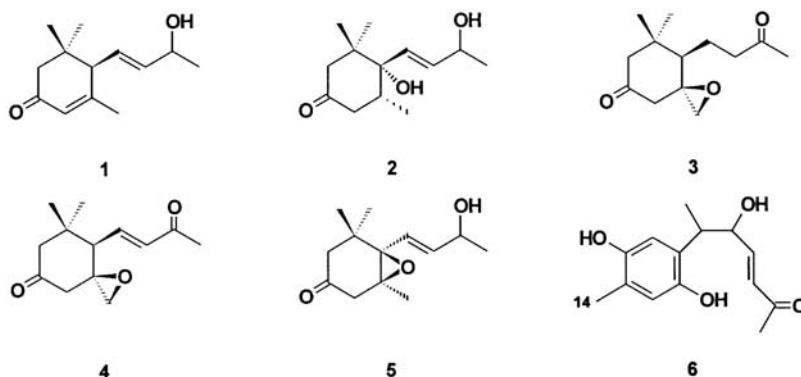


FIGURE 2.1
Selected bioactive norsesquiterpenes.

We reported the isolation of related compounds, three new bioactive ionone type bis-norsesquiterpenes annuionones A-C (3-5) and the new norbisabolene helinorbornene (6) (Figure 2.1), from a sunflower cultivar.¹⁶ As result of allelopathic bioassays, the most relevant effects on dicotyledoneous species (*Lactuca sativa* and *Lepidium sativum*) are those shown by 4 which stimulated root growth of *L. sativum* at low concentration (10^{-8} M, 47%; 10^{-9} M, 32%), and 6 which showed a strong inhibitory effect on the germination of *L. sativa* at all tested concentrations (average -50%) (Figure 2.2).

Clear selectivity (parameters and species) on monocotyledon species was observed. 1 and 3 induced inhibitory effects (1, 10^{-4} M, -38%; 3, 10^{-4} M, -47%) on germination of *Allium cepa*, but exhibited clear stimulatory activity (1, 10^{-4} M, 63%; 10^{-8} M, 54%; 3, 10^{-4} M, 42%; 10^{-5} M, 48%; 10^{-6} M, 49%) on root growth. Nevertheless, only stimulatory effects on root and shoot growth of *Hordeum vulgare* were observed. In this case, 5 and 6 provoked an average of 35% for 5 and 40% for 6 on root growth in a range of concentrations of 10^{-5} to 10^{-9} M. Only 6 showed effects on shoot growth of *Hordeum vulgare* (average of 30%).

2.3 Sesquiterpenes

Sesquiterpenes are, together with monoterpenes, the most frequent terpenes implicated in allelopathic processes. The number and structural variability make it difficult to establish a structure-activity relationship. The number of different skeletons with reported phytotoxic activity is around 50.¹⁷ Open-chain sesquiterpenes such as farnesol¹⁸ and nerolidol,¹⁹ bisabolene types such as β -bisabolene,²⁰ guaiane types such as α -bulnesene,²¹ aromadendrane types such as (+)-espathulenol,²² eudesmane types such as ciperol and ciperone, as well as the recently described skeletons heliannane^{23a,b} and heliespirane²⁴ have been reported to have allelopathic properties.

A number of compounds from the novel sesquiterpene family heliannuol has been isolated from a sunflower cultivar (Figure 2.3).^{1,23a,b} To evaluate their potential allelopathic activity and to obtain information about the specific requirements needed for their bioactivity, the effect of aqueous solutions with concentrations 10^{-4} to 10^{-9} M, were evaluated on root and shoot growths of lettuce, barley, wheat, cress, tomato, and onion seedlings.

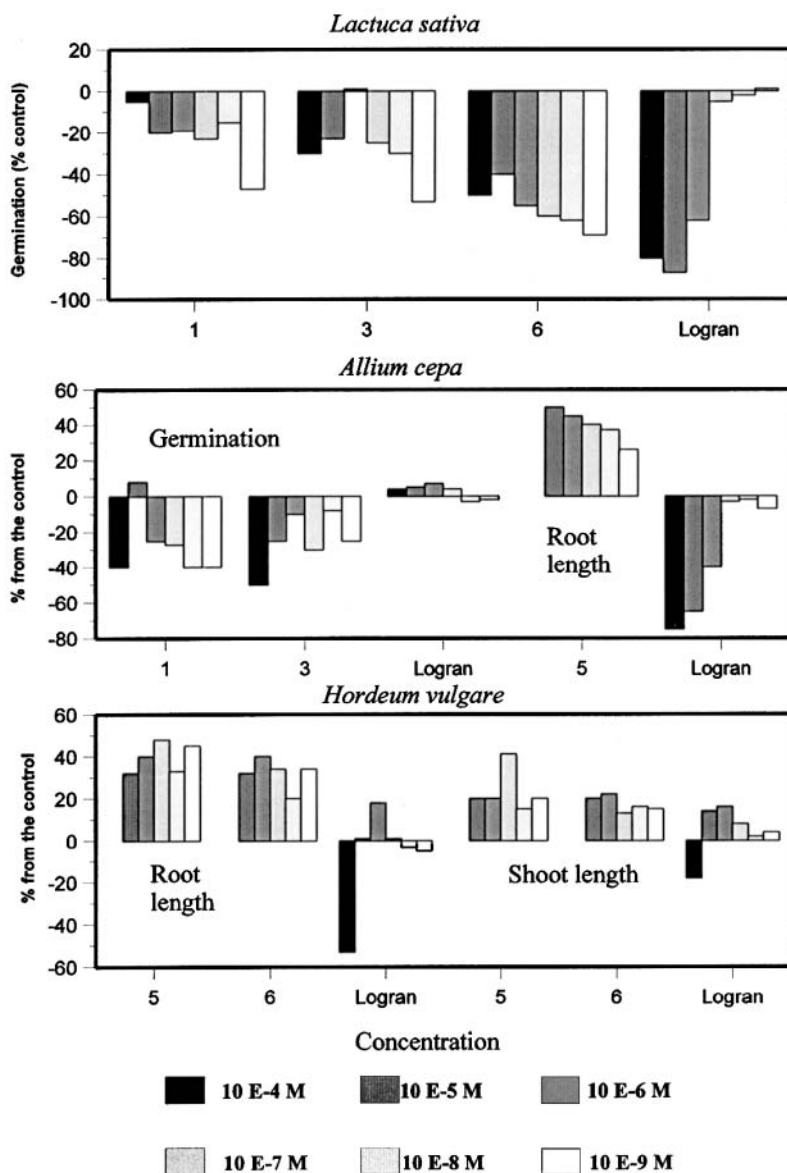


FIGURE 2.2

Selected bioactivity data of norsesquiterpenes in comparison with Logran.

Figure 2.4, where selected examples are presented, showed that compounds **7**, **9**, **12**, **13**, and **15** inhibited germination and root growth of lettuce better than Logran, and **10** and **14** inhibited germination and root growth of onion, while **11** inhibited root growth of barley.

The main observed effect on lettuce is the strong inhibition of germination. This activity is very intense at high concentrations of Logran but decreases quickly at concentrations lower than 10^{-6} M. This fact is clearly observed in root growth of this species.

The effect on germination induced by natural compounds is similar, but less intense, at high concentrations and more persistent with dilution. Indeed, we observed significant values of activity at 10^{-7} M with compounds **12**, **13**, and **15** [**12** (–43%), **13** (–52%), and **15** (–80%)]

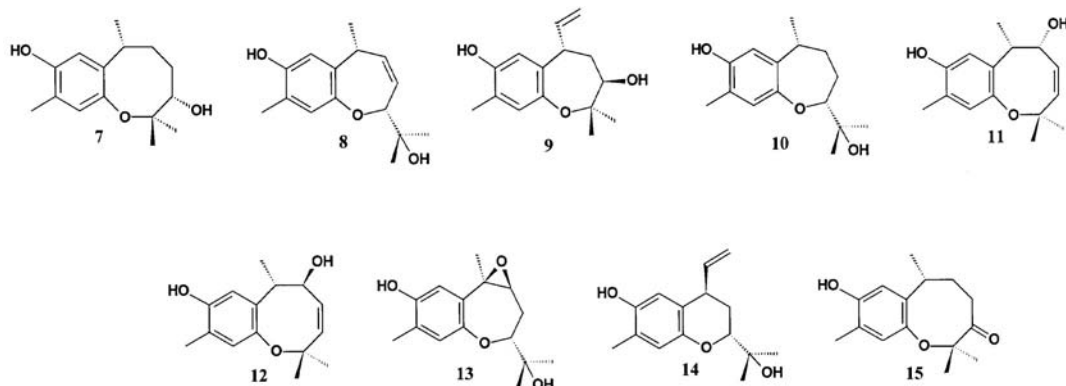


FIGURE 2.3
Selected bioactive heliannuols.

and a homogeneous inhibitory profile of activity for heliannuol A (7) with an average of –40% inhibition on the germination of lettuce with 10^{-4} to 10^{-9} M, whereas, heliannuol D (10) showed a strong stimulation on the germination of lettuce (average 50%) as well as inhibition on root and shoot length with averages of –22% and –30%, respectively.

Heliannuol B (8) has a strong inhibitory effect on shoot length of cress (*Lepidium sativum*) (–60%, 10^{-4} M; –40%, 10^{-5} M; –30%, 10^{-6} M; –40%, 10^{-7} M; –38%, 10^{-8} M); inhibition of root growth was not observed.

Effects on onion are small except for the inhibition of root growth induced by Logran at high concentration. Heliannuol D (10) showed a similar inhibitory activity on root length (–40%, 10^{-3} M; –50%, 10^{-4} M; –40%, 10^{-5} M; –50%, 10^{-6} M) and shoot length (–45%, 10^{-3} M; –40%, 10^{-4} M; –35%, 10^{-5} M) of onion (*Allium cepa* L.) seeds.

The effect on barley was not significant, except for stimulation of root growth induced by 14 with an average range of 40%.

2.4 Sesquiterpene Lactones

There are several references about the regulatory activity on the germination and plant growth of sesquiterpene lactones.^{25,26} This has been correlated with the presence of an α -methylene- γ -lactone moiety, other functionalities and the different spacial arrangements that the molecule can adopt.²⁷ It seems that the accessibility of groups which can be alkylated plays an important role in the activity.

We have isolated 16 sesquiterpene lactones from *Helianthus annuus*.^{28a,b} They have different carbon skeletons: guaianolides, germacranolides, heliangolide, *cis,cis*-germacranolide and melampolides (Figure 2.5).

Guaianolides 16, 17, and 18 with few functional groups showed a high inhibitory activity on the germination of *Lactuca sativa* seeds in high as well as in low concentration [–71% (17, 10^{-5} M); –62% (18, 10^{-6} M)] that had only a small effect on root and shoot length (Figure 2.6). However, guaianolides 20 and 21 that present a second α,β -unsaturated system, an angeloyl ester at C-8, show stimulatory effects on the germination of lettuce (average 40%) and inhibitory effects on root (20, –33%, 10^{-5} M; –29%, 10^{-9} M; 21, –25%, 10^{-7} M) and shoot

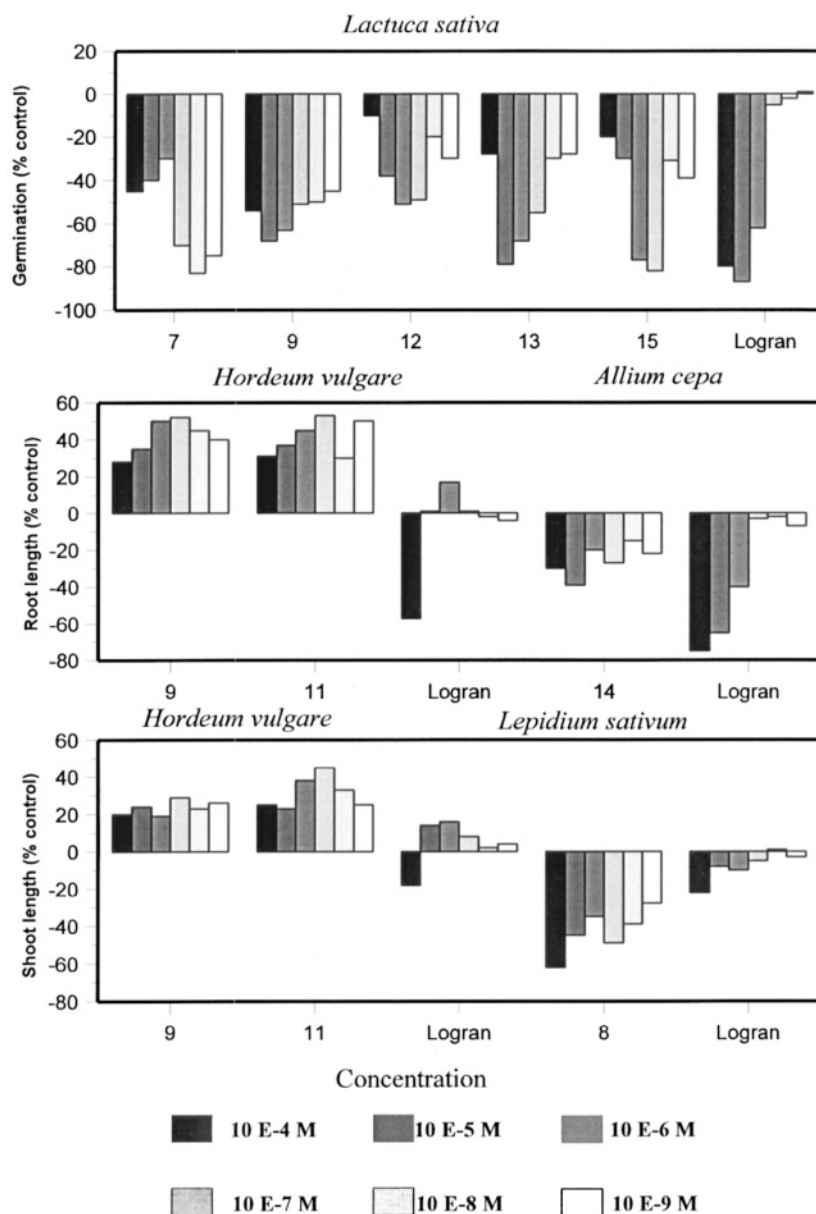


FIGURE 2.4

Selected bioactivity data of heliannuols in comparison with Logran.

length (20, -34%, 10^{-5} M; -34%, 10^{-7} M; -34%, 10^{-9} M; 21, -24%, 10^{-5} M; -30%, 10^{-7} M). Compounds 16 and 18 showed an inhibitory effect on the germination, while 20 to 23 showed an stimulatory effect on the germination and inhibitory effects on the shoot and root length. The differences in activity observed for compounds 16 to 19 and those for 20 to 23 may be attributed to the presence of an ester at C-8 that provokes steric hindrance on the β side of the molecule and, consequently, less accessibility of the α -methylene- γ -lactone moiety.

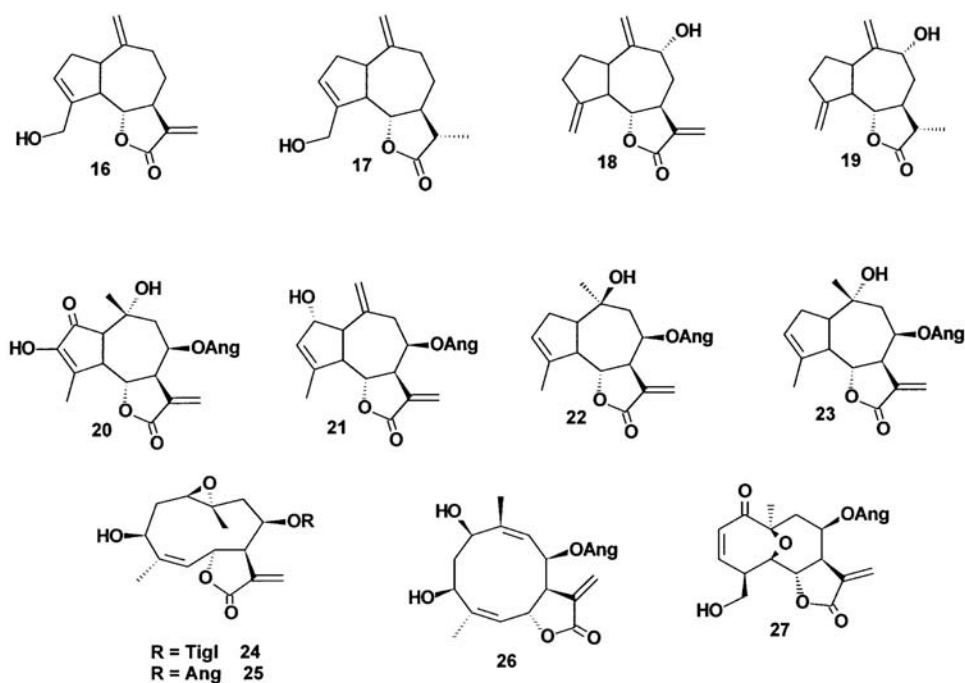


FIGURE 2.5
Selected bioactive sesquiterpene lactones.

The effects of guaianolides on the germination and growth of *L. sativum* and *L. aesculentum* are, in general, of no significance, except for **20** and **23** where inhibitory effects have been found on the shoot length of *L. esculentum*. These compounds are epimers at C-10. Both present similar profiles of activity, nevertheless the most persistently active compound dilution (–30%) is **20** which has an α -orientated hydroxyl group at C-10.

These compounds have low effect on the germination and growth of *Hordeum vulgare* seeds, except **16** and **19**. **16** has an inhibitory effect on the radicle length (–19%, 10^{-4} M) and there are stimulatory effects on germination induced by **16** (27%, 10^{-5} M) and **19** (17%, 10^{-5} ; 23%, 10^{-6} M).

Germacranolides have more flexibility in their skeleton and, therefore, a number of different possibilities of conformations are possible. The most notable effects are the following: **24** and **25** have related structures and both showed strong inhibitory effects at high concentration on germination (**24**, –78%, 10^{-5} M; **25**, –50%, 10^{-4} M) and shoot (**24**, –35%, 10^{-6} M; **25**, –24%, 10^{-4} M) and root growth (**24**, –47%, 10^{-5} M; –60%, 10^{-6} M). **24** showed inhibitory effects on the shoot growth (–21%, 10^{-5} M) of *L. esculentum*. The activity on *H. vulgare* are, in general, stimulatory, especially at low concentrations. **26** and **27** exhibited inhibitory effects on germination (**26**, –53%, 10^{-9} M; **27**, –48%, 10^{-8} M) and shoot (**27**, –24%, 10^{-6} M) and root growth (**26**, –27%, 10^{-8} M, **27**, –22%, 10^{-8} M) of *T. aestivum*.

All germacranolides tested possess an α -methylene- γ -lactone moiety; therefore, the different profiles of activity should be attributed to the presence of a second or a third receptor site for alkylation in the molecule. These could be α,β -unsaturated carbonyl groups together with the conformational change inherent to the particular functionality of the molecule which will allow or hinder accessibility to the receptor sites. These effects fundamentally influence root and shoot growth more than germination.

Those compounds that possess a double bond with Z geometry between C-4 and C-5 (**24**, **25**, and **26**) are more active on root and shoot growth of dicotyledonous species. The effects of conformational changes are so much more important due to greater flexibility of the molecule.

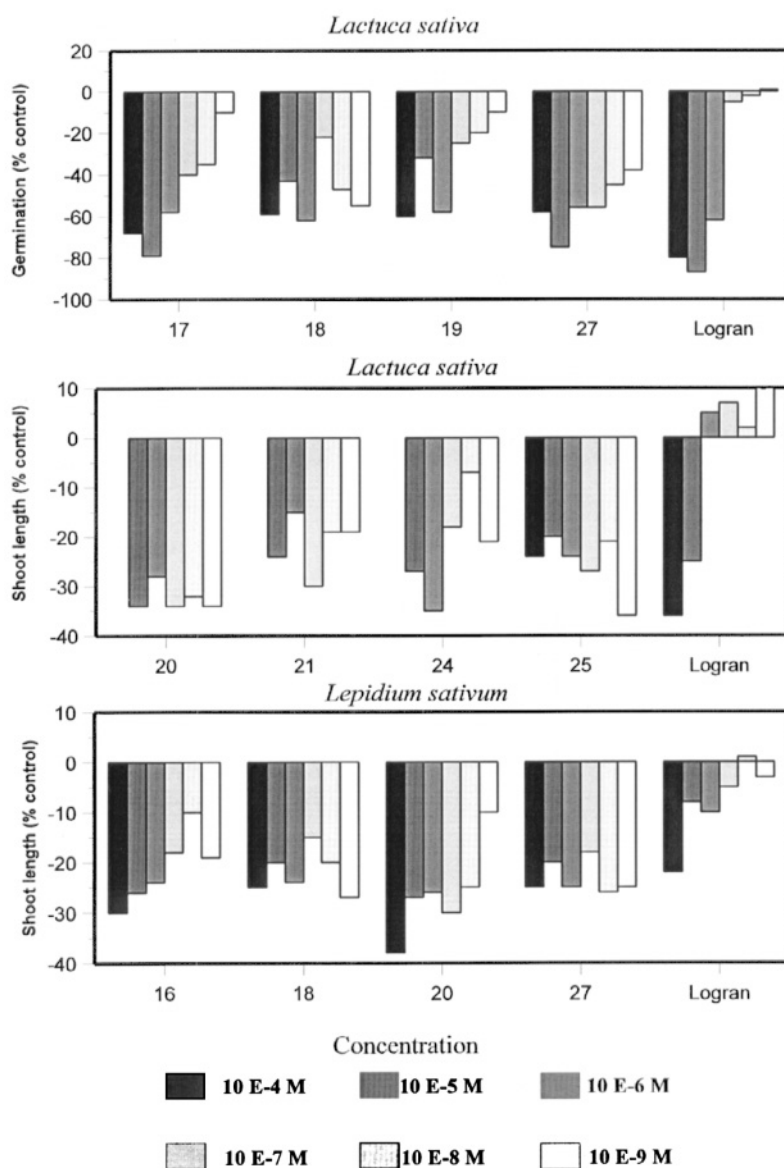


FIGURE 2.6

Selected bioactivity data of sesquiterpene lactones in comparison with Logran.

This factor more strongly influences germacranolides than guaianolides. The presence of electrophilic groups and conformational changes could be considered the reasons for increase in the bioactivity of these compounds.

2.5 Diterpenes

There are not many references about the effects on seed germination and plant growth of diterpenes with drimane, labdane, abietane, and clerodane skeletons.

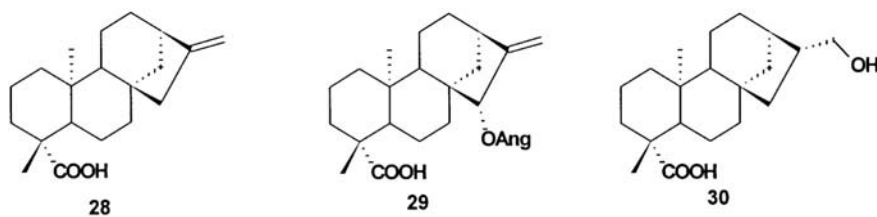


FIGURE 2.7

Selected bioactive diterpenes.

A few drimanes were examined for plant growth regulatory properties²⁹ and only at concentrations of *ca.* 100 to 500 ppm, at which they completely inhibited seed germination and promotion of the root growth on rice (*Oryza sativa*). However, at a concentration of less than 25 ppm a dramatic promotion of root elongation was observed. The root elongation of lettuce was completely inhibited at 100 ppm.

Bioactivity studies with labdanes and abietanes were made on *Peronospora tabacina* (ADAM) sporangia^{30,31} and in *in vitro* experiments a total inhibition at 10 $\mu\text{g}/\text{cm}^2$ and a stimulation in germination upon dilution was found. *In vivo*, the sporangium germination was never completely inhibited, a 78% reduction in germination was observed when sporangia were exposed to 30 $\mu\text{g}/\text{cm}^2$ and no differences were found when individual isomers or a mixture were applied.

The clerodanes tested were isolated from *Chrysoma pauciflosculosa*,³² a common shrub of the Florida scrub with alleged allelopathic potential. Biological studies were made on three Florida sandhill species and lettuce. They showed activities at concentrations of 12 to 48 ppm, reducing the germination and radical growth of two native species, but they had no significant effects on germination and only a slight stimulatory effect on radicle growth of *Rudbeckia hirta* and lettuce. The low activity observed with lettuce confirms earlier observations with allelochemicals obtained from other scrub species that lettuce is less sensitive to such compounds than are the native sandhill species. In this case, the higher activity has been related to the presence of alkylating groups.

Extraction of the fresh leaf aqueous extract of *H. annuus* L. var. VYP³³ with methylene dichloride afforded from low polar fractions, after chromatography on silica gel using hexane-EtOAc, mixtures of increasing polarity consisting of four kaurenoid carboxylic acids: (–)-kaur-16-en-19-oic acid (**28**), (–)-grandifloric acid, (–)-angeloylgrandifloric acid (**29**), and the (–)-17-hydroxy-16 β -kauran-19-oic acid (**30**) (Figure 2.7).

In general, clear inhibitory effects were observed on germination and shoot length, and a stimulatory effect on the radical length of *L. sativa*, *L. sativum*, and *A. cepa* (selected effects are presented in Figure 2.8). The most active compound was (–)-kaur-16-en-19-oic (**28**) which, at a concentration of 10^{-3}M , reduced germination (–36%) and root length (–29%) of *L. sativa*. At low concentration, **28** presents a clear inhibitory profile of activity on the germination and shoot length of *A. cepa* (germination, 10^{-8}M , –38%). The observed activity on *L. sativum* is very similar with significant inhibition of germination (10^{-8}M , –30%) and shoot growth (10^{-7}M , –29%; 10^{-8}M , –42%; 10^{-9}M , –23%) at low concentration.

The observed effects on the germination and growth of *L. esculentum* and *H. vulgare* are, in general, not significant, except for **29** and **30** where inhibitory effects on radicle (**29**, 10^{-6}M , –16%; **30**, 10^{-4}M , –18%) and shoot length (**29**, 10^{-6}M , –24%; **30**, 10^{-4}M , –24%) of *L. esculentum* and inhibitory effects on germination (**29**, 10^{-7}M , –24%; **30**, 10^{-7}M , –28%) and root length (**29**, 10^{-9}M , –14%; **30**, 10^{-8}M , –20%), and stimulatory effects on shoot length (**30**, 10^{-5}M , 29%) of *H. vulgare* were observed.

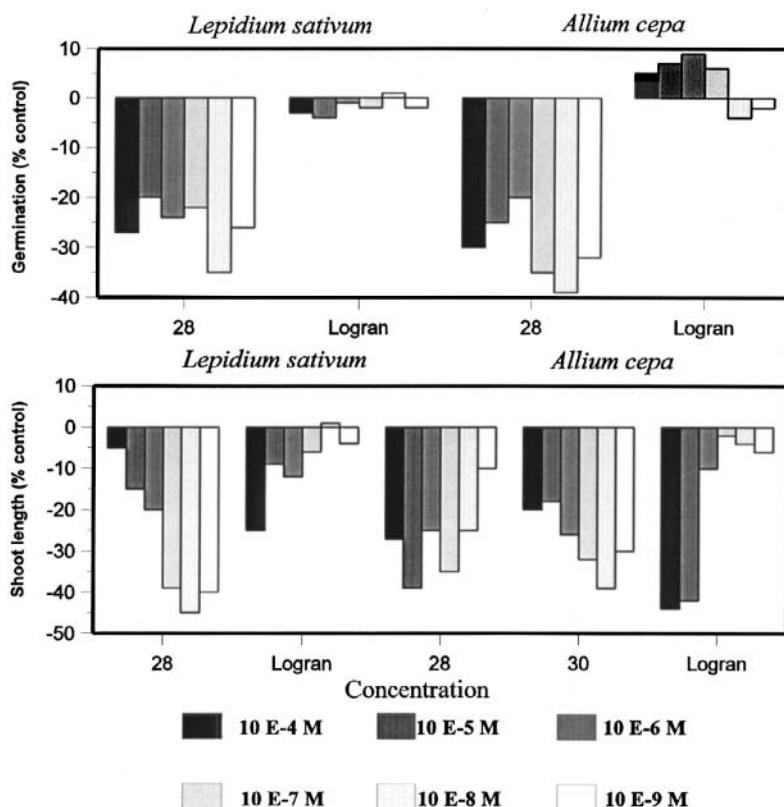


FIGURE 2.8
Selected bioactivity data of diterpenes in comparison with Logran.

2.6 Triterpenes

Only limited data are available concerning triterpene activity and growth development. We have isolated a number of triterpenes with a variety of structures (lupane, oleanane, nor-lupane, and gammacerane) from *Melilotus messanensis*.^{34a-d}

Lupane triterpenes (Figure 2.9) showed, in general, high stimulatory activity on the germination of *Lactuca sativa* seeds in high and low concentration, especially **32**, **37**, and **41** (**32**, 10^{-4} M, +38%, 10^{-7} M, +38%; **37**, 10^{-6} M, +75%; **41**, 10^{-6} M, +73%). The effects on the radical and shoot length are, in general, low or not significant (Figure 2.10).

These compounds have a small effect on the germination and growth of *Lepidium sativum*. The most powerful stimulatory effects on the radical and shoot length are those shown by **41** with a CH_2OH group at C-17 (radicle length, 10^{-4} M, +44%; shoot length, 10^{-4} M, +36%). These data suggest that the bioactivity of these compounds can be related to the presence of a free hydroxyl group at C-3, a CH_2OH at C-17 as shown by **32**, **41**, and **37**, and this is increased when a methyl and ketone groups, and CH_2OH and methylene are attached at C-20.

Looking at the root growth of *Lycopersicon esculentum*, (Figure 2.10) compounds **41** and **36** have a promising activity profile: from nonsignificant negative values at 10^{-5} M to a stimulatory effect as the concentration falls (**36**: 27%, 10^{-6} M; 32%, 10^{-9} M; **44**: 29%, 10^{-6} M; 24%,

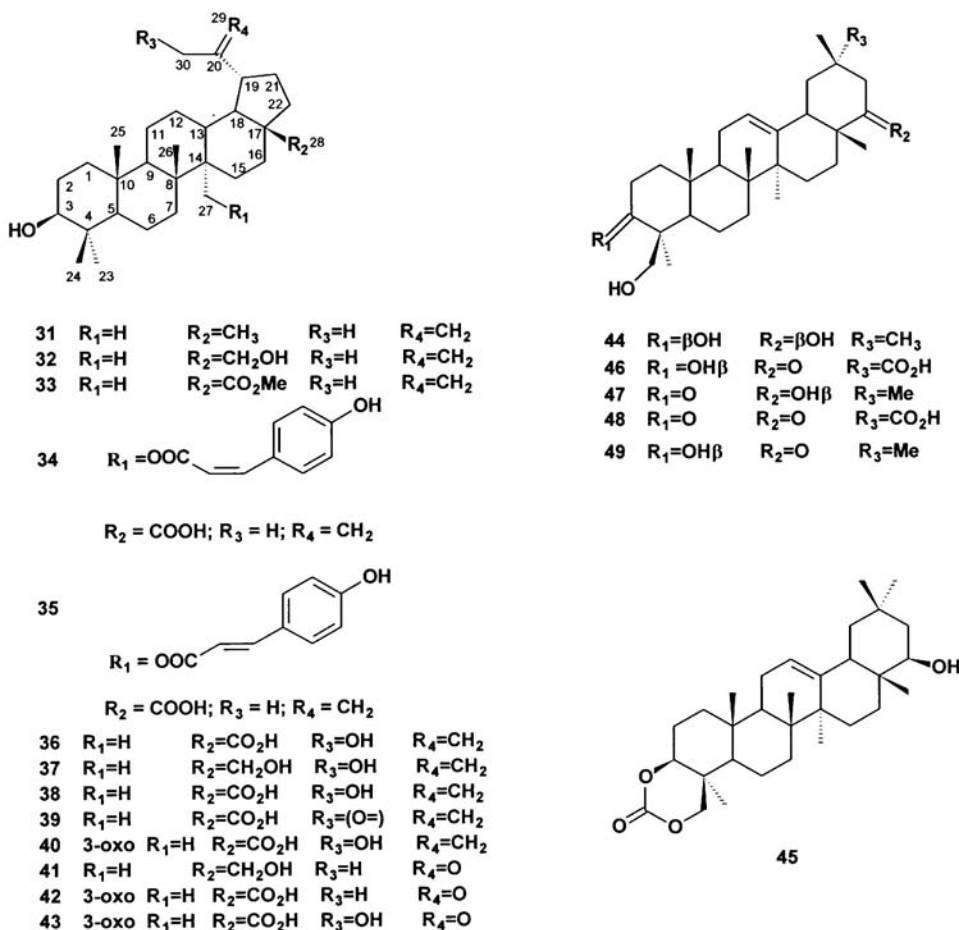


FIGURE 2.9

Selected bioactive triterpenes.

$10^{-8}M$). Shoot growth does not show any general trend. Although all values are low, the behavior of **34** and **36** at the highest concentrations should be pointed out (**34**: 14%, $10^{-5}M$; 23%, $10^{-6}M$; **36**: -12%, $10^{-5}M$; -19%, $10^{-6}M$).

With *Allium cepa*, the triterpenes showed a moderate stimulation in germination pattern. At $10^{-5}M$ some compounds show values greater than 20%; in particular were compounds **38** (35%), **40** (26%), and **43** (37%). The overall effect was less than that observed with *H. vulgare*. Oleanic triterpenes (**46**, **47**, **48**) appear to inhibit root growth (**46**: -29%, $10^{-5}M$; **47**: -31%, $10^{-7}M$; **45**: -40%, $10^{-7}M$).

In the case of germination of *Hordeum vulgare*, triterpenes can be classified into two groups — those showing a good level of stimulatory activity within all ranges of concentration such as compounds **32**, **33**, **35**, **44**, and **45** and those with no significant activity like compound **34**. The only difference in the stereochemistry of compounds **34** and **35** is the double bond in the cinnamoyloxy moiety. The *E* isomer (**35**) behaves as a growth promoter at all concentrations, while the *Z* isomer (**34**) has no activity except at the lower concentration. Germination data are homogeneous and highly inhibitory for lupane and nor-lupane acids and remain active at the lower concentrations, e.g., compounds **38** (-38%, $10^{-6}M$; -69%,

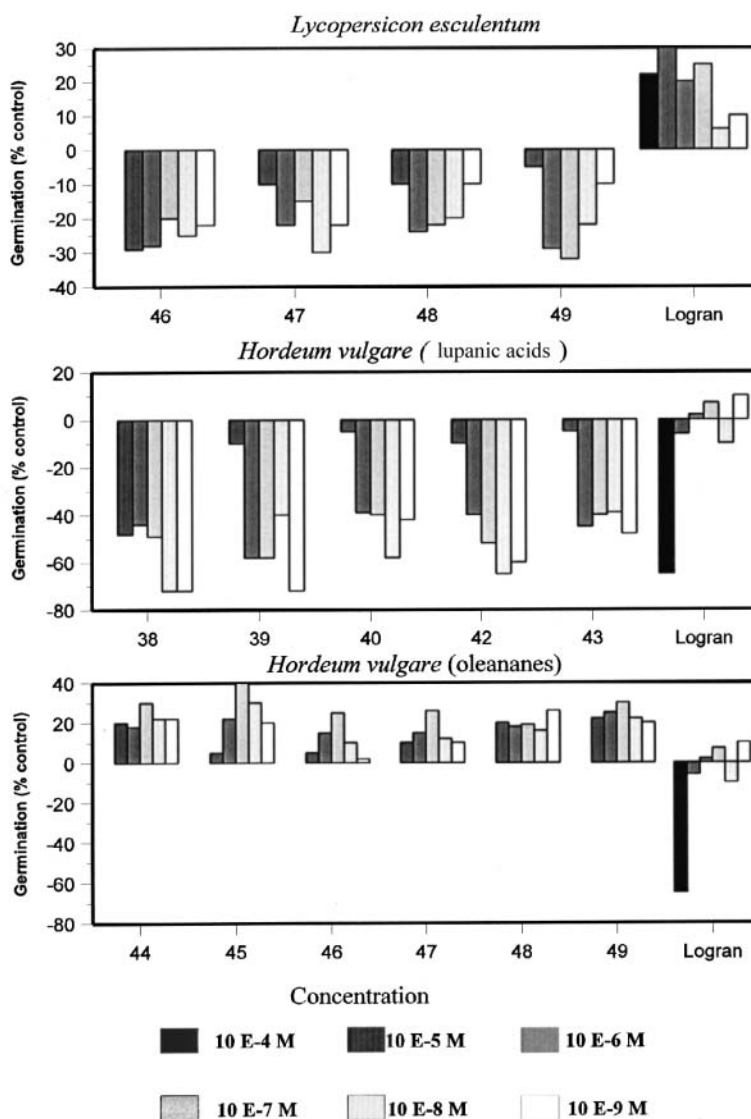


FIGURE 2.10

Selected bioactivity data of triterpenes in comparison with Logran.

10^{-8}M ; -69% , 10^{-9}M), **39** (-15% , 10^{-5}M ; -69% , 10^{-9}M), and **42** (-15% , 10^{-5}M ; -62% , 10^{-8}M). The oleanic triterpenes tested (**46** to **49**) exerted a moderately stimulatory effect on germination. No significant effects or general trends were shown on root or shoot length except for **32** and **33** with *H. vulgare*. Compound **32** had an inhibitory effect on the shoot length (10^{-6}M , -42% ; 10^{-7}M , -44%) and **32** (10^{-9}M , $+30\%$), and **33** (10^{-7}M , $+36\%$) stimulated germination.

Most of the major lupane and nor-lupane acids stimulate root length. No significant differences were observed except for messagenic acid G (**40**) which had the most homogeneous activity profile (31% , 10^{-6} ; 39% , 10^{-7}M ; 28% , 10^{-8}M ; 25% , 10^{-9}M), and **42** which had the highest values (30% , 10^{-5}M ; 59% , 10^{-7}M ; 24% , 10^{-8}M). The rest of the triterpenoid acids showed rather dispersed values and oleanic triterpenes showed no significant activity.

We have found that, in general, sensitivity increased in the following order: *Lactuca sativa*, *Lycopersicon esculentum*, *Allium cepa*, and *Hordeum vulgare*. From these data we conclude that the triterpenoid acids are more active principally on monocotyledon species and show higher levels of activity than the other compounds. As the pH of the solutions is buffered in a range of 6.0 to 6.5, triterpenoids acids are in their carboxylate form. It has been shown for other triterpene and saponins that their sodium salts are more active than the non-ionized acids.^{35a,b}

2.7 Steroids

Some steroids, such as chondrillasterol or amasterol, are reported to affect germination.^{36,37} The only steroids that have been reported with potent plant growth promoter effects at levels as low as 1 ng^{38a,b} are the brassinosteroids, acting in many cases in a synergistic manner with auxin.³⁹ Saponins are another group of closely related compounds that have exhibited important inhibitory activities, e.g., several isolated from alfalfa roots with sapogenins such as medicagenic acid, hederagenin, lucernic acid, zhanic acid, and soyasapogenol B have been tested and proved to be active to some weeds and wheat.⁴⁰

Only small effects were noted on the steroids from *Melilotus messanensis* that were tested on germination and growth of *Lactuca sativa*. *Lycopersicon esculentum* was slightly more sensitive. In this case, the steroids tested had a low and homogeneous inhibitory activity profile. Typical examples include: **51** (–14%, 10^{–5}M; –17%, 10^{–7}M) and **52** (–15%, 10^{–6}M; –19%, 10^{–7}M; –17%, 10^{–9}M). Shoot growth was affected in the opposite manner being stimulated by steroids. For example, **50** shows stimulatory activity (14%, 10^{–5}M; 28%, 10^{–6}M; 27%, 10^{–8}M; 24%, 10^{–9}M) (Figure 2.11).

Hordeum vulgare is the species that was the most sensitive to most of the compounds. The most active steroids are **50** and **53** that exert a stimulatory effect on germination with similar profiles: high levels of stimulatory activity at higher concentrations (**50**: 48%, 10^{–5}M; 40%, 10^{–6}M; 44%, 10^{–7}M; **53**: 44%, 10^{–4}M; 76%, 10^{–5}M; 48%, 10^{–7}M). Compounds **51** and **52** are the most active at lower concentrations (**51**: 72%, 10^{–9}M; **52**: 52%, 10^{–9}M) (Figure 2.12).

In attempting to elucidate roles for both triterpenes and steroids in plants, there are at least three aspects that need further study.

1. The compounds may act as self germination modulators, since they stimulate germination at low concentrations.
2. They may form micromicelles that facilitate transport of low polar terpenes through cell membranes. Ursolic acid is present in high quantities in the leaves

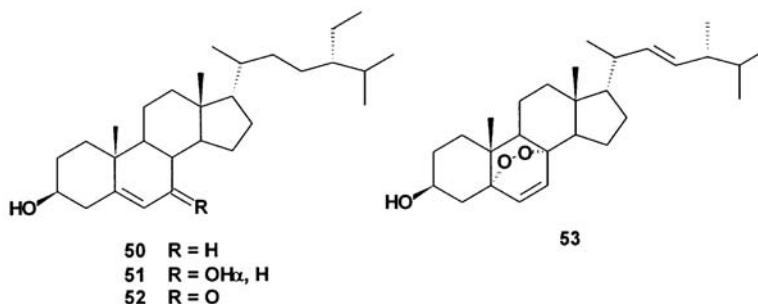


FIGURE 2.11
Selected bioactive steroids.

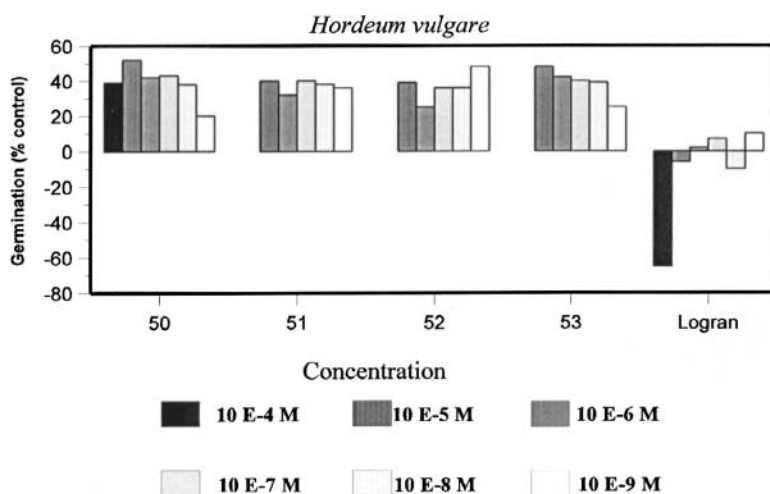


FIGURE 2.12
Selected bioactivity data of sterioids in comparison with Logran.

of some allelopathic shrubs of the Florida scrub community⁴¹ and has been proven to enhance monoterpene phytotoxicity. Betulinic acid is a major constituent of *M. messanensis* and it is the precursor of many other terpenoid acids, thus, it is possible that it or any of its products may play a role as natural detergents that facilitate transport of low polar compounds towards cell membranes of competitor species.

3. Saponins are known to be growth regulators and have been associated with the allelopathic potential of alfalfa (*Medicago sativa*). If saponins exhibit reasonable plant growth regulatory activity, their precursors will may demonstrate activity in the same systems.

In conclusion we note that (1) allelochemicals have better profiles (wide range and consistent activity at very low levels on a wide range) of activity in terms of concentration and intensity, and (2) allelochemicals show more sensitivity and selectivity against parameters and species.

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Allelopathy of Velvetbean: Determination and Identification of L-DOPA as a Candidate of Allelopathic Substances

Yoshiharu Fujii

CONTENT

- 3.1 Introduction
- 3.2 Materials and Methods
- 3.3 Results and Discussion
- References

ABSTRACT Among the 65 plants tested for allelopathic properties, velvetbean (*Mucuna pruriens* var. *utilis*) was found to be the most promising candidate. It is recognized that this tropical legume grown for green manure, has a special ability to smother weeds. The field test showed that test plots containing velvetbean had the smallest weed population compared to that of tomato, egg plant, upland rice, and fallow conditions. HPLC and seed germination and seedling growth bioassays showed that the growth inhibiting substance was L-3,4-dihydroxyphenylalanine (L-DOPA). L-DOPA is a well known precursor of the neurotransmitter dopamine and is an intermediate of many alkaloids. This study revealed that velvetbean leaves and roots contain large amounts of L-DOPA (about 1% of the fresh weight). L-DOPA suppressed the growth of some broad leaf weeds, while little effect was observed on grasses. It was concluded that in addition to its usefulness as a green manure, velvetbean could be utilized as an allelopathic crop to control weeds.

KEY WORDS: *green manure, phytotoxicity, companion plants, allelopathy, bioassay, weed control, intercropping*

3.1 Introduction

Velvetbean (*Mucuna pruriens* (L.) DC. var. *utilis* or *Stizolobium deeringianum* Piper et Tracy) is a tropical legume grown generally for green manure. It is recognized that velvetbean increases the yield of its companion graminaceous crops and that it smothers the growth of harmful weeds such as nutsedge (*Cyperus* spp.) and alang-alang (*Imperata cylindrica*).^{1,2}

A series of experiments was performed for the purpose of screening allelopathic plants with special emphasis on chemical interactions among them. The results of these experiments indicated that velvetbean was the most promising candidate.^{3,4} A field test showed that velvetbean stands minimized the size of weed populations compared with those of tomato, egg plant, upland rice, and fallow.^{5,6}

The genus *Mucuna* consists of about 100 species growing in the tropics and subtropics.^{7,8} There are two subgenera in *Mucuna*: one is *Mucuna* which is perennial and woody and the other is *Stizolobium* which is annual or biennial and herbaceous. In cultivars of *Stizolobium*, the total plant is utilized for green manure and/or cover crop, the leaves for fodder, the grains for food and seeds, and the stems for medicine in Africa and China.⁹ Grain yield reaches as high as 1.5 to 2.0 t/ha, and fresh leaves and stems weigh 20 to 30 t/ha, indicating that velvetbean is one of the most productive crops in the world. If the physiological mechanism of its allelopathic activities are identified, the use of velvetbean could be further developed. For example, it can be cultured in larger areas in the tropics, and it can have a greater utilization as green manure and/or weed-control crop. This chapter reviews the results of studies on allelopathic activities of *Mucuna pruriens* with special emphasis on L-DOPA as a potential allelochemical with weed-suppression properties.

3.2 Materials and Methods

Survey on allelopathic plants^{3,4,10} — Seventy plant species were tested for their allelopathy following the Richards' function method,¹¹ which proved to be suited to germination tests of lettuce and some weed plants.¹² In order to destroy the enzymes which degrade some constituents of a plant and to minimize the changes of the organic chemicals they contain, the leaves, stems, and roots were dried at 60°C for 24 h. One hundred mg of each of the dried samples was extracted with 10 ml water. Extraction mixtures were sonicated for 60 sec to complete the migration of chemicals. The extracts were filtered through Whatman No. 4 filter paper. Ten lettuce seeds were placed in 4.5 cm diameter Petri dishes containing 0.5 ml of test solution on Whatman No. 1 filter paper. The Petri dishes were incubated in the dark at 25°C. The number of germinated seeds was counted and hypocotyl and radicle growth were measured on the fourth day. The parameters for germination tests were: onset of germination (Ts), germination rate (R), and final germination percentage (A).¹² A simplex method was applied for the computer simulation of germination curves with the Richards' function.

Velvetbean cultivar — A dwarf cultivar of velvetbean, *Mucuna pruriens* var. *utilis* cv. *ana*, was used for the field test and the extraction of allelochemicals. The seed was a gift from Dr. Shiro Miyasaka and purchased at Pirai Seed Company in Brazil.

Incorporation of velvetbean leaves into soil — Two treatments of velvetbean were added to the volcanic ash soil in Tsukuba; one was leaves oven-dried at 60°C overnight, the other was fresh leaves. One gram of oven-dried leaves was added to 100 g of soil. The same weight of cellulose powder was added to other pots as a control. Fertilizers added to each pot were as follows: N, P, K of 50, 100, 50 mg/100 g soil d.w., respectively. Available nitrogen contained in the velvetbean residues (1.2%) was supplemented to control pots.

Weed appearance in the fields with velvetbean stands — Planting of velvetbean and some other plants was repeated for a period of 2 to 3 years.⁵ Plants were grown in lysimeters; each size being 10 m² with six replications where the 10 cm deep surface soil was replaced with uncultivated soils in the starting year. Each plot received a standard level of chemical fertilizers: N, P, K of 80, 80, 80 g/10 m², except for fallow.

Mixed culture of velvetbean by allelopathy discrimination methods — Allelopathy of velvetbean in the field was confirmed using stairstep^{13,14,15} and substitutive experiment.^{6,16,17} The stairstep experiment was designed according to the method of Bell and Koeppel¹⁸ with three replications within two mixed plants. Circulation of the nutrients solution was about 600 to 800 ml/h per pot, and a half strength of Hoagland's solution was used. The substitutive experiment was modified from the methods of References 6, 16, and 17.

Isolation and identification of allelopathic substances — Some fractions were extracted from fully expanded leaves and roots of velvetbean with 80% ethanol. The acid fraction of the extract inhibited the growth of lettuce seedlings. This fraction was subjected to silica gel column chromatography and HPLC on an ODS column, and the major inhibitor was identical to L-3,4-dihydroxyphenylalanine(L-DOPA).¹⁹ The identification was confirmed by co-chromatography with an authentic sample using two HPLC column systems (silica gel and ODS) equipped with an electro-conductivity detector.

Mechanism of action of L-DOPA and their analogs — Sixteen analogs of L-DOPA, mainly catechol compounds (see Figure 3.6), were tested for their inhibitory activity to radicle and hypocotyl growth of lettuce and the effect on lipoxygenase from soybean.

3.3 Results and Discussion

Survey of allelopathic plants — Sixty-five plants were investigated with lettuce seed germination tests. It was observed that the activity of velvetbean was distinctive (Table 3.1). Some other plants such as *Artemisia princeps*, *Houttunia cordata*, *Phytolacca americana*, and *Colocasia esculenta* also show inhibitory response. Further study of the allelopathic nature of these plants also is important.

Incorporation of velvetbean leaves into the soil — An experiment was performed to examine the effects of velvetbean on the growth of other plants in a mixed culture. The treatment also included an incorporation of velvetbean leaves into soils. Fresh leaves incorporation to soils (1.0% W/W in dry weight equivalent) reduced succeeding emergence of kidney bean (*Phaseolus vulgaris*) up to 60%, and plant biomass up to 30% of the control (Table 3.2). This effect diminished 2 weeks after the incorporation. Dried leaf incorporation showed no inhibition.

Weed appearance in the fields of velvetbean stands — Table 3.3 shows weed populations in the spring in continuous cropping fields grown in lysimeters. The velvetbean plot showed a lower population of weeds dominated by sticky chickweed (*Cerastium glomeratum*) than did the other plots of egg plant, tomato plant, upland rice, and fallow.

Mixed culture of velvetbean with stairstep apparatus — The stairstep method is a type of sand culture with a nutrient solution recirculating system on a staircase bed. Through this method, the presence of velvetbeans reduced lettuce shoot growth to the level of 70% of the control (Table 3.4). This result indicates that velvetbean root exudates have allelopathic activity.

Allelopathic compound in velvetbean — The analysis on effective compounds of velvetbean in restraining the growth of companion plants confirmed its association with L-3,4-dihydroxyphenylalanine (L-DOPA). It is well known that velvetbean seeds contain a high concentration of L-DOPA (6 to 9%),^{20,21} which plays a role as a chemical barrier to insect attacks.^{22,23} In the mammalian brain, L-DOPA is the precursor of dopamine, a neurotransmitter, and also important alkaloids intermediates. In animal skin, hair, feathers, fur, and insect cuticle, L-DOPA is oxidized through dopaquinone to produce melanin. As L-DOPA

TABLE 3.1

Screening of Allelopathic Plants with Lettuce Germination/Growth Test

Plant (Part ¹)	Germination Test					Growth Test		Extraction Ratio ⁸
	A ²	R ³	Ts ⁴	I ⁵	T ₅₀ ⁶	Hypocotyl ⁷	Radicle ⁷	
Compositae								
<i>Ambrosia elatior</i> (R)	87	141	1.7	78	1.2	146	63	10
<i>Ambrosia elatior</i> (S)	94	74	2.1	34	1.6	139	54	10
<i>Artemisia princeps</i> (S)\$\$\$	65	20	2.9	5	3.3	51	50	20
<i>Carthamus tinctorius</i> (W)	100	173	0.9	206	0.7	141	65	8
<i>Erigeron canadensis</i> (L)	89	80	1.3	56	1.2	114	50	25
<i>Erigeron canadensis</i> (R)	94	66	1.2	55	1.2	121	67	25
<i>Helianthus annuus</i> (R)	100	191	1.2	167	0.7	130	52	12.5
<i>Helianthus annuus</i> (S)\$	86	38	1.2	27	1.5	102	33	10
<i>Helianthus tuberosus</i> (R)	94	99	1.3	71	1.2	114	63	25
<i>Helianthus tuberosus</i> (S)	91	96	1.4	62	1.3	104	67	25
<i>Ixeris debilis</i> (W)	85	96	1.3	71	1.6	114	63	10
<i>Saussurea carthamoides</i> (R)	90	78	2.1	36	1.7	112	64	10
<i>Saussurea carthamoides</i> (S)	97	74	2.1	34	1.7	139	63	10
<i>Senecio vulgaris</i> (W)	86	70	1.4	62	1.8	104	67	10
<i>Solidago altissima</i> (L)\$	67	39	1.4	19	1.5	90	70	25
<i>Solidago altissima</i> (R)	89	59	1.3	42	1.3	109	78	25
<i>Taraxacum officinale</i> (R)	99	32	0.5	64	1.6	108	66	10
<i>Taraxacum officinale</i> (S)	97	37	0.4	94	1.3	105	79	6.3
Gramineae								
<i>Alopecurus geniculatus</i> (R)	91	78	1.8	39	1.5	127	94	10
<i>Alopecurus geniculatus</i> (S)	95	89	2.5	34	1.8	138	62	10
<i>Avena sativa</i> (L)	98	117	1.4	88	1.0	105	105	2.5
<i>Avena sativa</i> (R)	98	84	1.2	70	1.2	131	126	5
<i>Digitaria sanguinalis</i> (R)	91	41	1.5	23	1.6	97	96	25
<i>Digitaria sanguinalis</i> (S)	90	25	1.6	15	2.1	98	42	10
<i>Hordeum vulgare</i> (L)	100	102	0.9	114	1.0	144	65	6.3
<i>Hordeum vulgare</i> (R)\$\$	99	84	1.4	62	1.3	72	36	25
<i>Miscanthus sinensis</i> (S)	97	70	3.3	20	3.4	118	52	25
<i>Oryza sativa</i> (L)	100	226	2.2	105	1.0	114	77	12.5
<i>Sasa sinensis</i> (S)	94	55	3.2	17	2.7	134	44	25
<i>Secale cereale</i> (L)\$\$	91	62	1.2	48	1.3	79	21	10
<i>Secale cereale</i> (R)	100	186	1.4	142	0.8	132	55	12.5
<i>Sorghum bicolor</i> (R)\$	98	131	1.0	133	0.8	84	43	12.5
<i>Sorghum bicolor</i> (S)	85	60	1.3	39	1.3	104	55	10
<i>Sorghum sudanense</i> (R)	100	132	1.0	135	0.8	106	58	12.5
<i>Sorghum sudanense</i> (S)\$	86	66	1.3	47	1.3	107	31	10
Legminosae								
<i>Arachis hypogaea</i> (L)\$	83	90	4.9	16	1.8	98	60	10
<i>Arachis hypogaea</i> (R)	94	93	3.3	21	1.9	97	57	16
<i>Glycine max</i> (S)	96	44	0.6	70	1.4	117	41	10
<i>Lupinus albus</i> (S)\$	95	98	2.8	33	1.6	100	37	12.5
<i>Mucuna pruriens</i> (L)\$\$\$	96	82	9.3	9	4.6	79	26	25
<i>Mucuna pruriens</i> (R)	95	98	1.8	49	1.1	95	51	6
<i>Mucuna pruriens</i> (stem)	96	45	1.1	38	1.6	96	54	10
<i>Pisum sativum</i> (S)	99	45	0.5	99	1.1	115	38	10
<i>Pueraria lobata</i> (L)\$\$	82	72	5.0	12	2.2	73	45	12.5
<i>Pueraria lobata</i> (R)	95	32	0.5	103	1.4	95	68	10
<i>Pueraria lobata</i> (stem)\$	98	57	3.4	17	3.5	111	32	10
<i>Trifolium repens</i> (S)	98	49	1.8	28	1.9	105	56	10
<i>Vicia angustifolia</i> (S)\$	97	60	3.6	16	2.8	126	22	6.7
<i>Vicia hirsuta</i> (S)\$	100	62	3.6	18	2.8	114	24	6.7

Plant (Part ¹)	Germination Test					Growth Test		Extraction Ratio ⁸
	A ²	R ³	Ts ⁴	I ⁵	T ₅₀ ⁶	Hypocotyl ⁷	Radicle ⁷	
Chenopodiaceae								
<i>Beta vulgaris</i> (R)\$\$	90	75	4.3	16	2.1	57	21	25
<i>Beta vulgaris</i> (S)	96	86	1.5	56	1.2	109	64	5
<i>Chenopodium album</i> (L)	98	43	1.0	44	1.9	90	48	10
<i>Chenopodium album</i> (R)	92	76	1.1	66	1.1	88	48	25
<i>Spinacia oleracea</i> (L)	94	68	2.4	28	1.7	119	38	5
<i>Spinacia oleracea</i> (R)\$	97	73	4.5	16	2.1	102	36	10
<i>Fagopyrum esculentum</i> (S)	100	235	2.4	100	1.0	107	60	12.5
<i>Polygonum blumei</i> (S)\$\$	84	48	1.3	31	1.5	86	37	25
Labiatae								
<i>Lamium amplexicaule</i> (W)\$	85	54	2.4	19	2.0	70	45	10
<i>Melissa officinalis</i> (L)\$\$	39	23	3.7	3	2.3	101	57	8
<i>Melissa officinalis</i> (R)	98	73	1.6	45	1.4	164	103	8
<i>Mentha spicata</i> (L)\$	99	51	1.9	27	1.9	121	28	8
<i>Mentha spicata</i> (R)	95	75	0.9	80	1.2	139	89	8
<i>Salvia officinalis</i> (L)	94	106	3.3	31	1.3	112	67	10
<i>Salvia officinalis</i> (R)	98	86	3.1	27	1.9	123	83	8
Solanaceae								
<i>Lycopersicon esculentum</i> (R)	98	123	3.3	38	1.4	131	45	10
<i>Lycopersicon esculentum</i> (S)	96	136	5.9	23	1.9	135	37	10
<i>Solanum carolinense</i> (S)	96	120	0.8	153	0.9	144	117	6
<i>Solanum melongena</i> (S)	86	83	4.9	15	1.9	125	51	10
<i>Solanum melongena</i> (R)	98	84	2.9	29	1.6	130	58	10
<i>Solanum tuberosum</i> (L)	99	75	1.3	127	1.3	127	62	6
<i>Solanum tuberosum</i> (stem)	99	72	0.4	167	0.8	148	88	2.5
Cucurbitaceae								
<i>Citrullus lanatus</i> (L)	95	102	3.7	26	1.3	133	69	6
<i>Citrullus lanatus</i> (R)	94	103	4.2	23	2.2	113	74	12.5
<i>Citrullus lanatus</i> (stem)	96	116	3.0	36	1.7	129	59	6
<i>Cucumis sativus</i> (R)	98	224	4.3	52	1.3	159	71	10
<i>Cucumis sativus</i> (S)	99	123	3.1	41	1.3	187	78	5
<i>Cucurbita maxima</i> (R)	100	109	2.3	48	1.1	113	84	17
<i>Cucurbita maxima</i> (S)	93	153	4.8	30	1.8	119	50	12.5
Other genus								
<i>Amaranthus tricolor</i> (L)	92	66	4.0	15	2.4	93	81	6
<i>Amaranthus tricolor</i> (stem)	94	100	4.0	23	2.1	116	97	10
<i>Brassica campestris</i> (L)	93	27	0.5	58	1.6	141	94	3
<i>Brassica oleracea</i> (L)\$	76	97	5.6	14	1.4	146	88	5
<i>Brassica juncea</i> (S)	87	61	1.6	34	1.5	154	71	3
<i>Brassica napus</i> (R)\$\$	76	60	1.3	37	1.3	98	37	10
<i>Brassica napus</i> (S)	84	85	1.3	56	1.2	108	98	10
<i>Calystegia hederacea</i> (R)	99	87	2.5	35	1.8	103	46	10
<i>Calystegia hederacea</i> (S)	96	66	2.4	27	1.9	94	60	10
<i>Cerastium glomeratum</i> (W)\$	90	74	2.1	31	1.7	103	29	10
<i>Colocasia esculenta</i> (L)\$\$\$	92	22	6.3	3	4.9	22	32	10
<i>Colocasia esculenta</i> (R)	98	95	4.9	20	1.9	149	42	10
<i>Colocasia esculenta</i> (stem)\$	99	74	3.1	24	1.8	133	35	5
<i>Commelina communis</i> (L)	91	62	4.5	12	1.8	132	65	10
<i>Garium spurium</i> (W)\$	92	65	2.1	29	1.8	85	58	10
<i>Houttuynia cordata</i> (R)	95	66	0.9	68	1.5	126	50	10
<i>Houttuynia cordata</i> (S)\$\$\$	98	33	3.6	9	3.4	62	26	5

TABLE 3.1 (continued)

Screening of Allelopathic Plants with Lettuce Germination/Growth Test

Plant (Part ¹)	Germination Test					Growth Test		Extraction Ratio ⁸
	A ²	R ³	Ts ⁴	I ⁵	T ₅₀ ⁶	Hypocotyl ⁷	Radicle ⁷	
<i>Impatiens balsamina</i> (L)	93	101	3.3	28	1.9	117	64	6
<i>Impatiens balsamina</i> (stem)	93	80	3.1	24	1.9	136	77	3
<i>Oenothera biennis</i> (R)	91	61	1.1	52	1.2	119	40	25
<i>Oenothera biennis</i> (S)	84	48	1.3	31	1.5	105	39	25
<i>Paederia scandens</i> (L)	97	46	1.5	86	1.2	123	92	12.5
<i>Paederia scandens</i> (stem)	98	52	0.5	96	1.1	143	98	10
<i>Paulownia tomentosa</i> (L)	100	53	1.2	45	1.5	119	61	12.5
<i>Paulownia tomentosa</i> (stem)	100	139	1.5	98	1.2	136	52	12.5
<i>Phytolacca americana</i> (L)\$	98	44	2.3	19	2.2	57	33	6
<i>Phytolacca americana</i> (R)\$	75	40	1.8	16	1.8	78	37	10
<i>Phytolacca americana</i> (stem)	93	61	1.6	37	1.5	124	39	6
<i>Plantago major</i> (L)	88	101	3.5	26	1.6	121	73	5
<i>Plantago major</i> (R)	84	75	3.3	19	1.8	138	74	12.5
<i>Portulaca oleracea</i> (W)	90	117	4.8	22	1.9	119	49	3
<i>Stellaria media</i> (W)	97	69	1.4	51	1.4	99	67	5
Average	92.3	78.7	2.4	47.0	1.7	113	58.4	
Standard deviation (σ_{n-1})	9.4	40.3	1.5	38.2	0.7	26.8	21.6	

Note Plant name with underline denotes strong inhibition in either of following parameters: hypocotyl elongation, radicle elongation, A (germination %), and I (germination index). \$ mark after plant name shows the degree of inhibition. When each value exceeds the criteria of average $\pm\sigma$, we judge the possibility of inhibition. The number of \$s is the number of inhibition in four criteria of the above.

¹ Abbreviations of plant parts are as follows: S: Shoot, R: Root, W: Whole plant (=S+R), L: Leaf, Stem: Stem.

² Germination percentage at the end of germination process speculated with cumulative germination curves fitted to Richards' function (% of control).

³ Germination rate (% of germinated seeds per day, % of control).

⁴ Start of germination (a time spent until one seed germinates, ratio-to-control).

⁵ Germination index ($I = A \cdot R/Ts$).

⁶ 50% germination time (a time spent until 50% of seeds germinate, ratio-to-control).

⁷ Percent of control (control dish is cultured with water).

⁸ Extraction ratio (mg-D.W./ml). Extraction ratio was determined in order that EC of the assay solution did not exceed 1 mS/cm.

is an intermediate and rapidly metabolized, usually normal tissues have little concentrations of L-DOPA.

HPLC and GC-MS analysis showed that fresh velvetbean leaves and roots contained as much as 1% of L-DOPA and exudation took place from their intact roots. L-DOPA strongly inhibits the radicle growth of lettuce, but its precursor, such as tyrosine and phenylalanine, have no inhibitory activities (Figure 3.1). L-DOPA is, however, less effective to the hypocotyl growth and has practically no effect on germination (Figure 3.2). L-DOPA actually exudes from the root and its concentration reaches 1 ppm in water-culture solution, and 50 ppm in the vicinity of roots (Figure 3.3). This concentration is high enough to reduce the growth of neighboring plants and the growth inhibition in a mixed culture is shown in an agar-medium culture.^{24,25} It also leaches out from leaves with rain drops or dew. Since velvetbean produces 20 to 30 tons of fresh leaves and stems per hectare, approximately 200 to 300 kg of L-DOPA may be added to soils a year.

Phytotoxic effects of L-DOPA — Some effects of L-DOPA on germination and growth of the selected crops and weeds are summarized in Table 3.5. L-DOPA suppresses the radicle growth of lettuce and chickweed to the level of 50% of the control at 50 ppm (2×10^{-4} mol/l).

TABLE 3.2

Plant Growth After the Incorporation of Velvetbean Leaves to Soils¹

(Condition) Cultivated Plant	Plant Height (Percent of Control)	Shoot D.W. ²	Root D.W. ²
(60°C oven-dried leaf)			
<i>Oryza sativa</i> (upland)	101	71	83
<i>Zea mays</i>	110	104	103
<i>Sorghum bicolor</i>	91	77	91
<i>Glycine max</i>	98	97	107
<i>Phaseolus vulgaris</i>	160	101	88
<i>Arachis hypogaea</i>	105	95	134
<i>Solanum melongena</i>	86	91	95
<i>Cucumis sativus</i>	82	83	102
(Fesh leaf)			
<i>Zea mays</i>	85	88	69
<i>Phaseolus vulgaris</i>	32	27	25
<i>Cucumis sativus</i>	96	86	57

¹ One gram of dried, or equivalent to dried, plant residue was added to 100 g of soil. The same weight of cellulose powder was added to the control pot.

² Shoot and root growth of each plant was calculated from the dry weight and compared to the growth of control.

TABLE 3.3

Weed Population in Continuous Cropping Fields

Crop	Treatment	Weed population (g Dry Weight per m ²)	Weed species observed ⁶
Upland rice	3-yr. crop ¹	5.11 (49.4) ⁴	1, 3, 5, 6, 7, 8, 9, 10, 11 ⁵
Egg plant	3-yr. crop	16.82 (40.1)	1, 2, 3, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14
Tomato	3-yr. crop	4.92 (64.9)	1, 5, 6, 9, 12, 13, 17
Velvetbean	2-yr. crop	0.00 (0.0)	No emergence
Velvetbean	1-yr. crop, 1-yr. fallow ²	3.05 (74.8)	1, 10, 12, 13, 16, 18
Fallow	3-yr. fallow ³	0.97 (37.3)	1, 2, 6, 10, 12, 13, 15, 16

¹ Continuous cropping for 3 years.

² Cultivated for 1 year, followed by fallow next year (test year).

³ Fallow for 3 years without fertilizer.

⁴ Numbers in parenthesis are percentages of chickweed, a dominant species.

⁵ Species appeared in each plot: (1) sticky chickweed (*Cerastium glomeratum*), (2) "Miminagusa" (*Cerastium vulgatum* var. *angustifolium*), (3) Annual fleabane (*Erigeron annuus*), (4) Philadelphia fleabane (*Erigeron philadelphicus*), (5) starwort (*Stellaria alsine* var. *undulata*), (6) floating foxtail (*Alopecurus geniculatus*), (7) narrowleaf vetch (*Vicia angustifolia*), (8) Flexuosa bittercress (*Cardamine flexuosa*), (9) "Inugarashi" (*Rorippa atrovirens*), (10) common dandelion (*Taraxacum officinale*), (11) Japanese mugwort (*Artemisia princeps*), (12) danadian fleabane (*Erigeron canadensis*), (13) "Hahakogusa" (*Gnaphalium affine*), (14) blady grass (*Imperata cylindrica*), (15) meadowgrass (*Poa annua*), (16) creeping woodsorrel (*Oxalis corniculata*), (17) shepherd's purse (*Capsella bursa-pastoris*), (18) prickly sowthistle (*Sonchus asper*).

⁶ Surveyed on April 14, 1988.

Source: Fujii et al. 1991.⁴

L-DOPA strongly inhibits the plant growth of *Cerastium glomeratum*, *Spergula arvensis* (both Caryophyllaceae), *Linum usitatissimum* and *Lacutuca sativa*, and moderately inhibits the growth of Compositae, while having very limited effects on Gramineae and Leguminosae

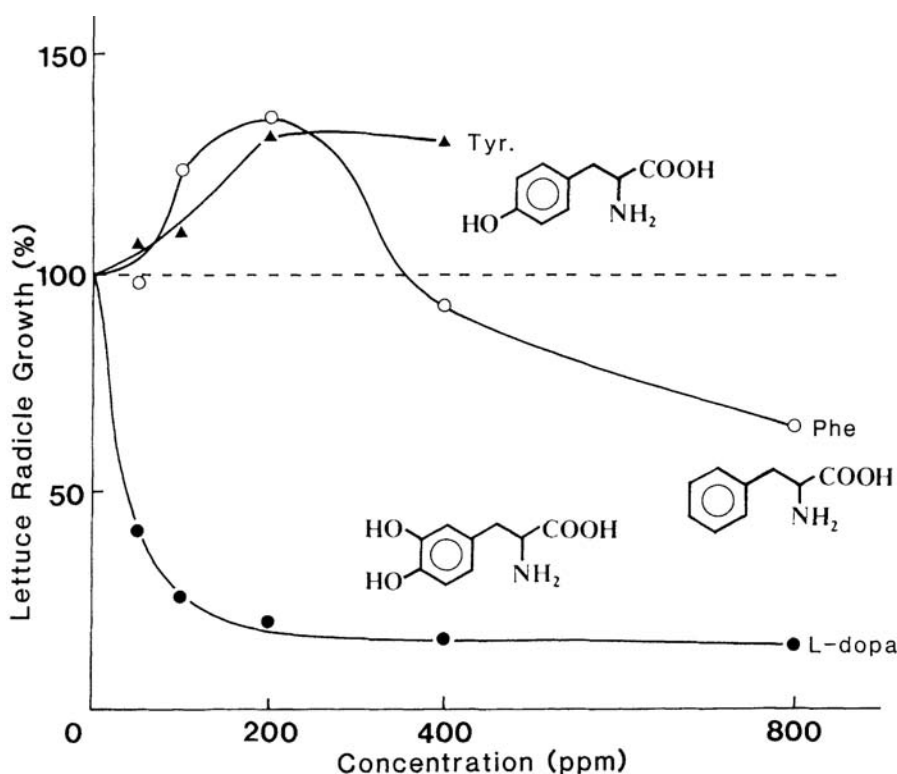
TABLE 3.4

Effect of Mixed Culture of Velvetbean to the Growth of Lettuce and Kidney Bean under a Stairstep Experiment

Receiver Plant	Donor Plant	Leaf Area (cm ²)	Shoot dry Weight (g)	Root dry Weight (g)
Lettuce	Lettuce	30.4 ^b (89)	53.9 ^b (96)	12.0 ^b (101)
	Velvetbean	21.5 ^c (63)	39.3 ^c (70)	5.7 ^c (48)
	(None)	34.2 ^a (100)	56.3 ^a (100)	11.9 ^a (100)
Kidney bean	Kidney bean	87.9 ^a (97)	343 ^a (96)	148 ^b (79)
	Velvetbean	81.4 ^a (90)	344 ^a (96)	153 ^b (81)
	(None)	90.3 ^a (100)	358 ^a (100)	188 ^a (100)

Note: Numbers in the parentheses are percent of control. Means in a column followed by the same letter (a, b, c) are not significantly different at the 1% level (Duncan's multiple range test).

Source: Fujii et al. 1991.¹⁵

**FIGURE 3.1**

Effect of L-DOPA, tyrosine (Tyr), and phenylalanine (Phe) on the radicle growth of lettuce.

(Table 3.5). Such selective effectiveness is comparable with other candidate allelochemicals.^{26,27} The L-DOPA exudated from the intact roots of velvetbean fully explained the radicle growth inhibition in the agar medium (Figure 3.3). The result showing that L-DOPA strongly suppresses the growth of chickweed agrees with weed inhibition in the velvetbean field (Table 3.3). All these data suggest that L-DOPA functions as an allelopathic substance.

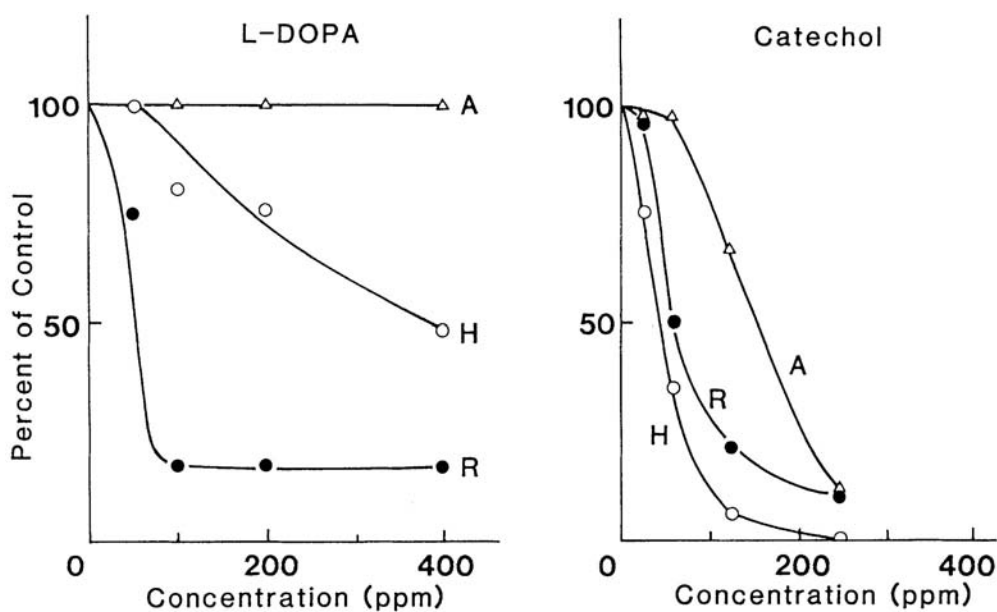


FIGURE 3.2

Effect of L-DOPA and catechol on the radicle growth (R), hypocotyl growth (H), and final germination percentage (A).

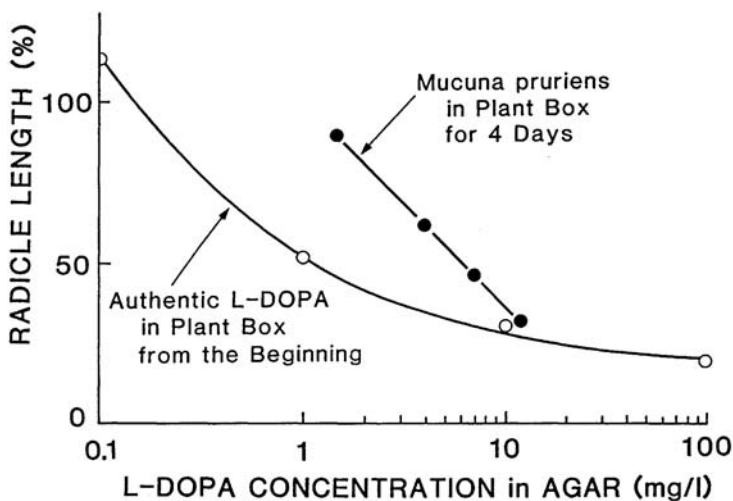


FIGURE 3.3

Comparison of the concentration of L-DOPA by the exudation from the root of velvetbean (*Mucuna pruriens*) and authentic L-DOPA in agar medium.

In the aged leaves, the content of dopamine increases and L-DOPA and dopamine are presumably changed to catechol in the litter as in the case of L-mimosine (Figure 3.4). The inhibitory activity of catechol to radicle growth is almost the same as to L-DOPA, but catechol is more toxic to hypocotyl growth and germination of lettuce (Figure 3.2). Table 3.6 shows activities of L-DOPA, dopamine, and catechol. In all plants tested, dopamine showed no practical inhibition to radicle growth, but catechol showed stronger or equal inhibitory effects to other weeds than did L-DOPA.

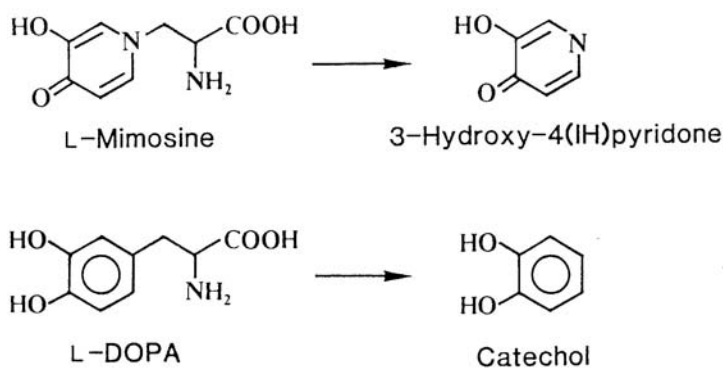
TABLE 3.5

Effect of L-DOPA on the Growth of Radicle of Some Weeds

Scientific Name (Family)	EC ₅₀ (mM)	English Name
<i>Cerastium glomeratum</i> (ck)	0.10	Sticky chickweed
<i>Spergula arvensis</i> (ck)	0.20	Corn spurrey
<i>Linum usitatissimum</i> (ln)	0.20	Flax
<i>Lactuca sativa</i> (co)	0.20	Lettuce
<i>Solidago altissima</i> (co)	0.46	Tall goldenrod
<i>Taraxacum officinale</i> (co)	1.30	Common dandelion
<i>Amaranthus lividus</i> (am)	0.76	Wild blite
<i>Miscanthus sinensis</i> (gr)	0.86	Chinese fairygrass
<i>Eleusine coracana</i> (gr)	1.00	African millet
<i>Setaria faberi</i> (gr)	1.60	Giant foxtail
<i>Plantago asiatica</i> (pl)	1.40	Asiatic plantain
<i>Trifolium pratense</i> (le)	2.00	Red clover
<i>Vicia villosa</i> (le)	2.00	Hairy vetch

Note: EC₅₀ (mM) = a concentration at which radicle length becomes 50% of the control. Abbreviations of family names are: co = Compositae, am = Amaranthaceae, gr = Gramineae, ck = Caryophyllaceae, pl = Plantaginaceae, le = Leguminosae, and ln = Linaceae.

Source: Fujii et al. 1991.¹⁹

**FIGURE 3.4**

Scheme of degradation of L-mimosine and L-DOPA to their degradation derivatives, 3-hydroxy-4(1H) pyridine and catechol.

As for the mechanism of action of L-DOPA and related catechol group compounds, 16 analogs of L-DOPA, mainly catechol compounds (Figures 3.5 and 3.6), were tested for their inhibitory activity to radicle and hypocotyl growth of lettuce and effect to soybean lipoxygenase. Figure 3.7 shows the high relationship ($r = 0.818$, $n = 16$, significant at 0.1%) between inhibition of plant growth and inhibition of lipoxygenase. It is known that the catechol group is a potent inhibitor for lipoxygenase,¹⁷ inhibitory allelopathic activity of catechol compounds might be attributed to the inhibition of lipoxygenase in plants. The real physiological role of lipoxygenase in plants is still unknown, but there is a hypothesis that this enzyme produces jasmonate, volatile compounds, and phytoalexins (Figure 3.8). As lipoxygenase is an enzyme that converts linoleic acid or linolenic acid to hydroperoxides, there might be a role for them as reducing agents for membrane and cell wall formation in roots. Here I would like to postulate the hypothesis that catechol compounds including

TABLE 3.6

Effects of L-DOPA and Related Compounds in Velvetbean on the Growth of Radicles of Lettuce and Some Weeds

Compounds	<i>Lactuca sativa</i> ³	<i>Solidago altissima</i> ⁴	<i>Taraxacum officinale</i> ⁵	<i>Amaranthus lividus</i> ⁶
	EC ₅₀ (mM) ¹			
L-DOPA	0.20	0.46	1.3	0.76
Dopamine	6.3	>3.2	1.6	>3.20
Catechol ²	0.73	0.36	0.73	<0.27

Compounds	<i>Miscanthus sinensis</i> ⁷	<i>Setaria faberi</i> ⁸	<i>Cerastium glomeratum</i> ⁹	<i>Spergula arvensis</i> ¹⁰
	EC ₅₀ (mM) ¹			
L-DOPA	0.86	2.0	0.10	0.20
Dopamine	>3.2	4.4	>3.2	1.6
Catechol ²	0.73	2.7	0.55	1.4

¹ 50% inhibition concentration. ² Pyrocatechol. ³ Lettuce. ⁴ Tall goldenrod.

⁵ Common dandelion. ⁶ Wild blite. ⁷ Chinese fairygrass. ⁸ Giant foxtail.

⁹ Sticky chickweed (mouse-ear). ¹⁰ Corn spurrey.

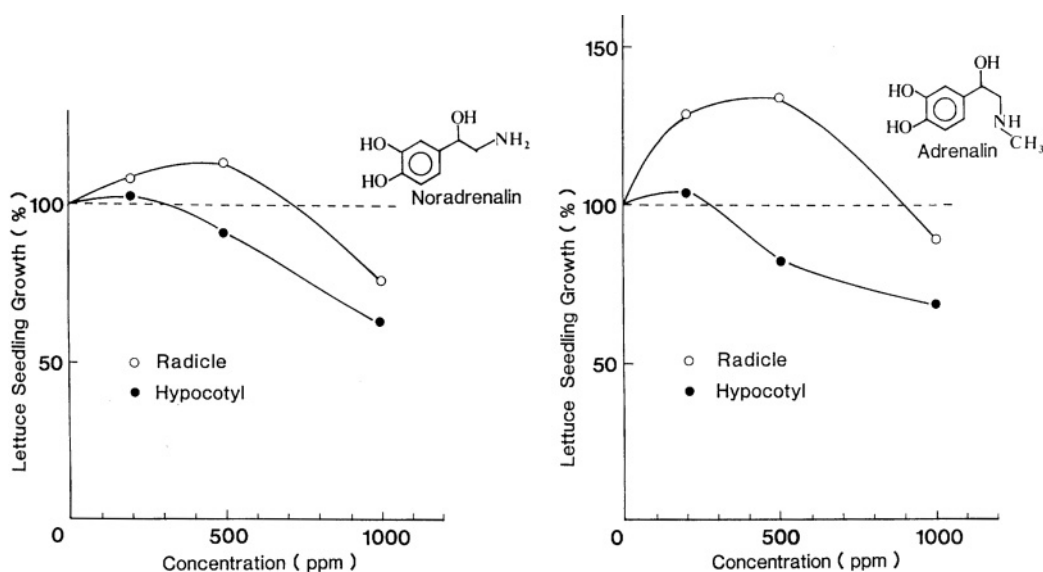


FIGURE 3.5

Effect of noradrenalin and adrenalin on the seedling growth of lettuce.

L-DOPA inhibit lipoxygenase reaction and, thus, inhibit the growth of roots in plants (Figure 3.8).

It is an earlier understanding that velvetbean smothers weeds by its rapid and thick covering effect with leaves. However, the above-noted results suggest that L-DOPA or its associate compounds, accumulated in an extremely high concentration in plants, function as an allelochemical in reducing weed population. The role of L-DOPA in velvetbean seeds was earlier regarded as a chemical barrier to insect attacks.²² It is now confirmed, however, that it plays another role in its allelopathic activity in weed control.

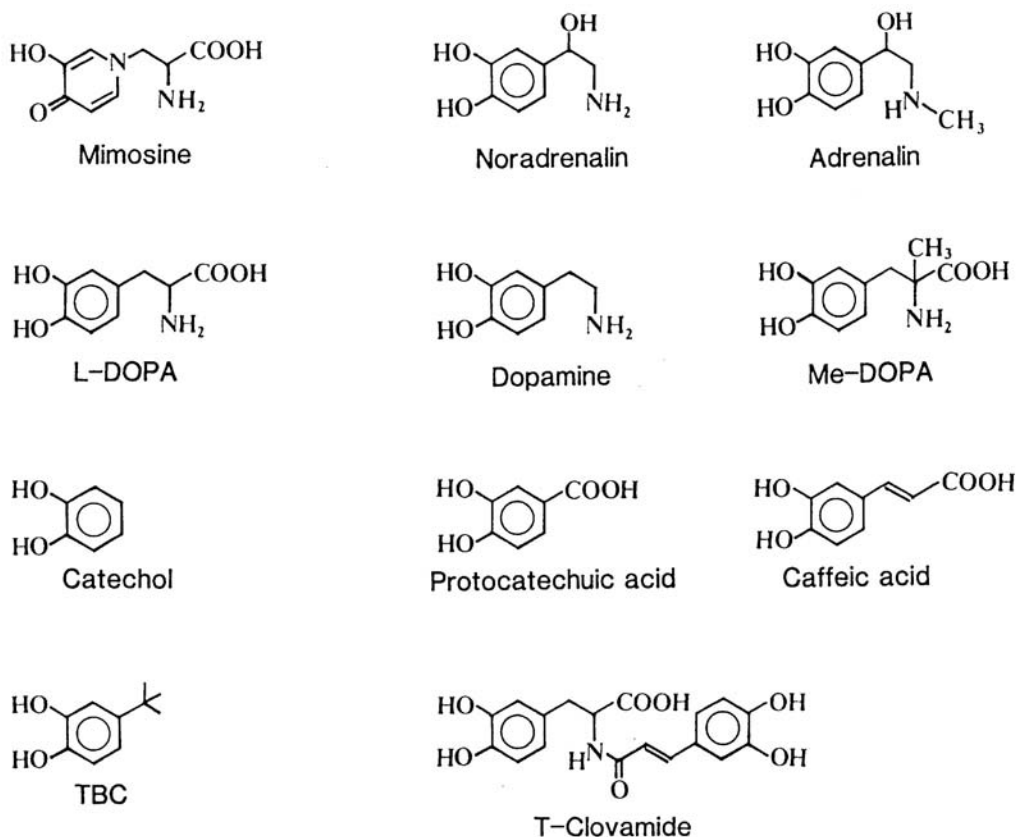


FIGURE 3.6

Analogues of L-DOPA tested for the mechanism of action in Figure 3.7.

Apart from the L-DOPA in velvetbean, caffeine in a coffee tree,²⁸ mimosine in *Lucaena* spp.,²⁶ nordihydroguaiaretic acid (NDGA) in a creosote bush,²⁷ each of which is contained in a high quantity in the respective plants, are well known to have physiological effects on animals, while their associations with other plants in terms of allelopathy have only recently been identified. It is expected that some secondary metabolites would be identified in the field of allelopathy.

Since velvetbean has special abilities such as weed smothering,^{5,19} tolerance to pests,^{22,29} suppression of nematode population,^{30,31,32} and soil improvement in its physical structure,²⁹ it could be more widely used to reduce applications of artificial chemicals to a lower level. Velvetbean seed yields are very high in the tropics, and the seed contains a high level of protein with a useful protein source. If detrimental factors such as L-DOPA and trypsin inhibitors could be eliminated through proper cooking,³³ it would also contribute to the alleviation of food problems in some tropical countries.

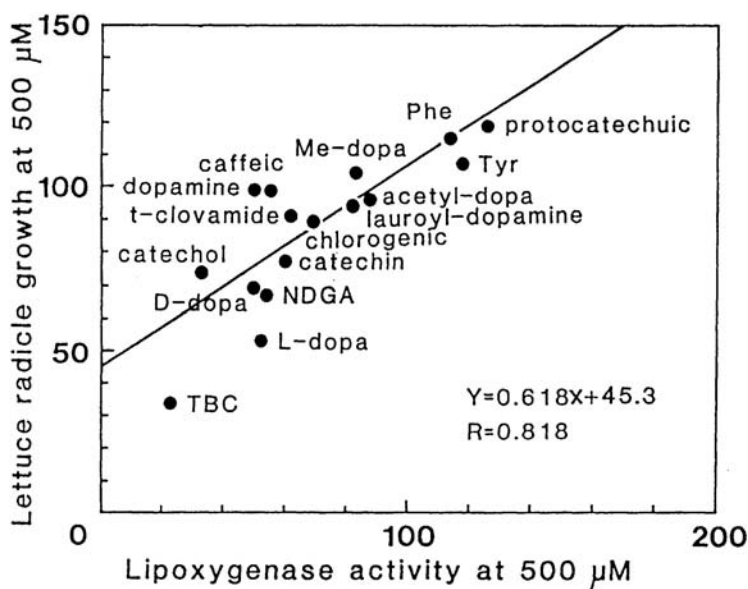
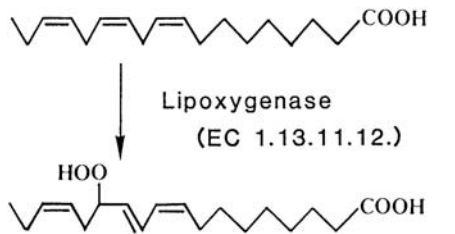


FIGURE 3.7
Relationship between the inhibitory activity of radicle growth of lettuce and inhibitory activity of lipoxxygenase by the analogous chemicals of L-DOPA.



Physiological Role in Plants

1. Root growth ?
2. Jasmonic acid
3. Volatile compounds
4. Phytoalexin

FIGURE 3.8
Postulate role of lipoxxygenase in plants.

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Phytochemical Inhibitors from the Nymphaeaceae: *Nymphaea odorata* and *Nuphar lutea*

Stella D. Elakovich, Stacy Spence, and Jie Yang

CONTENTS

- 4.1 Introduction
- 4.2 *Nuphar lutea*
- 4.3 *Nymphaea odorata*
- 4.4 Conclusions
- Acknowledgments
- References

KEY WORDS: *Nymphaeaceae*, *Nymphaea odorata*, *Nuphar lutea*, *hydrophytes*, *allelopathy*, *growth inhibition*, *alkaloids*, *gallotannins*.

ABSTRACT From a survey for potential allelopathic activity of some 25 herbaceous hydrophytes native to the southeastern U.S., we found two species in the Nymphaeaceae to possess the greatest inhibitory activity. *Nuphar lutea* was the most active species and was some 10 times more active than *Nymphaea odorata*, the second most active as measured by lettuce seedling and *Lemna minor* growth assays. Bioassay-directed isolation of plant growth inhibitory constituents from *N. odorata* led to the isolation of five allelochemicals of moderate activity. These included gallic acid, myricitrin, myricetin, 1,2,3,4,6-pentagalloyl-D-glucose, and 2,3,4,6-tetragalloyl-D-glucose. Examination of alkaloids from *N. lutea* showed 6,6'-dihydroxythiobinupharidine to be highly inhibitory of lettuce seedling radicle elongation at concentrations greater than 2 ppm. Nupharolutine, a second alkaloid isolated that was of similar structure, was inactive. Literature reports of phytochemicals present in *N. odorata* and *N. lutea*, as well as in other members of the Nymphaeaceae, are examined.

4.1 Introduction

In our work in the area of allelopathy (the influence of one plant on another), we have been working with aquatic and wetland plants native to the southeast U.S. We have examined more than 25 different aqueous extracts from these plants to determine their allelopathic potential.¹⁻³ The plant species examined are listed in Table 4.1. Species of *Eleocharis* were included as they often appear in monoculture in their undisturbed natural habitats and

TABLE 4.1

Plants Native to the southeastern U.S. Examined for Allelopathic Potential

<i>Brasenia schreberi</i> Gmel.	<i>Juncus repens</i> Michx.
<i>Cabomba caroliniana</i> Gray	<i>Limnobium spongia</i> (Bosc.) Steud.
<i>Ceratophyllum demersum</i> L.	<i>Myriophyllum aquaticum</i> (Vell.) Verdc.
<i>Eleocharis acicularis</i> (L.) R. and S.	<i>M. spicatum</i> L.
<i>E. equisetoides</i> (Ell.) Torr.	<i>Najas guadalupensis</i> (Spreng.) Maagus
<i>E. flavescens</i> (Poir.) Urban	<i>Nuphar lutea</i> (L.) Sibth. and Sm.
<i>E. montana</i> (HBK.) R. and S.	<i>Nymphaea odorata</i> Ait.
<i>E. obtusa</i> (Willd.) Shultes	<i>Nymphoides cordata</i> Ell.
<i>E. quadrangulata</i> (Michx.) R. and S.	<i>Potamogeton foliosus</i> Raf.
<i>E. tuberculosa</i> (Michx.) R. and S.	<i>Sparganium americanum</i> Nutt.
<i>Hydrilla verticillata</i> (L.F.) Royal	<i>Vallisneria americana</i> Michx.

have been observed to “displace” other hydrophytes.⁴ Some species, including *Hydrilla verticillata* and *Myriophyllum aquaticum*, were chosen because they are among the most noxious of aquatic weeds. Other species were included based on their observed growth patterns and their propensity toward monocultures, and some species were selected based on previous reports of allelopathic activity. Aqueous extracts of each of the plants were subjected to lettuce seedling and *Lemna minor* bioassays. Lettuce seedlings are perhaps the most widely used plant growth inhibition target system.^{1,5,6} We pregerminated the seeds, ran each assay at three extract concentrations using 20 seedlings per plate, and ran duplicate plates. Radicle lengths were compared to controls.¹ *Lemna minor* bioassay allows the use of this small floating aquatic plant, with the common name duckweed, as the target species for exploration of the allelopathic nature of aquatic hydrophytes.⁷ We maintained axenic *L. minor* cultures, ran the assay in 24-well sterile tissue culture plates with 1.5 mL of growth medium per well. We then ran each assay at concentrations corresponding to the three concentrations of the lettuce seedling assays, ran six replications per 24-well plate, and ran duplicate plates which agreed to within $\pm 5\%$.¹ Control and extract wells were chosen such that position was random, and the assay was read when the control wells reached 20 fronds. Advantages of this assay are that it required only 1.5 mL of growth medium to accommodate the entire target *L. minor* plant, and that aquatic plant targets are more appropriate for evaluation of aquatic plant allelopathy. Results of these initial bioassays showed species in the Nymphaeaceae to possess the highest allelopathic potential. Worldwide members of the Nymphaeaceae are listed in Table 4.2. Some botanists also include the two genera of Cobombaceae, *Brasenia schreberi* and *Cabomba* (about seven species), in the Nymphaeaceae. Of the six genera listed in Table 4.2, we examined only *Nuphar* and *Nymphaea*, as these were the only plants readily available to us in the southeastern U.S. The others are native to Southeast Asia, Australia, or South America. We did, however, also examine *Cabomba caroliniana* and *Brasenia schreberi*, two species sometimes included in the Nymphaeaceae. Table 4.3 gives lettuce seedling bioassay results of only the most inhibitory

TABLE 4.2

Worldwide Members of the Genera in the Nymphaeaceae Family

<i>Barclaya</i> (two species — tropical Southeast Asia)
<i>Euryale ferox</i> (one species — Southeast Asia)
<i>Ondinae purpurea</i> (one species — northwestern Australia)
<i>Nuphar</i> (7 to 20 species — Northern temperate)
<i>Nymphaea</i> (~40 species — cosmopolitan)
<i>Victoria</i> (two species — South America)

TABLE 4.3

Bioassay Results of Inhibition of Lettuce Radicles
by Aqueous Extracts of Selected Hydrophytes

<i>Nuphar lutea</i> (roots and rhizomes)	100%
<i>Nuphar lutea</i> (leaves)	100%
<i>Nymphaea odorata</i> (leaves)	95%
<i>Juncus repens</i>	86%
<i>Vallisneria americana</i>	83%
<i>Brasenia schreberi</i>	82%
<i>Ceratophyllum demersum</i>	80%
<i>Eleocharis acicularis</i>	78%
<i>Nymphaea odorata</i> (roots and rhizomes)	78%

Note: Tested concentrations were 250 ppt of a filtered solution prepared by extraction of a mass of dried plant material with an equal mass of sterile water. Results are expressed as percent inhibition as compared to the control.

TABLE 4.4

Bioassay Results of Inhibition of *Lemna minor* Growth
by Aqueous Extracts of Selected Hydrophytes

<i>Nuphar lutea</i> (leaves)	100%
<i>Nuphar lutea</i> (roots and rhizomes)	100%
<i>Nymphaea odorata</i> (leaves)	98%
<i>Myriophyllum aquaticum</i>	83%
<i>Nymphaea odorata</i> (roots and rhizomes)	72%
<i>Cabomba caroliniana</i>	68%

Note: Tested concentrations were 250 ppt of a filtered solution prepared by extraction of a mass of dried plant material with an equal mass of sterile water. Results are expressed as percent inhibition as compared to the control.

extracts tested at 250 ppt. Both *N. lutea* and *N. odorata* were divided into above ground and below ground parts before testing. Table 4.4 gives *Lemna minor* bioassay results of only the most inhibitory extracts tested at 250 ppt. Clearly, *Nuphar lutea* completely inhibits all growth of both lettuce seedlings and *L. minor* at this concentration. *Brasenia schreberi* and *Combomba caroliniana*, two of the occasional members of the Nymphaeaceae, also are among these most inhibitory extracts towards lettuce seedling and *Lemna minor* as target plants, respectively.

Table 4.5 summarizes the results of *N. lutea* inhibition of lettuce seedlings and *L. minor* at much lower concentrations than the 250 ppt of Tables 4.3 and 4.4. *N. lutea* was significantly inhibitory even at 2.5 ppt. It was 10 fold more inhibitory in these two bioassays than the overall next most active plant, *Nymphaea odorata*.

4.2 *Nuphar lutea*

The genus *Nuphar* has been examined rather extensively and others have reported anti-fungal activity⁸ and the medical utility⁹ of *Nuphar* extracts. The genus is such a rich source of alkaloids that these are known as *Nuphar* alkaloids.¹⁰ *N. lutea* produces alkaloids having a regular sesquiterpenic skeleton incorporated into 3-furyl-substituted piperidines or quinolizidines, as well as C30 alkaloids that contain a sulfur atom along with two 3-furyl substituents.¹¹⁻¹³ As none of these alkaloids had previously been examined for allelopathic

TABLE 4.5

Inhibition of *Lemna minor* and Lettuce Seedling Growth by Various Concentrations of Leaf, Root, and Rhizome Extracts of *Nuphar lutea*

Bioassay	<i>N. lutea</i> Part	Concentration (ppt)	Inhibition (%) (control = 0%)
<i>Lemna minor</i>	leaf	25	66
	leaf	12.5	47
	leaf	2.5	22
	root and rhizome	25	67
	root and rhizome	12.5	35
	root and rhizome	2.5	22
Lettuce seedling	leaf	12.5	100
	leaf	2.5	71
	root and rhizome	12.5	100
	root and rhizome	2.5	69

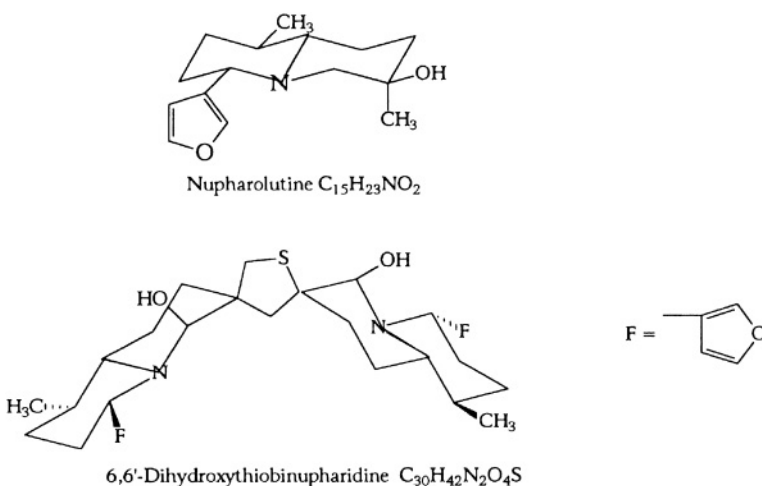


FIGURE 4.1

The structures of nupharolutine (top) and 6,6'-dihydroxythiobinupharidine (bottom), alkaloids isolated from *Nuphar lutea*.

activity, we isolated two of them and examined their allelopathic potential by the lettuce seedling assay.¹⁴ Isolated were 6,6'-dihydroxythiobinupharidine and nupharolutine (Figure 4.1). Of these, nupharolutine was inactive up to 60 ppm, the highest concentration we could test due to solubility constraints of this isolated, water-insoluble compound. The second compound, 6,6'-dihydroxythiobinupharidine, was highly inhibitory of lettuce seedling growth at concentrations greater than 2 ppm as shown in Figure 4.2. It is possible that other of the many *Nuphar* alkaloids also are plant-growth inhibitory, but they have not yet been isolated and tested.

4.3 *Nymphaea odorata*

Süttfeld and co-workers have recently reported finding resorcinol as one of the exudates of *N. lutea* seedlings.¹⁵ Although resorcinol-related compounds are widespread products of

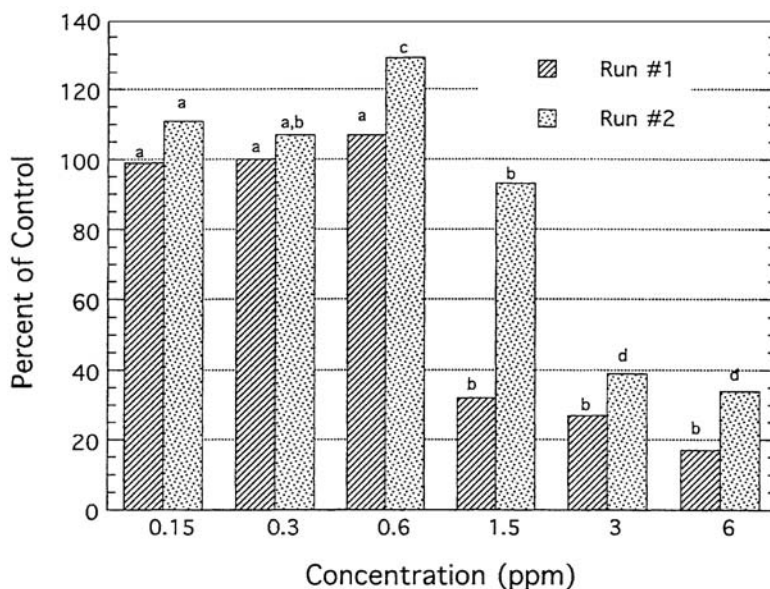


FIGURE 4.2

The results of bioassay of 6,6'-dihydroxythiobinupharidine with lettuce seedlings as the target. Results of replicate runs are shown. Bars with the same letter (within each run) are not significantly different according to the Duncan multiple range test at $P < 0.05$. The control was different from the inhibitory effect of 6,6'-dihydroxythiobinupharidine at >2 ppm. At 0.6 ppm the alkaloid was significantly stimulatory in Run 2. (From Elakovich, S.D. and Yang, J., *J. Chem. Ecol.*, 22:12, 2209-2219, 1996. With permission.)

plant metabolism, this is the first report of nonderivatized resorcinol from a higher plant and is the first evidence for exudation of structurally defined phenol from an undisturbed aquatic macrophyte. Activity testing at the concentration resorcinol that is exuded from *N. lutea* showed the zooplankton *Daphnia* to be sensitive to resorcinol, but two (of five tested) phytoplankton organisms, *Cryptomonas* and *Rhodomonas*, were able to metabolize resorcinol and, in doing so, to increase their starch granules.¹⁵ This suggests that resorcinol acts as an allelochemical in an aquatic medium. Its specificity indicates it may be detrimental to *Daphnia* but be beneficially used by *Cryptomonas* and *Rhodomonas* or other organisms. Three additional species tested were uninfluenced by the levels of resorcinol applied.¹⁵ Sütfield et al.¹⁵ support the earlier conclusion of Weidenhamer et al.¹⁶ that although a given species may produce and exude only small amounts of allelochemicals, a constant flux of these compounds may produce a significant deleterious effect on target species.¹⁶

Nymphaea odorata has not received the same intensive investigation that *Nuphar* (with its abundant and important *Nuphar* alkaloids) has, but some reports exist. The commonly found β -sitosterol and the long chain alcohol 1-hexacosanol have been reported¹⁷ along with reports of the sterols campesterol, stigmasterol, and the triterpenes α - and β -amyrin, lupeol, and taraxasterol.¹⁸ *Nymphaea* species, other than *N. odorata* which have been examined, are listed in Table 4.6.

Saeed et al.²⁵ have examined the lipoidal matter of *N. hybrida* and identified β -amyrin, α -amyrin, campesterol, cholesterol, stigmasterol, and β -sitosterol, as well as the hydrocarbons squalene and dotriacontane. They also identified a dozen fatty acid methyl esters. The lipoidal material showed antimicrobial activity against some gram-positive bacteria and insecticidal activity against cotton leaf worm.

None of the reports of *Nymphaea* constituents examined the plant growth-inhibiting nature of the identified compounds. We carried out a bioassay-directed examination of the

TABLE 4.6

Compounds Reported from *Nymphaea* Species Other than *N. odorata*

<i>N. alba</i> Linn.	Flavonoids including isokaempferide, apigenin, isoquercitrin, hyperoside, quercetin 4'- β -xyloside, 3-methylquercetin 3'- β -xyloside ¹⁹ Dienoic, trienoic, and tetraenoic acids ²⁰ α -aminoadipic acid ²¹ β -sitosterol, gallic acid, 1-hexacosanol ²² Nupharin, nymphaeine, nymphalin ²³ Tocopherylesters ²⁴
<i>N. hybrida</i>	Lipids ²⁵ Quercetin, myricetin ²⁶
<i>N. mexicana</i>	Alkanes of 28 and 29 carbons ²⁷
<i>N. stellata</i>	β -sitosterol, coclaurine ²⁸
<i>N. tetragona</i> ^a	Aminopropylhomospermidine ²⁹
<i>N. tuberosa</i> Paine	Alkaloids, flavones, triglycerides ³⁰
<i>N. variegatum</i> Engelm.	Alkaloids, flavonols, saponins ³⁰

^a Spelled *Nymphaca tetragona* in the original publication.

TABLE 4.7

Compounds Identified in *Nymphaea odorata*
by Bioassay-Directed Isolation

Compound	Concentration	Percent of Control	
		Lettuce	Lemna
Gallic acid	0.59 mM	10	50
Myricitrin	0.21 mM	Inactive	79
Myricetin	0.31 mM	Inactive	60
1,2,3,4,6-PGG	0.11 mM	52	63
2,3,4,6-TGG	0.13 mM	51	52

Note: Bioassay results for lettuce seedling and *Lemna minor* assays are given. PGG is pentagalloylglucose, TGG is tetragalloylglucose.

plant growth inhibiting (allelopathic) constituents of *N. odorata* and isolated the components shown in Table 4.7.³¹ Listed are the isolated compounds, their activities in the two bioassays applied, lettuce seedling and *Lemna minor*, and their millimolar concentration in the assays. Activities are shown for isolated compounds at 100 mg/L or 100 ppm. Structures of these compounds are shown in Figure 4.3. Although the activities are modest, we are not aware of other gallotannins, present in *Nymphaea* species, reported to possess plant growth inhibitory properties. There are reports of the plant growth inhibitory activity of myricitrin and gallic acid isolated from other sources. Nicollier and Thompson³² reported myricitrin, a flavonol glycoside, to be the key factor in the multiple bioactivities of *Desmanthus illinoensis*, a member of the Leguminosae being examined for its allelopathic activity. Myricitrin was allelopathic at less than 10 ppm against tomato root growth and also antibacterial against two *Bacillus* species. Price³³ reported the leaves of *Kalanchoe glassfeldiana* contained a specific flowering inhibitor shown to be gallic acid. Geraniin, an antimicrobial hydrolyzable tannin, similar in structure to the penta- and tetragalloylglucose compounds we isolated from *Nymphaea odorata*, was isolated from *N. tetragona* and found to be active against fish pathogenic bacteria.³⁴

Tannins have been isolated from *Nuphar* species, but not from *N. lutea*. From *N. japonicum* there have been four dimeric and trimeric hydrolyzable tannins isolated, called nupharins A, B, C, D, E, and F that possess an α -D-glucopyranose core with a hexahydroxydiphenoyl

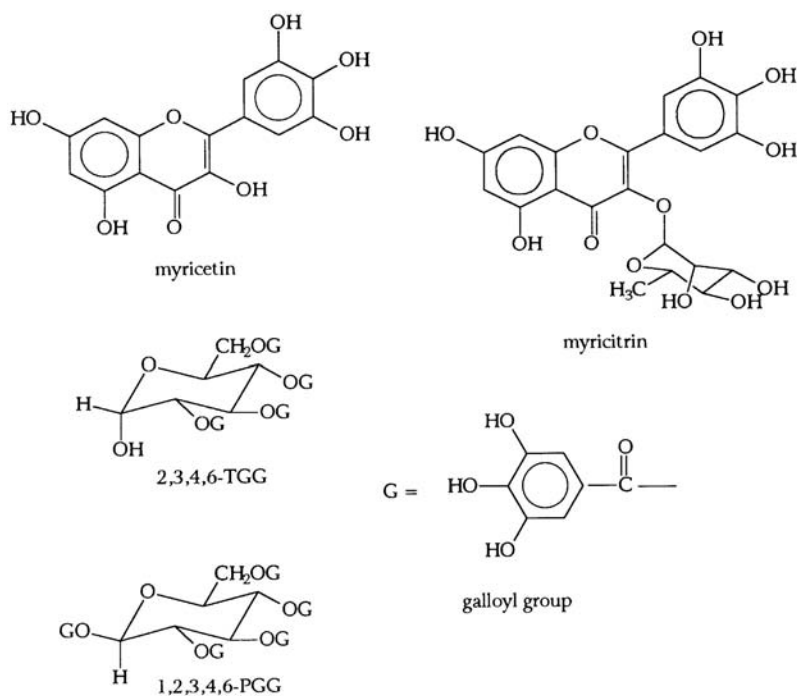


FIGURE 4.3

Compounds identified from *Nymphaea odorata* by bioassay-directed isolation. PGG is pentagalloylglucose, TGG is tetragalloylglucose.

group at the 3,6-position.³⁵⁻³⁷ These nupharins are similar in structure to the 1,2,3,4,6-pentagalloylglucose we found in *Nymphaea odorata*. Nishizawa et al.³⁸ found two gallotannins and two ellagitannins with antibacterial activity in *Nuphar variegatum*.

4.4 Conclusions

This examination of phytochemical inhibitors from the Nymphaeaceae leads to the conclusions that

- Members of the Nymphaeaceae appear to possess potent growth-inhibitory compounds.
- Both *Nuphar* and *Nymphaea* possess gallotannins and related compounds of moderate activity.
- The *Nuphar* alkaloid we isolated from *N. lutea*, 6,6'-dihydroxythiobinupharidine, has by far the greatest plant growth inhibitory properties reported for compounds isolated from members of the Nymphaeaceae.
- Other members of the Nymphaeaceae must be examined to determine the extent of family characteristics.
- Hydrophytes should be considered as an important source of useful secondary metabolites.

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*Development of an Allelopathic Compound From Tree-of-Heaven (*Ailanthus altissima*) as a Natural Product Herbicide*

Rod M. Heisey

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ABSTRACT Since 1959, it has been known that *Ailanthus altissima* produces one or more phytotoxic compounds. The major toxin was recently identified as ailanthone, a member of the quassinoid group. In greenhouse trials, purified ailanthone exhibited moderate preemergence herbicidal activity and strong postemergence activity. It also had potent herbicidal activity under field conditions. In one field trial, ailanthone was sprayed postemergence onto seedlings of 17 species of weeds and crops. The lowest application rate (0.3 kg/ha) reduced shoot biomass of six species to $\leq 10\%$ of the control and 10 species to $\leq 50\%$ of the control. Ailanthone is relatively nonselective, but cotton, yellow nutsedge, and *A. altissima* seedlings show a high level of tolerance. Ailanthone also was tested in the field for its ability to control weeds in several vegetable crops (beans, tomato, cauliflower, corn). A marked reduction in weed population occurred for a few weeks after application, but the herbicidal activity was short-lived. Nine weeks after spraying, weed biomass in the most effective treatment (0.6 kg/ha) was 60% of the weed biomass in the untreated control. Ailanthone is rapidly degraded by soil microorganisms. It killed many of the weed seedlings present at the time of application, but its herbicidal activity was rapidly degraded. As a result, weeds that were not killed were able to recover and a new crop of weeds was able to emerge and grow. Ailanthone exhibits a number of the same problems that have prevented other natural products from being developed as commercial herbicides; however, its striking herbicidal activity under field conditions justifies further investigations.

5.1 Introduction

Much interest exists in using natural products to control weeds in agroecosystems, but few natural products have actually been developed into commercial herbicides.¹⁻⁵ Bialaphos and glufosinate (= phosphinothricin) are the most successful natural product herbicides to date.⁶⁻⁹ Both are metabolites of bacteria in the genus *Streptomyces*, although glufosinate is currently produced synthetically. These compounds are now available as commercial herbicides and are the only two agriculturally important herbicides to have resulted from phytotoxins produced by microorganisms.⁵

From plants, no single compound in its naturally occurring form has yet been developed into a commercial herbicide, to the best of my knowledge.⁵ Many plants reportedly cause allelopathic effects, and numerous metabolites from plants are phytotoxic in laboratory bioassays,¹⁰ but few possess the level of activity necessary for development as herbicides. Corn gluten meal, which was recently patented for use as a preemergence herbicide, may be somewhat of an exception.¹¹ The most active herbicidal components of this product are dipeptides released during hydrolysis of the corn meal,¹² suggesting the involvement of microorganisms in producing the phytotoxic effects. The hydrolysate is a potent inhibitor of root system development in germinating grass seedlings.¹³

A number of synthetically derivatized metabolites from plants have been developed, or have potential for development, as commercial herbicides. Benzoic acid, which is produced by many plant species, has been derivatized to yield the herbicides dicamba (3,6-dichloro-2-methoxybenzoic acid) and chloramben (3-amino-2,5-dichlorobenzoic acid). The allelopathic terpenoid 1,8-cineole has served as a model for the development of cinmethylin, an experimental herbicide that has undergone field trials, but has not yet been released.¹⁴⁻¹⁶

5.2 Tree-of-Heaven (*Ailanthus altissima*)

Tree-of-heaven (*Ailanthus altissima*, Simaroubaceae) has been known since the work of Mergin in 1959 to produce one or more phytotoxic and potentially allelopathic compounds.¹⁷ *A. altissima* was introduced into the U.S. from China about 300 years ago.¹⁸ It has since become naturalized in many parts of the U.S., and is especially abundant in the milder regions of the Northeast.^{19,20} *A. altissima* is considered an undesirable woody weed having almost no value for timber, paper pulp, or fuelwood.

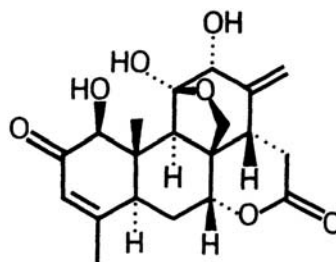
In the southern parts of Pennsylvania, New York, and Connecticut, *A. altissima* often colonizes disturbed sites such as abandoned fields or vacant lots^{20,21} and seems to inhibit the invasion of other tree species, suggesting an allelopathic effect.²² It commonly forms dense, nearly monospecific thickets, which may persist for decades.¹⁹ *A. altissima* is a vigorous competitor. It grows rapidly and reproduces prolifically from root sprouts, stump sprouts, and abundantly produced samaras.²³⁻²⁵ Aqueous extracts of *A. altissima* tissues are strongly phytotoxic in laboratory bioassays.²⁶⁻²⁸ The question of whether the invasiveness of *A. altissima* is due to allelopathy or to its superior competitive ability, or both, remains unanswered.²⁹

5.3 Ailanthone

The major phytotoxin produced by *A. altissima* was recently identified as ailanthone (Figure 5.1). This compound was first isolated and identified from *A. altissima* in the

FIGURE 5.1

Structure of ailanthone (molecular mass = 376), the major phytotoxic compound produced by *A. altissima*. (From Heisey, R. M., *Am. J. Bot.*, 83, 194, 1996. With permission.)



1960s,³⁰⁻³² but it was not recognized to be the major phytotoxic component until the 1990s.³³⁻³⁵ Ailanthone belongs to the class of bitter-tasting compounds called quassinoids that are commonly found in members of the Simaroubaceae. Quassinoids are heavily oxygenated lactones. The majority have a C-20 basic skeleton, but C-18, C-19, and C-25 quassinoids also exist.^{36,37} Quassinoids are degraded triterpenoids and are believed to be synthesized in plants from Δ 7-euphol or Δ 7-tirucallol. Over 75 quassinoids are known.³⁸

Quassinoids exhibit a wide range of biological activity. Soon after its discovery, ailanthone was reported to have activity against the amoeba *Entamoeba histolytica*.³⁹ Interest in the quassinoids was greatly stimulated by the discovery in the 1970s of antileukemic effects and other forms of anticancer activity.^{38,40} Additional effects have since been discovered, including activity against the malarial parasite *Plasmodium falciparum*,⁴¹ antifeedant and growth inhibitory activity against insects,⁴²⁻⁴⁴ antiviral activity against the Rous sarcoma virus,⁴⁵ and antifungal activity against *Plasmopara viticola*, the cause of grape downy mildew.⁴⁶

The phytotoxic effects of quassinoids were not reported until recently. In 1993, holocanthone was mentioned to have pronounced phytotoxicity at 100 ppm on grape plants.⁴⁶ The herbicidal effects of purified ailanthone were first reported in 1993³³ and were described more extensively a few years later.^{34,35}

In mature *A. altissima* trees, ailanthone is most abundant in the bark of roots, bark of branches, and the inner bark of trunks.²⁸ Concentrations are intermediate in leaves and low in the wood and the outer bark of the trunk. Ailanthone is soluble in polar solvents such as water and methanol, and it can be easily extracted from roots and bark with pure methanol or aqueous methanol. To purify ailanthone for use in greenhouse bioassays of herbicidal activity, a crude extract of macerated roots and root bark was made with aqueous methanol. The extract was concentrated under vacuum to remove the solvents, and the concentrate was redissolved in water and sequentially partitioned against hexane, diethyl ether, dichloromethane, and ethyl acetate. The phytotoxicity of the resulting fractions was monitored with a bioassay using seeds of garden cress (*Lepidium sativum*). The dichloromethane and ethyl acetate fractions were the most phytotoxic. These two fractions were combined and purified by low-pressure column chromatography on silica gel and C18 packing to yield purified ailanthone.³⁵

5.4 Pre- and Postemergence Effects of Ailanthone

Ailanthone exhibits potent herbicidal activity and appears to be a promising candidate for development as a natural product herbicide. The purified compound was tested for pre- and postemergence activity in the greenhouse using seeds and seedlings of weeds and crops planted in field soil in small flats.³⁵ For preemergence tests, purified ailanthone was sprayed onto the soil surface after the seeds had been planted. The soil was immediately watered to carry the ailanthone down into the seed zone. For postemergence tests, the

seeds of weeds and crops were sown in flats. The resulting seedlings were sprayed with ailanthon 4 to 6 days after emergence. The purified ailanthon was dissolved in distilled water containing 0.36% X-77 surfactant and sprayed at rates ranging from 0 (control) to 8 kg/ha.

5.5 Effects of Ailanthon on Weeds and Crops

Even the lowest application of ailanthon (0.5 kg/ha) slowed or reduced seedling emergence. Emergence was completely inhibited for at least 6 days after spraying in treatments receiving 4 and 8 kg/ha (Figure 5.2). Eventually some seedlings emerged, even in the 8 kg/ha treatment, but the number was greatly reduced relative to the control.

The postemergence effects of ailanthon were more striking (Figure 5.3). Even the lowest dose (0.5 kg/ha) killed all seedlings except those of velvetleaf and *A. altissima*. Velvetleaf was somewhat resistant to ailanthon, but at the higher rates it was severely injured or killed. *A. altissima* seedlings were remarkably tolerant to ailanthon and showed no visible injury even at the highest application (8 kg/ha). This result indicates *A. altissima* possesses one or more mechanisms to avoid autotoxicity.

The strong postemergence activity of ailanthon suggested its herbicidal effects should be tested under actual field conditions. Much larger quantities of ailanthon were needed for field trials. Because of the difficulties associated with purifying such a large amount of ailanthon, a crude extract prepared from the inner bark of the trunk was used as the spray material. The amount of ailanthon in the crude extract was first quantified with a bioassay. Appropriate quantities of the crude extract were then applied to the field plots to deliver the desired amounts of ailanthon.

The first field trial was designed to examine the herbicidal effects and selectivity of ailanthon on 17 species of weeds and crops under actual field conditions. A randomized block design containing four replicates (only two in 4.5 kg/ha treatment) was used. Each of the 17 plant species was sown in two adjacent rows running the length of the blocks. Each block was randomly subdivided into six treatments (0.4 × 7.8 m) containing two adjacent rows of all 17 species.



FIGURE 5.2

Preemergence effects of purified ailanthon shown 6 days after application to seeds planted in soil. Application rates (left to right) are 0 (control), 0.5, 1, 2, 4, and 8 kg of ailanthon per hectare. The plant species, as shown by the control, are (front to rear of flats) redroot pigweed, garden cress, velvetleaf, foxtail, barnyard grass, and corn (cv. Silver Queen). (From Heisey, R. M., *Am. J. Bot.*, 83, 196, 1996. With permission.)



FIGURE 5.3

Postemergence effects of purified ailanthone shown 5 days after spraying on emerged seedlings. The application rates and species are identical to those in Figure 5.2, except an additional row at the rear of the flats contains *A. altissima* seedlings. (From Heisey, R. M., *Am. J. Bot.*, 83, 197, 1996. With permission.)

The crude extract of *A. altissima* bark was sprayed onto the seedlings 3 weeks after planting. Application rates were adjusted to provide 4.5, 2.2, 1.1, 0.6, 0.3, and 0.0 (control) kg/ha of ailanthone, based on the 1.2% content of ailanthone in the crude extract. For the treatments of 2.2, 1.1, 0.6, and 0.3 kg/ha, the extract was dissolved or suspended in distilled water containing 0.36% X-77 surfactant. These solutions were applied with a double-nozzle spray boom on a knapsack sprayer adjusted to deliver 935 L of solution per hectare at 30 psi. The control received a similar application of distilled water containing 0.36% X-77. Only two replications of the 4.5 kg/ha treatment were possible because of the limited amount of extract available. Because of difficulties in dissolving and spraying such a high concentration of extract, the 4.5 kg/ha treatment was sprayed twice with a solution having the same concentration of extract as the 2.2 kg/ha treatment (but half the concentration of X-77). The effects of the treatments were evaluated 19 days after spraying. At this time, all plants were cut at soil level, dried at 55 to 60°C, and weighed for biomass determination.

The results of this investigation showed that the potent postemergence effects of ailanthone previously observed in the greenhouse also occurred under field conditions (Table 5.1). Symptoms of phytotoxicity were evident on certain species in less than 24 h after spraying. Even the lowest application rate tested (0.3 kg/ha) reduced shoot biomass in 10 of the 17 species to $\leq 50\%$ of the control. Shoot biomass of the six most sensitive species (redroot pigweed, common lambsquarters, bindweed, sunflower, tomato, and radish) was reduced to $\leq 10\%$ of the control by 0.3 kg/ha of ailanthone.

A wide range in sensitivity to ailanthone occurred (Table 5.1). Yellow nutsedge and cotton were the most resistant and showed no reduction in shoot biomass, even at the highest application (4.5 kg/ha). Velvetleaf, large crabgrass, and Johnson grass also exhibited a high level of resistance. Broadleafed species (dicots) generally were more sensitive to ailanthone than grasses and yellow nutsedge (monocots). The resistance of cotton and velvetleaf suggested species in the Malvaceae may be tolerant to ailanthone and indicated additional members of this family should be examined in future tests. All crop species other than cotton (oats, soybean, sunflower, tomato, squash, radish, corn) exhibited high-to-moderate sensitivity.

TABLE 5.1

Applications of Ailanthone (kg/ha) Needed to Reduce Shoot Biomass of Weeds and Crops to 50% (ID50) and 10% (ID10) of Control Biomass

Plant species	ID50	ID10
Velvetleaf (<i>Abutilon theophrasti</i>)	1.1	>4.5
Redroot pigweed (<i>Amaranthus retroflexus</i>)	<0.3	<0.3
Oats (<i>Avena sativa</i> cv. Ogle)	<0.3	0.6
Common lambsquarters (<i>Chenopodium album</i>)	<0.3	<0.3
Field bindweed (<i>Convolvulus arvensis</i>)	<0.3	<0.3
Yellow nutsedge (<i>Cyperus esculentus</i>)	>4.5	>4.5
Large crabgrass (<i>Digitaria sanguinalis</i>)	1.1	>4.5
Barnyard grass (<i>Echinochloa crus-galli</i>)	<0.3	2.2
Soybean (<i>Glycine max</i> cv. Agripro 4420)	<0.3	4.5
Cotton (<i>Gossypium hirsutum</i> cv. Koker 315)	>4.5	>4.5
Sunflower (<i>Helianthus annuus</i> cv. Black Oil Hybrid)	<0.3	0.3
Tomato (<i>Lycopersicon esculentum</i> cv. Roma)	<0.3	<0.3
Squash (<i>Cucurbita pepo</i> cv. Black Beauty zucchini)	<0.6	4.5
Radish (<i>Raphanus sativus</i> cv. Cherry Belle)	<0.3	<0.3
Foxtail (<i>Setaria faberii</i>)	0.6	>4.5
Johnson grass (<i>Sorghum halepense</i>)	0.6	>4.5
Corn (<i>Zea mays</i> cv. Iochief)	<0.3	<0.6

Note: Applications used in this investigation were 4.5, 2.2, 1.1, 0.6, and 0 (control) kg/ha. Data were collected 19 days after ailanthone application.

These results provided strong evidence that ailanthone has significant herbicidal activity under field conditions. Therefore, a second field trial was conducted to test the effectiveness of ailanthone in controlling weeds in an actual cropping system. This trial was based on the following rationale. Ailanthone is very expensive to produce, even in the form of a crude extract. If it is going to be developed as a herbicide, its most likely market would be as a specialty product for organic growers or home gardeners who want to control weeds chemically but without synthetic pesticides. This field trial had two goals: (1) to evaluate the ability of ailanthone to control weeds in an actual agricultural system, and (2) to determine what effects ailanthone would have on certain vegetable crops that might be planted by a home gardener or organic grower.

The field trial was set up in a randomized block design with four replicates. Four vegetable crops (green bean, *Phaseolus vulgaris* cv. Tendergreen; tomato cv. Roma; cauliflower, *Brassica oleracea* cv. Snow Crown Hybrid; and sweet corn cv. Silverado) were planted in rows running the length of the blocks. The tomatoes and cauliflower were planted as transplants and the beans and corn were direct seeded. The plots were sprayed about 1 week after planting. At this time a high population of recently-emerged weed seedlings ranging from 0 to 5 cm tall was present. The crude extract was dissolved or suspended in distilled water containing 0.36% X-77 surfactant and sprayed directly onto the weeds and crops at rates calculated to deliver 1.1, 0.6, 0.3, 0.15, and 0 (control) kg/ha of ailanthone. Weed density and crop injury were monitored for several weeks after spraying. Crop yield and weed biomass were determined 6 to 9 weeks after spraying, when the investigation was ended.

The initial results demonstrated ailanthone did have very impressive herbicidal effects. The major weeds in the plots were galinsoga (*Galinsoga ciliata*), black nightshade (*Solanum nigrum*), common lambsquarters, redroot pigweed, carpet weed (*Mullugo verticillata*), and several grasses (Figure 5.4a). Even at the lowest application (0.15 kg/ha), a reduction in the weed population was noticeable and the crops were not seriously harmed. A greater reduction in weed population occurred at 0.3 kg/ha, but damage to cauliflower was evident (Figure 5.4b). Weed control increased in the 0.6 kg/ha treatment, but crop damage became



(a)



(b)

FIGURES 5.4a-d

Effects of ailanthon on weeds and crops shown 21 days after spraying. (a) Control (0 kg/ha ailanthon), (b) treatment receiving 0.3 kg/ha ailanthon (≈ 0.25 lb/acre), (c) treatment receiving 0.6 kg/ha ailanthon (≈ 0.5 lb/acre), and (d) treatment receiving 1.1 kg/ha ailanthon (≈ 1.0 lb/acre). The four crops present in rows running the width of the plots are (front to rear) corn, cauliflower, tomato, and green beans.



(c)



(d)

FIGURE 5.4 (continued)

TABLE 5.2

Shoot Biomass of All Weed Species Present in Plots
9 Weeks after Spraying with Ailanthone

	Ailanthone Application Rate (kg/ha)				
	0	0.15	0.3	0.6	1.1
Weed biomass	100a	84ab	74b	60b	76b

Note: Data are expressed as percentage of biomass in control plots (0 kg/ha). Data followed by different letters differ at 0.05 probability level in Duncan's multiple range test.

more serious (Figure 5.4c). Very strong suppression of weeds occurred at 1.1 kg/ha, but moderate to severe injury was present on all crops except beans (Figure 5.4d). Cauliflower was the most sensitive of the four crops tested and some of the plants were killed at the higher applications of ailanthone. Corn and tomato were intermediate in sensitivity, and beans were the most tolerant.

Although the weed suppression caused by ailanthone initially was impressive, it eventually became apparent that the herbicidal effects were short-lived. Weed biomass in the treated plots was reduced throughout the duration of the investigation, but the results at the end of the investigation (9 weeks after spraying) were not as striking as those observed initially (Table 5.2). The reduction in weed biomass for the 0.3, 0.6, and 1.2 kg/ha treatments was statistically significant ($P = 0.05$) compared to the 0 kg/ha control. The greatest reduction was in the 0.6 kg/ha treatment, where biomass was reduced to 60% of that in the control. The reason why 0.6 kg/ha reduced weed biomass more than 1.1 kg/ha was not readily apparent.

The reason the suppression in weed biomass at the end of the investigation was less impressive than the reduction in weed population immediately after spraying is because ailanthone degrades rapidly in the field. Ailanthone killed many of the weeds that were present at the time of spraying, but its toxicity disappeared quickly. As a result, weeds that were not killed were able to recover and a new crop of weeds was able to emerge and grow normally. This result was not surprising because previous work had shown ailanthone is degraded by microorganisms within several days of application to soil.^{28,35} The microbial transformation of other herbicidal natural products also has been reported for juglone from black walnut,⁴⁷ 2(3H)-benzoxazoline from rye,⁴⁸ and phenolic compounds.⁴⁹

It is difficult to compare the herbicidal effects of ailanthone with those of other natural products from plants because few of these compounds have been tested as herbicides under actual field conditions. Shettel and Balke investigated the pre- and postemergence herbicidal effects of salicylic acid, coumarin, and caffeine.⁵⁰ Their postemergence effects were stronger than the preemergence effects, but even at 11.2 kg/ha these compounds showed only mild herbicidal action. Ailanthone appears to be considerably more active under field conditions. The comparatively high phytotoxicity of ailanthone under field conditions justifies further investigations of its potential for development as a herbicide.

Despite its potential, ailanthone exhibits several of the problems that have traditionally inhibited the development of natural product herbicides. Ailanthone and many other natural products are degraded rapidly in the environment by microbial or physicochemical processes. Short persistence may be advantageous from the standpoint of safety to humans and the environment, but it can limit herbicidal efficacy. Ailanthone, like many other secondary plant products, is produced only in small amounts and has a relatively complex molecular structure. The ailanthone content of the bark used to produce the crude extracts

in these investigations was about 0.06% of bark fresh weight. These characteristics limit yields of the compound from natural sources and hinder commercial synthesis at a price competitive with artificial pesticides.

The question of how to produce aianthone in large quantities at a competitive price must be answered if aianthone is to be developed as a commercial herbicide. Aianthone has not yet been synthesized artificially in the laboratory, to the best of my knowledge. Numerous attempts have been made to synthesize related quassinoids. Only a few have been successful, the procedures are complex, and the yields are low.^{37,51-53}

Extraction from trees currently is the best way to obtain aianthone, but doing so would be expensive on a large scale. For example, a typical 15 year-old tree will have a trunk diameter of about 25 cm and will yield about 25 kg (fresh-weight) of easily harvestable bark with an aianthone content of about 0.06%. A tree this size, therefore, would yield 14 g of pure aianthone which is enough to treat 100 m² (0.01 ha) at 1.2 kg/ha. If one were to grow *A. altissima* trees 4 m apart in plantations, giving a population of 625 trees per hectare, the yield of aianthone would be 8.75 kg in 15 years. This would be enough to treat about 7 ha at 1.2 kg/ha. Obviously, producing aianthone this way would be expensive and not economically competitive with synthetic herbicides. This is a good example of why the pesticide industry currently favors artificial synthesis over biosynthesis.³

Cell culture is another possibility for producing aianthone. *A. altissima* cells have already been cultured by other investigators. Aianthone production has been demonstrated, but the concentrations have been below those in the bark of intact trees.^{54,55} Therefore, if aianthone is to be developed as a herbicide, large-scale production is an issue that must be addressed.

5.6 Conclusions

Aianthone exhibits strong postemergence herbicidal activity, not only in the greenhouse, but also under actual agricultural conditions in the field. Its herbicidal activity, however, appears to be degraded within several days under field conditions. Weed seedlings present in the field at the time of spraying are killed or injured, but after several days the aianthone is degraded and a new cohort of weeds is able to emerge. More research is needed to develop ways to extend the period of activity. Multiple applications over the growing season might alleviate the problem, but also would increase costs. The phytotoxicity of aianthone to certain crops is another issue. One solution would be to develop ways to apply aianthone without getting it on the crops. Methods to produce aianthone in large quantities at a competitive price also must be investigated. The fact that aianthone is a natural product with potent herbicidal activity under field conditions makes these topics worthy of further investigation.

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Triterpenoids and Other Potentially Active Compounds from Wheat Straw: Isolation, Identification, and Synthesis

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6.1 Introduction

Straw consists of the above-ground fractions (normally cut at a height of around 20 cm) of cereal plants after removal of the grain.¹ Depending on the harvesting system, part of the cut straw is left in the field together with the stubble. The straw length and diameter vary greatly and, consequently, also the biomass yield both within and between species. The biomass production and its chemical composition are important parameters since straw and other fibrous by-products from cereals that are available in the world amount to approximately 3 trillion tonnes per year.² Part of the straw is utilized for feed,³ paper,⁴ and fuel,⁵ but a major part of the straw is discarded as a waste product. In some regions of the world straw is used in mulch-tillage in no-till cropping systems,^{6,7} a common agricultural practice credited with a number of ecological advantages such as reduction of soil compaction, good erosion control, better water retention, and conservation of organic matter. In many instances, inhibitory effects on germination and growth of other plant species were observed.

The direct or indirect, harmful or beneficial effects of plants on other plants of the same or of different species by means of chemicals released from living, or decaying, plant material has been broadly defined as allelopathy.⁸ The presence of chemical signals in ecosystems has been attributed to numerous types of secondary metabolites⁹ which are able to

induce physiological and morphological changes in plants: inhibition of respiration, germination and growth, perturbation of nutrient uptake, chlorosis, and death.

Although allelopathic effects of wheat straw upon some weeds have been known for some time, few compounds have been associated with these effects. However, due to their potential application in the understanding and developing of natural herbicides and to their economical and ecological importance in agriculture, there is a need for research on the active principles present in straw to properly understand the allelopathic phenomenon and clearly establish a cause–effect relationship.

6.2 Active Extracts from Wheat Straw

A systematic extractive workup of wheat straw yielded two major fractions that showed allelopathic activity in lettuce bioassays: a weak acidic fraction, mainly composed of phenolic compounds and a “neutral” fraction which was shown to be composed of triterpenoid structures and fatty acid derivatives.

6.2.1 Phenolic Compounds

In order to establish the composition of phenolics from cereal straws, packed column GC and GC-MS have been used in the analysis of ethylated phenolic compounds and their mass spectra discussed, but only a few compounds have been identified.¹⁰ Today HPLC is the most commonly used method for analyses of phenolics of plant origin.^{11,12} However, resolution is highly dependent upon eluant composition and the analytical conditions must be carefully optimized according to expected phenolic structures.¹³ Some authors have studied the application of HPLC with electrochemical detection to chromatographic assays of phenolic compounds from wheat straw.¹⁴ Only six compounds (*p*-hydroxybenzoic acid, vanillic acid, vanillin, syringic acid, *p*-coumaric acid, and ferulic acid) were identified as free phenols.

In the author’s laboratory, the high resolution, speed of analysis, and sensitivity of capillary gas chromatography, coupled with mass spectrometry (HRGC-MS) and Fourier transform infrared spectrometry (HRGC-MS-FTIR), was used to assess the composition of the phenolics extracted from mature wheat straw.¹⁵ In the case of some phenolic compounds of low molecular weight, distinction between isomers is particularly difficult by HRGC-MS. Also, phenolic compounds frequently appear partially methylated in nature. Furthermore, the position of the nonalkylated OH groups cannot be assessed after permethylation. In order to distinguish between free and methylated OH, the straw extract was ethylated with diazoethane in methanol and the resulting mixture analyzed by HRGC. Under these conditions, salicylic, gentisic, and β -resorcylic acids are only partially ethylated as is shown by the presence of a free OH band (3547 cm^{-1}) in the corresponding HRGC-FTIR spectra. The *ortho* OH groups are not derivatized. The fact that the mass spectrum of syringic acid showed a molecular ion at m/z 254, corresponding to the ethylation of the hindered OH group in position 4, seemed to suggest that hydrogen bonding effects, rather than steric effects, are responsible for the incomplete ethylations. Ethylation with diazoethane is, therefore, a useful method for the distinction of isomeric phenolic acids by GC-MS. The ethyl derivative of 3,4-dihydroxybenzoic acid (protocatechuic acid) showed a molecular ion at m/z 238, but the derivatives 2,4 (β -resorcylic acid) and 2,5 (gentisic acid) yielded a molecular ion at m/z 210. The same distinction can be made between salicylic acid and *p*-hydroxybenzoic acid.

Individual organic compounds possess characteristic infrared spectra with unique absorption patterns in the “fingerprint” region (below 1600 cm⁻¹). The FTIR, with its facility for electronic retrieval and spectral comparison, offers a powerful tool for distinction between isomers. For instance, gentisic and β -resorcylic acids can be differentiated by means of the corresponding FTIR spectra. Combination of HRGC-MS results and HRGC-FTIR spectral data led us to the identification of the most significant components in the extract.

Ferulic, protocatechuic, gentisic, *p*-hydroxybenzoic, trans-*p*-coumaric, and azelaic acids were obtained on a preparative scale from raw extracts by droplet countercurrent chromatography (DCCC).¹⁵ This technique has many advantages in preparative chromatography. Sample losses due to compound adsorption are not observable, as all the process takes place in the liquid phase.¹⁶ This is particularly important in the case of phenolic compounds. Its main drawbacks are the large amounts of solvents used and the length of time. Raw extracts could be directly used for DCCC. The isolated components could be recovered in high purity after solvent evaporation. Their identity was confirmed by HNMR, IR, UV, and MS. Table 6.1 shows the identified components. The majority of these compounds

TABLE 6.1

Phenolic Acids Identified in the Acidic Extract of Mature Wheat Straw

Compound	m/z (ITD) (Ethyl Derivatives)	cm ⁻¹ (FTIR) (Ethyl Derivatives)
Benzoic acid	150 (M ⁺), 149, 122, 106, 105, 77	3034, 2982, 2984, 1740, 1448, 1369, 1270, 1107, 709
Succinic acid	174 (M ⁺), 147, 130, 129, 101	2988, 2944, 1755, 1373, 1345, 1270, 1210, 1180, 1037, 960
Fumaric acid	172 (M ⁺), 143, 142, 114, 69	2988, 2943, 2913, 1743, 1647, 1471, 1401, 1370, 1296, 1259, 1227, 1154, 1103, 1041, 983
<i>m</i> -Toluic acid	164 (M ⁺), 163, 120, 119, 91	3030, 2940, 1720, 1590, 1450, 1390, 1280, 1210, 1110, 1090, 790, 750, 690
Salicylic acid ^a	166 (M ⁺), 165, 120, 93, 92	3547, 3261, 2985, 1690, 1615, 1475, 1400, 1374, 1310, 1253, 1208, 1159, 1133, 1084, 755, 686
Maleic acid	172 (M ⁺), 127, 117, 99, 84	2990, 1730, 1640, 1410, 1380, 1290, 1210, 1160, 1020, 860
<i>p</i> -Hydroxybenzoic acid	194 (M ⁺), 166, 149, 138, 121, 93, 65	2958, 2942, 2924, 1745, 1600, 1483, 1452, 1375, 1291, 1242, 1126, 1080, 1049, 752, 681
Gentisic acid ^a	210 (M ⁺), 164, 136, 135, 111	3547, 2988, 1736, 1605, 1509, 1269, 1247, 1168, 1104
Vanillic acid	224 (M ⁺), 196, 168, 151, 123, 97	3090, 2972, 1736, 1604, 1512, 1432, 1373, 1283, 1216, 1101, 1032, 767
β -Resorcylic acid ^a	210 (M ⁺), 181, 136	3547, 2958, 2942, 2924, 1745, 1600, 1483, 1452, 1375, 1291, 1242, 1126, 1080, 1049, 752, 681
Protocatechuic acid	238 (M ⁺), 210, 182, 165, 154, 137	2984, 2943, 1735, 1600, 1505, 1477, 1413, 1369, 1284, 1214, 1180, 1127, 1101, 1032, 761
Azelaic acid	244 (M ⁺), 199, 152, 135, 125, 111, 83, 69, 55	2983, 2940, 2868, 1752, 1372, 1304, 1172, 1161, 1114, 1098, 1079, 1047
α,β -Dihydroferulic acid	252 (M ⁺), 224, 151, 150, 137	2982, 1735, 1604, 1425, 1372, 1255, 1216, 1186, 1104, 975
trans- <i>p</i> -Coumaric acid	220 (M ⁺), 175, 164, 148, 147	2988, 1736, 1605, 1509, 1269, 1274, 1168, 1104
Syringic acid	254 (M ⁺), 226, 211, 198, 181	2984, 2946, 1735, 1584, 1492, 1468, 1413, 1369, 1330, 1215, 1184, 1111, 1037
cis-Ferulic acid	250 (M ⁺), 222, 194, 177	2985, 1733, 1637, 1607, 1508, 1306, 1259, 1210, 1163, 1045, 1037, 672
3,4-Dihydroxyphenyl-propanoic acid	266 (M ⁺), 238, 224, 210, 165, 147	3647, 3574, 3081, 3022, 1736, 1604, 1404, 1251, 1164, 1118, 905, 825 ^b
trans-Ferulic acid	250 (M ⁺), 236, 205, 136, 121	3008, 2987, 2965, 2948, 1735, 1697, 1639, 1609, 1466, 1425, 1372, 1323, 1255, 1216, 1186, 1167, 1104, 975
1-Naphthoic acid	171 (M ⁺ -29), 127, 99	3574, 3055, 1750, 1550, 1504, 1344, 1164, 1105, 938, 772, 712 ^b

^a Partial ethylation was observed.

^b Spectrum corresponding to the underivatized acid.

show significant biological activity. They are the most cited allelopathic agents possessing very significant allelopathic effects towards weeds.¹⁷

6.2.2 Fatty Acid Derivatives

Malic, malonic, citric, acetic, tartaric, fumaric, propionic, butyric, isobutyric, pentanoic, isopentanoic, nonanoic, decanoic, lauric (12:0), myristic (14:0), palmitic (16:0), stearic (18:0), oleic (18:1; 9c), linoleic (18:2; 9c,12c), linolenic (18:3; 9c,12c,15c), arachidonic (20:0), 11,14-eicosadienoic, heneicosadienoic, behenic (22:0) acids, and (Z,Z)-9,12-8-hydroxyoctadecadienoic are the most frequently reported carboxylic acids present in active allelopathic extracts, or as biologically active compounds in the literature.^{9,18-20} McCracken et al.²¹ reported that the unsaturated compounds were the most inhibitory; their toxicity increasing with the number of unsaturated bonds.

Classical methods for isolating, separating, and identifying fatty acids and derivatives comprise distillation (steam distillation, fractional reduced pressure, and molecular distillation), recrystallization, countercurrent distribution, and the chromatographic methods: column partition chromatography (CC), paper chromatography (PC), and thin-layer chromatography (TLC).²² Nowadays, because of its high resolution power, GC is the most popular method.^{23,24} Usually acids are separated as methyl esters derivatives. The more volatile ones (C3-C9) are frequently derivatized as butyl, decyl, and other ester derivatives of higher molecular weight. There is a growing interest in HPLC,²⁵ especially when other functionalities rather than unsaturated bonds are present.

The classical structure methods comprise the spectroscopic ones: UV,²² to determine conjugated bonds; IR,²² to recognize special functionalities and also *trans* double bonds; nuclear magnetic resonance spectroscopy (NMR),²⁶ to assign structures; and also mass spectrometry.

GC-MS is the most commonly used method for separation and identification of medium and long chain carboxylic acid derivatives. The mass spectra afford direct information regarding chain length, degree of unsaturation, position of branching, and the nature and position of functional groups. Yet, double bond positions cannot be unequivocally assigned due to the frequent migration of double bonds on fragmentation. The use of negative ion chemical ionization (CI⁻) is ineffective in that regard.^{27,28} A more sophisticated method using collisional activated decomposition (CAD) of negative ions (M-H)⁻ was successfully used to determine the position of double bonds in unsaturated compounds.²⁷ A tandem triple mass spectrometer analyzer (MS/MS) must be used, but it is not available in the majority of laboratories. The best solution has been the use of double bond derivatives which can be analyzed in current electron impact mass spectrometers.

The neutral fraction, resulting from a systematic acid-base solvent extraction procedure, was shown to be composed of fatty acid methyl esters and triterpenoids. Carboxylic methyl esters were identified by GC analysis, by comparison with retention times of standards, and by analysis of their mass spectra obtained by HRGC-MS.²⁹ The differences in chain length, branch position, degree of unsaturation, position, and configuration of double bonds required careful GC and MS analysis with capillary columns. Generally, separation and identification of *cis-trans* mixtures require specialized chromatographic conditions, including the use of very long capillary columns or more polar stationary phases. However, these phases have lower temperature stability and the mass spectra of positional and geometrical isomers are difficult to differentiate. The dimethyl disulfide derivatives (DMDS)³⁰⁻³⁴ offered us an excellent method for the analysis of the monounsaturated fatty acid esters present. This is a single-step derivatization procedure. The mass spectra of DMDS adducts showed molecular ions (M)⁺ and two main ions (A⁺ and B⁺) derived from the cleavage of carbon-carbon bond where the double bond was originally

TABLE 6.2

Methyl Fatty Esters Identified in a Neutral Fraction of Mature Wheat Straw

Methyl Fatty Esters	m/z
Decanoate	214 (M ⁺), 188, 173, 145, 116, 99, 87, 74 (100%)
Azelate	220 (M ⁺), 206, 190, 178, 161, 146, 145, 131, 119, 105, 57
Syringic aldehyde	196 (M ⁺), 181, 153, 137, 111, 83
Tetradecanoate	242 (M ⁺), 199, 186, 157, 143, 129, 100, 87, 74, 55
10-Methylpentadecanoate	256 (M ⁺), 228, 213, 199, 185, 157, 143, 129, 115, 101, 87, 74, 55
Pentadecanoate	256 (M ⁺), 213, 199, 143, 129, 101, 87, 74, 55
12-Methylpentadecanoate	285 (M ⁺), 241, 227, 199, 171, 157, 129, 101, 87, 74, 55
Palmitate	270 (M ⁺), 237, 171, 143, 129, 115, 101, 87, 74, 55
3-Methylpalmitate	284 (M ⁺), 255, 241, 213, 199, 185, 171, 157, 143, 101, 87, 73, 55
Heptadecanoate	284 (M ⁺), 255, 241, 213, 199, 185, 143, 129, 101, 87, 74, 55
Stearate	298 (M ⁺), 255, 241, 199, 185, 143, 129, 101, 87, 74, 55
2-Methylstearate	312 (M ⁺), 269, 255, 226, 213, 199, 171, 157, 143, 101, 88, 73
Eicosanoate	326 (M ⁺), 283, 269, 227, 213, 171, 157, 143, 129, 88, 74
(Z)-9-Hexadecenoate ^a	315 (M ⁺ -47), 267, 217, 201, 185, 169, 137, 99, 71, 61
Docosanoate	354 (M ⁺), 311, 279, 213, 199, 168, 143, 115, 101, 87, 74
Oleate ^a	390 (M ⁺), 343, 325, 295, 253, 217, 204, 185, 173, 137, 87, 69, 55
Tricosanoate	368 (M ⁺), 325, 291, 269, 253, 213, 185, 143, 101, 87, 74, 55
Linoleate	294 (M ⁺), 262, 99, 95, 81
(Z)-10-Nonadecenoate ^a	404 (M ⁺), 372, 339, 294, 267, 231, 213, 199, 185, 173, 167, 156, 137, 97, 55
(Z)-19-Eicosenoate ^a	418 (M ⁺), 403, 373, 357, 241, 193, 139, 109, 95, 67, 61, 55
(Z)-9-Eicosenoate ^a	418 (M ⁺), 403, 371, 357, 339, 325, 217, 201, 187, 155, 137, 121, 87, 71, 55
Nonadecadienoate	308 (M ⁺), 279, 262, 220, 135, 95, 81

^a Dimethyl disulfide derivatives.

located and also an important fragment C⁺ derived from B⁺ via loss of methanol (B⁺-32). Alkylthiolation of Z- and E-isomers as a specific anti-addition led, respectively, to the *threo* and *erythro* adducts which were well separated by gas chromatography in a nonpolar stationary phase column (OV-101). This allowed a stereochemical identification. Table 6.2 lists the fatty acid methyl esters and their mass spectra identified in the neutral fraction of wheat straw. The branch position was achieved by comparison of mass spectra with the mass spectra of linear compounds with similar carbon number. The DMDS derivative is not effective for double bonds located at C2 and with polyunsaturated acids.³⁰ The reaction with deuterodiimide (N₂D₂) is a possible derivative for polyunsaturated locations.²⁸

Some of the identified acids were described as allelopathic or constituting a fraction having allelopathic activity. This is in good agreement with the activity demonstrated by the neutral fraction of wheat straw. Some authors³⁵ believe that the biosynthetic evolution of carboxylic acids of plant origin is strictly related to the biochemical necessity of their components having low melting points and this is the reason why plants have developed a great variety of unusual carboxylic acids, in contrast with animals. Perhaps this also explains why in a total of 21 identified esters, 7 are unsaturated and 4 are branched. These structural features, double bonds, and especially *cis* isomers and branches, lead to a decrease in the melting point.

6.2.3 Triterpenoids

Triterpenoids constitute a large group of compounds with a broad range of physical properties and biological activities with their nomenclature being well described.³⁶ The triterpenoids present in wheat straw are cycloartane and cholestane derivatives, both tetracyclic derived structures.

With respect to toxicity, the phytotoxic effects of triterpenoids in higher plants are relatively unknown. Only two compounds, digitoxigenin and estrofantidin, were mentioned as having proven antimicrobial activity.^{9,37} Macias et al.³⁸ referred allelopathic properties for some oxidized triterpenoids as having interesting inhibitory activities at low concentrations.

There is no such thing as a universal extraction method for triterpenoids. The extraction technique is usually determined by the material to be extracted and is conditioned by available information for the class structure present (free, glycosidic, and/or esterified). The most commonly used solvents are chloroform: methanol mixtures, acetone, chloroform, dichloromethane, petroleum ether, and ethanol. Usually a small quantity of water (2 to 7%) is added if the material is dry because its presence increases the yield of triterpenoids.³⁹

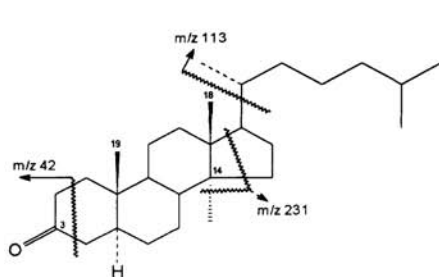
After extraction, the first step is usually a triterpenoid class separation. Again, there are several different methods employed. Solvent fractionation, precipitation with digitonin, and chromatographic separation using different techniques such as adsorption column chromatography CC (silica gel and/or alumina), reverse phase or argentation, or, in the case of a small quantity of extract, by adsorption or reverse phase thin layer chromatography TLC and/or by HPLC, are among the most common reported in the literature.⁴⁰

With respect to the individual separation of tetracyclic triterpenoids, the most often used methods are adsorption CC (silica gel or alumina) or reversed phase (Sephadex LH 20), reverse phase or argentation TLC, and HPLC, with HPLC being the most popular technique because it results in lower losses, produces fewer artifacts, and has a greater number of theoretical plates (higher resolution). GC is generally used as an analytical technique to evaluate the purity of isolated compounds.

The application of spectroscopic techniques NMR, MS, UV, IR, and x-ray is fundamental for final unequivocal identification.³⁶ However, the chromatographic and spectral information accumulated during the extraction, separation, and isolation process are very important, particularly if the compounds are present in very small quantities and the more sophisticated spectroscopic tools as bidimensional NMR techniques and x-ray spectroscopy, or even ¹³CNMR spectroscopy cannot be used. In these cases, the use of coupled techniques such as GC-MS or HPLC-MS is of paramount importance.

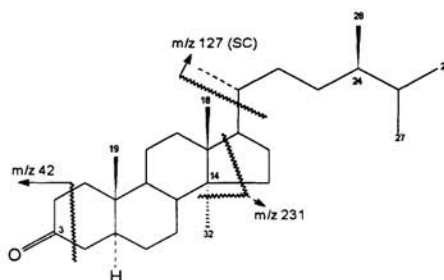
The isolated neutral fraction of wheat straw also is composed of triterpenoids: cholestane and cycloartane derived structures. Also included are seven new natural compounds: (24R)-14 α -methyl-5 α -ergostan-3-one; 14 α -methyl-5 α -cholestan-3-one; cycloart-5-ene-3 β ,25-diol; cycloart-3 β ,25-diol; 4 α -methylergostan-5-ene-3 β -ol; stigmast-4,22-dien-6 β -ol-3-one and 24-phenylethyl-cholest-3,6-dione were identified; together with several known compounds: (24R)-stigmast-4-ene-3-one; ergost-4-ene-3-one; (24R)-stigmast-4-ene-3,6-dione; stigmast-4,22-diene-3,6-dione; ergost-4-ene-3,6-dione; (24R)-5 α -stigmast-3,6-dione; stigmast-22-ene-3,6-dione; ergost-3,6-dione; cholesterol; ergosterol; campesterol; stigmasterol; β -sitosterol; spinasterol; stigmastanol; 7 β -hydroxysitosterol; stigmast-4-ene-6 β -ol-3-one; and ergost-4-ene-6 β -ol-3-one.^{29,41} Some of them were identified for the first time in higher plants.

The diethyl ether soluble material, specifically from the acetone–water extract of straw, was chromatographed on a silica gel column. The four fractions eluted with diethyl ether: ketones, monohydroxysterols, tetracyclic triterpenoids, dihydroxysterols, and ketohydroxysterols, were further separated by alumina column chromatography. The individual purification was done by preparative TLC and/or recrystallization from methanol or ethanol. The spectroscopic tools ¹HNMR, ¹³CNMR, FTIR, UV, LRMS, HRMS were used to make structural assignments. Figure 6.1 shows the new triterpenoid compounds identified in mature wheat straw and the most important spectroscopic data for their identifications. All these compounds were present in very small quantities (1 to 2 mg), and some of them were present in “trace” amount (≤ 0.1 mg). In those cases coupled techniques HRGC-MS, HPLC-MS, and HRFTIR were used and structures were proposed based on correlations with similar



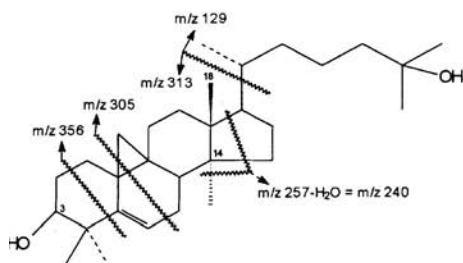
14α-Methyl-5α-cholest-3-one

MS: 400 (M)⁺, 385 (M-15)⁺, 231 (100%), 217, 213.



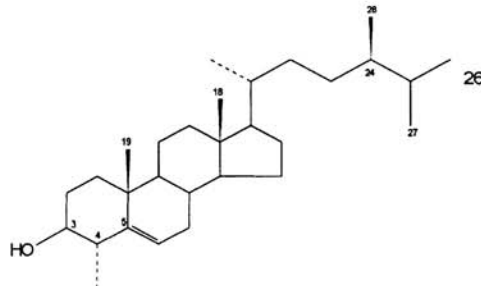
(24R)-14α-Methyl-5α-ergost-3-one

MS: 414 (M)⁺, 399 (M-15)⁺, 272 (M-SC-Me)⁺, 245 (M-SC-42)⁺, 231 (100%), 217, 213.
¹HNMR δ 0.681 ppm (H-18, 3H, s), 1.009 (H-19, 3H, s),
 δ 0.848 (H-32, 3H, s).



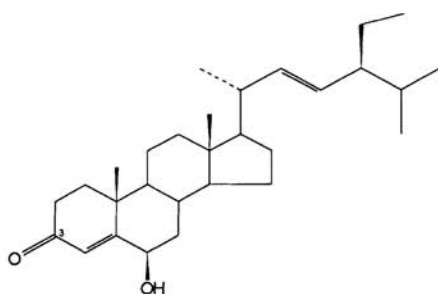
Cycloart-5-ene-3β,25-diol

MS: 442 (M)⁺, 399 (M-15)⁺, 396 (M-18)⁺, 381 (M-15-18)⁺,
 273 (M-SC-15)⁺
¹HNMR: ABq signal δ 0.33 and δ 0.55 ppm (J = 4.41 Hz)
 HRMS: 442.381118



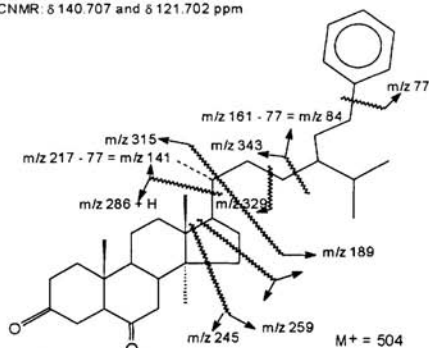
4α-Methylergost-5-ene-3β-ol

MS: 414 (M)⁺, 399 (M-15)⁺, 396 (M-18)⁺, 381 (M-15-18)⁺,
 273 (M-SC-15)⁺
 Ms silyl deriv.: 486 (M)⁺, 361 (M-90-15)⁺, 357 (M-129)⁺, 255 (M-SC-90-15)⁺,
¹³CNMR: δ 140.707 and δ 121.702 ppm



Stigmast-4,22-dien-6β-ol-3-one

¹HNMR δ 5.033 (H-22, dd), δ 5.160 (H-23, dd)
¹³CNMR δ 138.078 and δ 129.478 ppm
 MS: 426 (M)⁺, 354 (M-72)⁺, 339 (M-72-15)⁺,
 281 (M-SC)⁺, 267 (M-SC-18)⁺, 243 (M-SC-42)⁺



24-Phenylethyl-cholest-3,6-dione

¹³CNMR: δ 211.25, 209.08, 132.54, 129.05, 127.01,
 116.53, 31.62, 29.66 ppm
¹HNMR: δ 7.868 (2H, d), δ 7.565 (3H, m),
 δ 2.965 (2H, dd), δ 2.598 (3H, m)

FIGURE 6.1

New triterpenoid compounds from wheat straw.

mass spectra, rationalizing fragmentation, and, when possible, comparing the chromatographic behavior with standards.

6.2.3.1 HPLC-MS of Ketosteroids

The advantage of HPLC-MS over HRGC-MS is its ability to provide separation of compounds unsuitable for HRGC analysis, without the necessity of preparing volatile derivatives. The limitations reside in the specifications of the available interfaces and lower

TABLE 6.3

PB-HPLC-MS Mass Spectra of Ketosterols from Wheat Straw

Ketosterols	m/z
(24R)-Stigmast-4-ene-3,6-dione	426 (M) ⁺ , 411, 408, 398, 385, 276, 243, 187, 162, 149, 137, 121, 109, 95, 55 (100%)
(24R)-5 α -Stigmast-3,6-dione	428 (M) ⁺ , 100%, 413, 399, 287, 259, 245, 231, 189, 149, 137, 121, 109, 98
14 α -Methyl-5 α -cholest-3-one	400 (M) ⁺ , 385, 231 (100%), 217, 123, 163, 124, 107, 95, 81, 67, 55
(24R)-14 α -Methyl-5 α -ergost-3-one	414 (M) ⁺ , 399, 272, 245, 231 (100%), 217, 213, 189, 163, 135, 124, 107, 95, 81, 67
Ergost-4-ene-3-one	398 (M) ⁺ , 356, 274, 271, 229, 187, 173, 159, 147, 124 (100%), 121, 105, 95
Stigmast-4,22-diene-3-one	410 (M) ⁺ , 367, 349, 297, 269, 253, 227, 147, 124 (100%), 105, 91
(24R)-Stigmast-4-ene-3-one	412 (M) ⁺ , 397, 370, 288, 271, 245, 229, 187, 173, 159, 147, 124 (100%), 121, 105, 95, 79, 55
Ergost-4-ene-3,6-dione	412 (M) ⁺ , 385, 371, 285, 267, 243, 187, 175, 149, 137, 136, 121, 109, 95, 79, 55 (100%)

sensitivity. The particle beam (PB) separator offers the advantage of a full range mass spectra, although it severely limits the maximum eluent flow (1 ml min⁻¹) and cannot be used with buffered eluents. We have used the PB interface in order to fully gather 70 EI mass spectral data from liquid chromatography runs of ketosteroid rich fractions. The components of this fraction could not be satisfactorily resolved by HRGC. Although the chromatography could be drastically improved after trimethylsilylation, the GC-MS runs gave complex mass spectra that were too difficult to interpret.²⁹

Table 6.3 shows the most important ions of PB mass spectra of ketosteroids present in wheat straw. Using the PB interface, the spectra seemed to be common EI spectra which offered the advantage of being superimposable on published spectra of known structures or rationalizing their fragmentation if they are uncommon compounds.

6.2.3.2 Synthesis

The current interest in the activity of steroid metabolites having a C-6 oxo or hydroxyl function with occurrence in low concentrations in mature wheat straw extract, led us to establish a direct synthesis for them for unequivocal identification. There are only a few examples in literature⁴²⁻⁴⁴ that refer to the introduction of a hydroxyl group in the C-6 position of the steroid skeleton. More generally it is the introduction of an oxo group in position 6 of cholesterol with pyridinium dichromate (PDC),⁴⁵ or sodium dichromate,⁴⁶ or in cholestenone by sodium peroxide.⁴⁷

Stigmasta-4,22-dien-6 β -ol-3-one was present in residual amounts in wheat straw and its structure was unequivocally proven by a new direct synthesis, via *m*-chloroperbenzoic acid (MCPBA) oxidation of the stigmast-4,22-dien-3-one enol-ether (Figure 6.2). The β position of the hydroxyl group was confirmed by synthesis of a C-6 hydroxy derivative of stigmasta-4,22-dien-3-one. The treatment of the crude dienol-ether of stigmasta-4,22-dien-3-one in a dichloromethane/ethanol solution that was easily prepared by reaction with methyl orthoformate in DMF and in the presence of a catalytic amount of *p*-toluenesulfonic acid monohydrate and methanol⁴⁸ by slow addition of a dichloromethane solution of *m*-chloroperbenzoic acid at 0°C, yielded a mixture of stigmast-4,22-dien-6 β -ol-3-one and stigmast-4,22-dien-6 α -ol-3-one that was easily separated by chromatography on silica gel. The stigmast-4,22-dien-6 β -ol-3-one with a 39% overall yield was the major product, while the 6 α isomer yield was 25% (Table 6.4).

A mixture of epimers, where the β -isomer also is the predominant one, was referred to in the literature for the attack of a peracid on the enol ether of a derivative of pregn-4-ene-3-one.⁴⁹

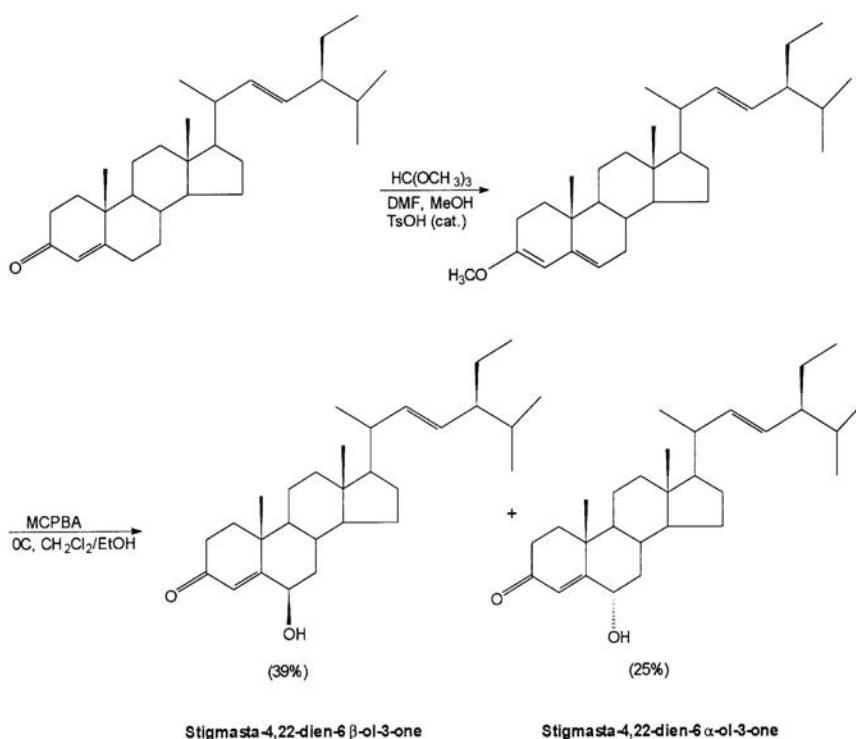


FIGURE 6.2
New triterpenoid synthesis.

TABLE 6.4
Physic and Spectroscopic Data of Synthesized Stigmast-4,22-dien-6-ol-3-one Isomers

Compound	m.p./°C	IR (cm ⁻¹)	¹ HNMR [δ (ppm)]	¹³ CNMR [δ (ppm)]
Stigmast-4,22-dien-6 β -ol-3-one	210–212	1690, 1660	5.79 (1H, s) (H-4)	200.56 (C-3), 168.75 (C-5),
			5.15 (1H, dd) (H-23)	138.09 (C-22), 129.44 (C-23),
			5.02 (1H, dd) (H-22)	126.21 (C-4), 73.12 (C-6)
			4.34 (1H, s) (H-6)	
Stigmast-4,22-dien-6 α -ol-3-one	166–168	1680, 1655	6.16 (1H, s) (H-4)	199.61 (C-3), 171.81 (C-5),
			5.14 (1H, dd) (H-23)	137.97 (C-22), 129.54 (C-23),
			5.02 (1H, dd) (H-22)	119.62 (C-4), 68.75 (C-6)
			4.33 (1H, m) (H-6)	

Also, Kirk⁴⁴ observed a mixture of epimers in peroxy-acid oxidation of 3-alkoxy-3,5-dienes in the presence of an ethanol/water mixture where the β -isomer predominated. He also stated that their proportions depend both upon the solvent and upon the method of mixing the reactants, aqueous-organic solvents, and gradual addition of peroxy-acid to the steroid, favoring the synthesis of the 6 β -hydroxy-compound. To the best of our knowledge, this is a new synthesis of stigma-4,22-dien-6 β -ol-3-one that can be applied to steroids having an analogous nucleus. This synthesis allowed us to confirm the structure of a minor compound.

6.3 Conclusions

Being predominantly a waste product, wheat straw proved to be an interesting and unexplored source for chemical structures or models. We have explored the triterpenoid and phenolic composition since these compounds appear to have a role in plant defense as signal transducers and play a regulatory role in interactions of plants with their biotic environment. Baas⁵⁰ commented that “qualitative compounds or toxins” are structures that are biologically active at much lower concentrations, many of them even at hormone levels and usually they are oxidized compounds. This is in good agreement with our experimental data.

Utilizing this naturally occurring chemical warfare among plants, wheat straw and other cover crops could play a more important role in controlling weeds in crops in the future. In some cases, it is possible that herbicide use may be reduced by partial substitution of the naturally occurring phytotoxic chemicals in mulches, the latter possessing known ecological and economical advantages. We believe that many no-till farmers are unconsciously receiving the benefits of allelopathy when they plant crops no-till into straw and certain cover crops. If ethnopharmacology and its cultural heritage can contribute to the development of clinical drugs, so in a parallel way, popular agricultural practices may lead to the discovery of new natural agrochemicals.

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Glucosinolates As Natural Pesticides

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ABSTRACT Glucosinolates are a group of secondary compounds produced by members of the Brassicaceae (the crucifer or mustard family) and at least 10 other dicotyledonous plant families. Glucosinolates are enzymatically hydrolyzed by thioglucosidase glucosylglucanase to form substituted isothiocyanates, nitriles, and thiocyanates. Glucosinolates and their hydrolysis products have long been of pharmacological and toxicological interest and are primarily responsible for the characteristic odors and flavors of cruciferous plants. Due to the potential loss of soil fumigants such as methyl bromide, the use of glucosinolate-containing plants as green manures or soil amendments to suppress weeds and soilborne pathogens is currently an area of active research. This chapter will focus on the pesticidal activity of glucosinolates and their degradation products.

7.1 Chemistry and Occurrence

Glucosinolates are a group of plant secondary compounds whose biologically active hydrolysis products are produced when cells containing them are ruptured and the glucosinolates (contained in vacuoles) are hydrolyzed by the enzyme myrosinase (α -thioglucosidase glucosylglucanase; EC 3.2.3.1) which is stored away from the vacuole in the cell walls, endoplasmic reticulum, Golgi vesicles, and mitochondria (Figure 7.1).¹⁻⁴ These products include substituted isothiocyanates, nitriles, thiocyanates, and oxazolidinethiones. Which

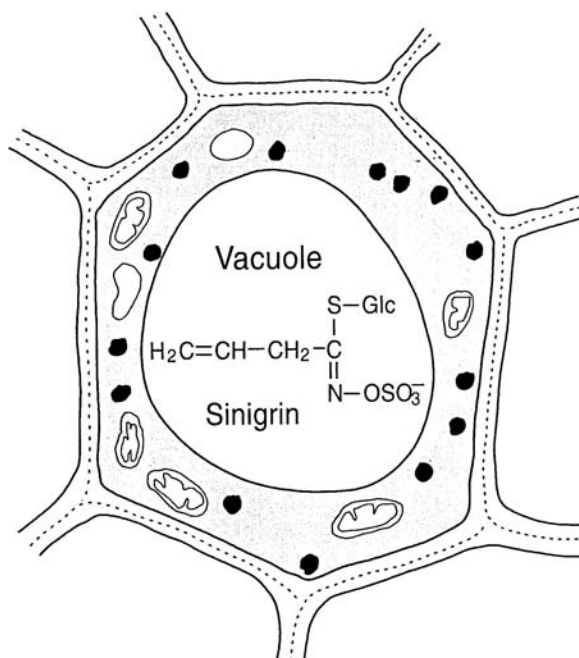


FIGURE 7.1

Localization of glucosinolates (e.g., sinigrin) and myrosinase (●) in horseradish root cells.²

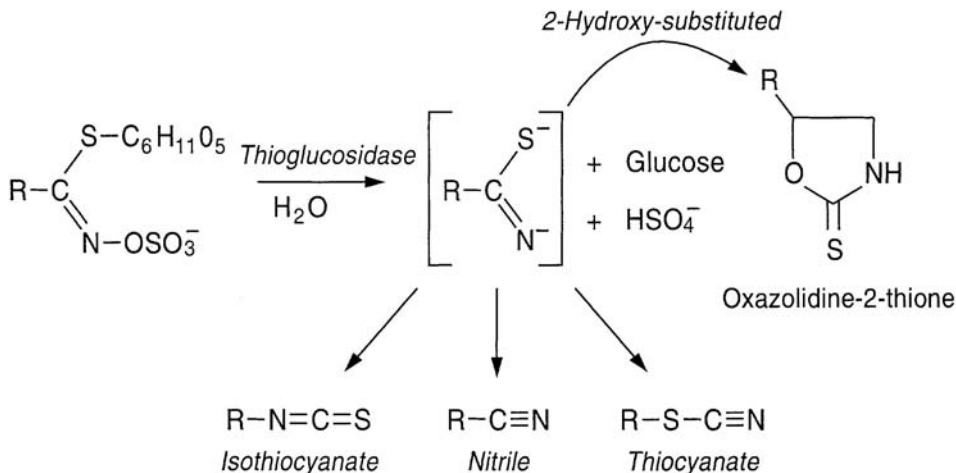


FIGURE 7.2

Major enzymatic hydrolysis products of glucosinolates.³

types are formed depend on several factors, including the side-chain substitution, pH, and iron concentration (Figure 7.2).⁵⁻¹⁰ Roughly 100 distinct glucosinolates have been identified in plants from at least 11 different plant families, principally in the order Capparales and the families Brassicaceae, Capparidaceae, and Resedaceae.¹¹ As many as 15 different glucosinolates have been identified in a single species, and concentrations of individual glucosinolates vary within different organs of the same plant and within populations of the same species.¹² Glucosinolates are biosynthesized from both protein and nonprotein amino

TABLE 7.1
Common Glucosinolates

R-Group	Trivial Name
Methyl	Glucocapparin
Allyl (2-Propenyl)	Sinigrin
3-butenyl	Gluconapin
2-hydroxy-3-butenyl	Progoitrin
4-pentenyl	Glucobrassicinapin
Benzyl	Glucotropaeolin
2-phenylethyl	Gluconasturtiin
4-hydroxybenzyl	Sinibin
3-methoxybenzyl	Glucolimnanthin
4-methylthiobutyl	Glucoerucin
4-methylsulfanylbutyl	Sulforaphene
Indol-3-ylmethyl	Glucobrassicin
1-methoxyindol-3-ylmethyl	Neoglucobrassicin

acid precursors, although the number of amino acids thus far identified as precursors is relatively small. Most aliphatic glucosinolates studied appear to have methionine as a precursor, while indole glucosinolates are synthesized from tryptophan, and aromatic glucosinolates from either phenylalanine or tyrosine.¹³⁻¹⁵ Several excellent reviews discuss in greater detail glucosinolate chemistry, biosynthesis, and toxicology.^{3,4,11,12,16} Chemical structures of some of the glucosinolates discussed in this chapter are presented in Table 7.1.

The Brassicaceae (the crucifer or mustard family) includes important food crops, ornamentals, and weeds, and it is the plant family generally associated with the presence of glucosinolates.¹⁷ Because viable alternatives to the use of synthetic chemical pesticides are needed for organic farming operations and for situations where public policies mandate reduced pesticide use, a significant amount of research has been conducted on the use of crucifers as green manures or soil amendments for the suppression of weeds, soil pathogens, nematodes, and insects. Alternatives to the use of methyl bromide as a fumigant also are needed, as industrialized nations face a phaseout of the compound by the year 2005. Identifying a cheap chemical fumigant that is as effective as methyl bromide may prove difficult. Organic soil amendments/green manures, possibly in combination with chemicals, may be good candidates as replacements for methyl bromide as a fumigant for the control of soilborne pathogens and weeds. Crucifers that have been examined for their ability to suppress pests, including cabbage (*Brassica oleracea* L. var. *capitata*), kale (*B. oleracea* var. *acephala*), brown or Indian mustard (*B. juncea*), white mustard (*B. hirta*), black mustard (*B. nigra*), charlock (*B. kaber*), and rapeseed (*B. napus*).^{18,19}

7.2 Pesticidal Activity

The pesticidal activity of crucifers is thought to be primarily due to glucosinolate degradation products, as intact glucosinolates generally exhibit much less biological activity than their corresponding hydrolysis products.^{20,21} Crucifers generally contain several different glucosinolates, and there are both qualitative and quantitative differences between tissue types.^{16,17} Volatile hydrolysis compounds produced from macerated/decaying tissues also are variable.^{22,23} Several of the volatile isothiocyanates, including allyl isothiocyanate (AITC) and benzyl isothiocyanate (BITC), have been shown to be toxic to a wide range of organisms at low levels.²³⁻²⁹ The toxicity of these compounds is probably due to several mechanisms.

Isothiocyanates are potent electrophiles which readily react with free amino groups of amino acids and proteins to form thiourea derivatives. In fact, the Edman degradation method for determining the amino acid sequences of proteins is based on the reaction of phenyl isothiocyanate with the α -amino group of the terminal amino acid.^{30,31} AITC has been shown to break the disulfide bond of cystine in proteins and glutathione through oxidative cleavage/scission.³²⁻³⁴ While much less studied, thiocyanates and nitriles also appear to contribute, at least for certain glucosinolates, to their toxicity. Thiocyanate ions have been shown to be toxic to a wide range of plants.³⁵⁻⁴³ Indolymethyl glucosinolates such as glucobrassicin form unstable indolymethyl isothiocyanates at normal cell pH ranges (4 to 7) which then decompose to form thiocyanate ion and indolymethanol. Under more acidic conditions (pH < 4) the formation of nitriles is favored.^{3,12} 2-hydroxy-substituted glucosinolates initially form an unstable isothiocyanate which cyclizes to form an oxazolidine-2-thione, although these glucosinolates may also form nitriles and epithionitriles.³

7.3 Stimulation/Repression of Glucosinolate Levels

Glucosinolate levels have been shown to vary due to both physical damage and attack by pathogens. Glucobrassicin levels in *B. rapa* roots and hypocotyls more than doubled over a period of 30 to 60 days after inoculation with the club root fungus *Plasmodiophora brassicae*, although levels of the chemically similar neoglucobrassicin decreased slightly over the same time period.⁴⁴ The concentration of progoitrin was higher in rutabaga (*B. napus* subsp. *napobrassica*) plants inoculated with turnip mosaic virus as compared to uninoculated plants.⁴⁵ Infestation by the cabbage stem beetle (*Psylliodes chrysocephala*) of *B. napus* tissues led to large increases of indole glucosinolates (including glucobrassicin, neoglucobrassicin, and 4-methoxyglucobrassicin) and concurrent reductions in aliphatic glucosinolates (progoitrin, gluconapin, glucobrassicinapin), while mechanical damage caused similar, although lower, changes in glucosinolate levels.⁴⁶ In a later paper by these researchers, mechanical wounding of rapeseed petioles was found to increase indole glucosinolate levels while lowering levels of aliphatic glucosinolates.⁴⁷ However, kale, black mustard, brown mustard, and white mustard responded quite differently than rapeseed. Wounded kale plants had higher levels of both indole and aliphatic glucosinolates, while total glucosinolate content in the mustards increased following wounding, but not levels of indole glucosinolates (mustards normally contain only trace levels of these compounds). Aliphatic glucosinolate levels increased in wounded black and brown mustard plants while aromatic glucosinolate levels increased in wounded white mustard plants. Rapeseed roots attacked by turnip root fly (*Delia floralis*) larvae also had higher indole and lower aliphatic glucosinolate content than control roots.⁴⁸ Total glucosinolates in roots of kale, rape, and rutabaga were higher after attack by *D. floralis* larvae, mainly due to a two-to-four fold increase in indole glucosinolates.⁴⁹ Further research by this group revealed that while *D. floralis* larval attack increased both total and indole glucosinolate levels in rapeseed, mechanical wounding (by cutting roots with scissors) resulted in a decrease in total glucosinolate content.⁵⁰ Both mechanical wounding or feeding by the flea beetle *Phyllotreta cruciferae* increased indole glucosinolates in rapeseed and brown mustard cotyledons without a concurrent increase in either aliphatic or aromatic glucosinolates.⁵¹ Grazing by rabbits (*Oryctolagus cuniculus*) increased total glucosinolate levels of rapeseed, although the proportion of aliphatic glucosinolates decreased in relation to indole and 2-phenylethyl glucosinolates.⁵²

7.4 Toxicity to Insects and Nematodes

Ecological studies support the hypothesis that the net effect of higher glucosinolate levels, especially of mixtures of glucosinolates, is defensive (i.e., higher glucosinolates = lower herbivory).⁵³ As previously mentioned, most of the activity associated with glucosinolates has been found to be caused by degradation products rather than the glucosinolates themselves. Ellenby⁵⁴ found that an extract from black mustard (which contained AITC) as well as pure AITC were toxic to the potato cyst nematode *Heterodera rostochiensis*. 2-phenylethyl isothiocyanate (PEITC) isolated from turnip roots was found to be a “natural” insecticide.⁵⁵ AITC was the most toxic compound studied of several glucosinolates and their degradation products to the nematode *Heterodera schachtii*, although the parent glucosinolate (sinigrin) had no activity.²⁷ Sinigrin was acutely toxic to eastern black swallowtail (*Papilio polyxenes*) larvae who feed primarily on plants of the carrot family (Apiaceae), while larvae of the cabbage butterfly (*Artogeia rapae*), a specialist crucifer pest, were unaffected by sinigrin levels much higher than found in nature.⁵⁶ Milled seed of meadowfoam (*Limnanthes alba*) added to the diet of fall armyworms at 3% wet weight resulted in 100% mortality within 8 days, with 3-methoxybenzyl isothiocyanate being identified as the primary toxin.⁵⁷ AITC was both a toxin and an antifeedant for *Limoniuss californicus* wireworms,²⁹ and was over three orders of magnitude more toxic to the nematode *Caenorhabditis elegans* than sinigrin.²⁰ BITC released by wounded green papaya fruit resulted in the mortality of both eggs and first instars of several insect pests of papaya.⁵⁸ High concentrations (up to 20 mM) of sinalbin in cotyledons and young leaves of *B. hirta* deterred feeding by both a specialized crucifer pest, the flea beetle (*P. cruciferae*), and a generalist pest, the Bertha armyworm (*Mamestra configurata*).⁵⁹ The feeding preferences of *M. configurata* neonate larvae were correlated to isothiocyanate-releasing glucosinolates, but not correlated to total glucosinolate levels.⁶⁰

7.5 Antimicrobial Activity

Walker et al.⁶¹ reported in 1937 that “mustard oils” (volatile sulfur-containing compounds including several volatile isothiocyanates) were toxic to pathogenic fungi, with AITC being the most potent compound tested. AITC also was the volatile isothiocyanate which was the most toxic to several pathogenic fungi.^{62,63} Foter and Golick⁶⁴ found that volatiles produced by crushed horseradish roots inhibited the growth of several bacteria, including the human intestinal bacteria *Escherichia coli* and *Mycobacterium tuberculosis* var. *hominis*, the causal agent of human tuberculosis. AITC, which is the major volatile released by crushed horseradish roots, was also toxic to the same group of bacteria when applied alone as a volatile.⁶⁵ Virtanen studied the antimicrobial activity of various isothiocyanates, thiocyanates, and other sulfur compounds, with BITC being the most active compound tested against *Staphylococcus aureus* and *Penicillium glaucum*.²⁴

In a study of chemical derivatives of BITC, there was an inverse correlation between water solubility and antifungal activity.^{66,67} Sinigrin had little or no effect on *Sclerotinia sclerotiorum* (stem rot), *Alternaria brassicae* (black spot), and *Phoma lingam* (black leg), important fungal diseases of rapeseed in Europe.⁶⁸ The minimum lethal concentration (MLC) of AITC to the yeast *Nematospora sinecauda* was 20 to 35 µg/mL, while the MLC for sinigrin was

found to be >1000 µg/mL.⁶⁹ AITC was the most inhibitory compound identified for the growth and zoospore germination of the pea root rot fungus *Aphanomyces euteiches*.⁷⁰ AITC was also the most potent glucosinolate degradation to several postharvest fruit pathogenic fungi.⁷¹

Mayton et al.⁷² determined that the release of AITC by green manure crops was the critical factor in the inhibition of growth of the potato tuber dry rot fungus *Fusarium sambucinum*, similar to the results found by Vaughn et al.²⁸ against the potato tuber silver scurf pathogen, *Helminthosporium solani*. Isothiocyanate levels (primarily allyl and phenyl) generated by decaying cabbage tissues in solarized soil were significantly correlated with a reduction in propagule numbers of *Pythium ultimum* and *Sclerotium rolfsii*.⁷³ 4-methylthio-3-butenyl isothiocyanate (MBTI), one of the pungent odor compounds released by radish (*Raphanus sativus*) tissues, strongly inhibited the growth of bacteria (both Gram positive and Gram negative), fungi, and yeasts when the microbes were exposed to the compound as a volatile.⁷⁴ A further study by these researchers uncovered a water-soluble antimicrobial compound, 2-thioxo-3-pyrrolidinecarbaldehyde, produced by the degradation of MBTI.⁷⁵ Practical use of these volatile isothiocyanates also has been studied. AITC added to apple juice, grape juice, and fresh cider at concentrations of 20 ppm inhibited several acid food spoilage microorganisms.^{76,77} BITC which is produced naturally by papaya fruit was used as postharvest treatment to prevent stem-end rot of papaya caused by *Rhizopus stolonifer*.⁷⁸ Interestingly, BITC treatment also caused the treated fruit to remain significantly greener than control fruit, which the authors hypothesized was due to inhibition of ethylene evolution. Steam-distilled AITC obtained from *B. juncea* seeds was investigated as a potential preservative for modified atmosphere packaging.⁷⁹ The minimum inhibitory dose of AITC for bacteria was generally higher than for yeasts or fungi. Several types of food in sealed plastic bags were kept unspoiled after 7 days, and while the authors stated that the packaged food samples had no AITC odor, they failed to mention how the food tasted. AITC also has been recently examined as a potential fumigant to prevent internal decay of wooden telephone poles.⁸⁰

7.6 Phytotoxicity

The decaying residue of crucifers has long been known to inhibit the germination of successive crops, especially small-seeded species.⁸¹ This inhibition may be caused by the release of volatile (mostly water-insoluble) or nonvolatile, water-soluble glucosinolate degradation products. Volatiles released by chopped tissues of *B. nigra* and *B. juncea* were much more phytotoxic to lettuce (*Lactuca sativa*) and barnyardgrass (*Echinochloa crus-galli*) seed germination and growth than volatiles from several other *Brassica* species, including *B. napus* and *B. hirta*.⁸² The primary volatile produced by both *B. nigra* and *B. juncea* is AITC, while *B. napus* and *B. hirta* primarily release other volatiles.^{22,23} PEITC and AITC were the most inhibitory to wheat germination and seedling growth, with other isothiocyanates (methyl, ethyl, butyl, benzyl, and phenyl) less active and the parent glucosinolates having little or no activity.²⁶ AITC was as inhibitory as the commercial fumigant methyl isothiocyanate (which also is formed in nature by the hydrolysis of glucocapparin), both of which were highly toxic as volatiles to several crop and weed species.²³ AITC, the major volatile produced by macerated *B. nigra* leaves, suppressed the seed germination and growth of annual grasses in a laboratory test.⁸³ BITC extracted from papaya (*Carica papaya*) seeds inhibited velvetleaf (*Abutilon theophrasti*) germination and seedling growth,²⁵ and was later investigated as a potential granular herbicide.⁸⁴

Although volatile isothiocyanates have generally been found to be the most toxic to a wide range of organisms, nonvolatile glucosinolate hydrolysis products also have been found to be active. Hirsutin (8-methylsulfinyl-octyl isothiocyanate), the major degradation product of the glucosinolate glucohirsutin, was identified as a potent phytotoxin released by the roots of yellow fieldcress (*Rorippa sylvestris*), one of the worst weeds of wet fields and pastures in Japan.⁸⁵⁻⁸⁷ In addition to hirsutin, Yamane et al.⁸⁷ also identified several other hydrolysis products released by *R. sylvestris* roots, including arabin (9-methylsulfinyl-nonyl isothiocyanate), camelinin (10-methylsulfinyl-decyl isothiocyanate), 8-methylsulfonyl-octyl isothiocyanate, and 9-methylsulfonyl-nonyl isothiocyanate that inhibited lettuce seed germination. Interestingly, the chemically similar 10-methylsulfonyl-decyl isothiocyanate had no effect on lettuce seed germination. Stock (*Matthiola incana*), an ornamental crucifer which had been reported anecdotally to be allelopathic, was found to contain as a phyto-toxin sulforaphene (4-methylsulfinyl-3-butenyl isothiocyanate).⁸⁸ Sulforaphene also was considerably more active than its parent glucosinolate, glucoraphenin.

7.7 Glucosinolate-Containing Plants as Pest-Suppressive Crops

Seminal work by Papavizas^{89,90} and Papavizas and Lewis,⁹¹ and recent research by Williams-Woodward and co-workers⁹² found effective suppression of the pea root rot pathogen *Aphanomyces euteiches* through the use of crucifer plant tissues as soil amendments. White mustard grown as a fall crop significantly decreased root rot severity ratings of peas grown the following year.⁹³ Soil populations of *Fusarium oxysporum* f. sp. *conglutinans* were markedly reduced by leaf and stem tissues of cabbage, kale, and black mustard.^{94,95} Soil incorporation of rapeseed tissues lowered population densities of the Columbia root-knot nematode *Meloidogyne chitwoodi*.^{96,97} Incorporated rapeseed residues suppressed several cereal root fungi but also injured the cereal crops.⁹⁸ Fall-planted *B. napus* greatly reduced weed biomass when incorporated as a green manure as compared to both sudangrass (*Sorghum sudanense*) green manure and fallow treatments.⁹⁹ Fall-planted/spring-incorporated *B. hirta* and *B. napus* green manures suppressed early (30 days after planting) weed emergence relative to a wheat green manure in green peas, although by harvest weed densities were similar in all green manure treatments.¹⁰⁰

7.8 Glucosinolate-Containing Seedmeals as Soil Amendments

Seedmeals from glucosinolate-containing plants may be useful as soil amendments for pest control.¹⁰¹⁻¹⁰⁶ Crambe (*Crambe abyssinica*) and meadowfoam are industrial crops grown primarily in North Dakota and Oregon, respectively, for their seed oils which are excellent sources of fatty acids for industrial uses.^{107,108} The residual seedmeals remaining after oil extraction would be expected to contain glucosinolates and/or their degradation products. Research by Walker¹⁰⁴ indicated that crambe meal was phytotoxic to tomato (*Lycopersicon esculentum*) and several other annual plants, while also suppressing plant parasitic nematodes. Tsao et al.¹⁰³ found that crambe seedmeal extracts were toxic to several important agricultural and public health insect pests, including mosquito (*Aedes aegypti*), house fly (*Musca domestica*), and western corn rootworm (*Diabrotica virgifera virgifera*) larvae. However, in both cases the active compound(s) were not identified. Research in the author's lab

has dealt with the possibility of using these seedmeals as herbicide alternatives. In both cases one major phytotoxin was identified: 1-cyano-2-hydroxy-3-butene for crambe seedmeal¹⁰⁵ and (3-methoxyphenyl)acetonitrile for meadowfoam seedmeal.¹⁰⁶ Further research is currently being conducted to determine the practical utilization of these seed-meals as soil amendments.

7.9 Involvement in Allelopathy

Glucosinolates and their breakdown products have been implicated in allelopathy. Bell and Muller⁸³ reported that black mustard formed pure stands on the slopes of annual grasslands in coastal California. Their studies suggested that allelopathy was involved in the establishment of these pure stands. While volatile toxins did not appear to be responsible for the observed effects, the greater invasion by mustard plants downhill of established mustard stands (and lowered numbers of other plants) led them to suspect the release of water-soluble toxins. Field plots containing broken mustard stalks had reduced stands of both annual grasses and mustard seedlings after an initial rainfall. Extracts from dead mustard stalks and leaves were very inhibitory to the root growth of several annual grasses, but the active components were not identified. Garlic mustard (*Alliaria petiolata*) is a herbaceous biennial member of the Brassicaceae which has invaded, and in some areas dominates, a large part of the hardwood forest understory in the eastern U.S. and Canada.¹⁰⁹ Populations of native plants such as cut-leaved toothwort (*Dentaria laciniata*) have declined in areas with extensive stands of garlic mustard, as well as several insect species which feed on these native plants.^{110,111} Decaying and/or crushed garlic mustard leaves emit high levels of AITC, while roots release primarily BITC (S. F. Vaughn, unpublished data). BITC could also be detected by solid phase microextraction from soil from areas heavily infested with garlic mustard. As previously noted, these isothiocyanates are potent inhibitors of seed germination and plant growth, and may be crucial in the dominance of garlic mustard in these forest ecosystems. Choesin and Boerner,¹¹² studying the possible allelopathic nature of rapeseed systems, concluded that the release of AITC by these plants was insufficient to inhibit neighboring plants. However, the major glucosinolates found in rapeseed are progoitrin, gluconapin, and glucobrassicinapin, with only trace amounts of sinigrin detected.^{102,113} Additionally, some of the most inhibitory hydrolysis products, such as AITC and BITC, are released only at very low levels by decaying rapeseed tissues, and may account for these results.^{22,23} Tall whitetop or perennial pepperweed (*Lepidium latifolium*) and dyer's woad (*Isatis tinctoria*), both members of the Brassicaceae, are problem invasive weeds in the intermontane regions of the western U.S. and exhibit allelopathic effects against neighboring plants.^{114,115} Dyer's woad has a potent water-soluble inhibitor that leaches from its winged fruits (silicles) that inhibits neighboring plants.¹¹⁶ Research is currently being conducted in the author's lab concerning the chemistry of these toxins.

7.10 Conclusions

As is evident from the amount of research conducted on the practical utilization of glucosinolate-containing plants for weed, disease, insect, and nematode control, additional research needs to be conducted, especially on the breeding of cold-hardy, high-glucosinolate

species that can be selected for use as cover/green manure crops in various crop production systems. Currently, there are several interdisciplinary research groups, including the author's, that are conducting research on this topic in the U.S.

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Coronatine: Chemistry and Biological Activities

Akitami Ichihara and Hiroaki Toshima

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8.1 Introduction

The phytotoxin, coronatine (**1**), was isolated from the phytopathogenic bacterium *Pseudomonas syringae* pv. *atropurpurea* which causes chocolate spot disease on the leaves of Italian ryegrass.¹ After structural determination of **1**, it was found that several pathovars of *Pseudomonas syringae* and *Xanthomonas campestris* pv. *phormiicola* produce **1** as a phytotoxic compound. Closely related analogues of **1** were isolated from *Pseudomonas syringae* pv. *glycinea*, pv. *tomato*, and *Xanthomonas campestris* pv. *phormiicola*. Recently, there has been much interest in **1**, since various biological activities similar to those of jasmonic acid were shown in assays for tuber-inducing activity, etc. Furthermore, the potent activities of **1** are 100 to 10,000 times higher than those of jasmonic acid. Therefore, **1** is the most valuable probe in the area of plant physiological studies relating to jasmonic acid. In order to supply a sufficient amount of **1**, an efficient synthetic method is urgently needed, since the productivity of **1** by *P. syringae* pathovars is rather fluctuated depending on the culture conditions and/or mutation. The total synthesis of optically active coronatine (**1**) was accomplished and made it possible to supply a practical amount of (+)-**1**. Analogues of **1** and jasmonic acid, and the compounds related to biosynthetic intermediates and amino acid conjugates of jasmonic acid were also synthesized and their biological activities were evaluated in some bioassay systems. In addition to the hormonal activities of coronatine, the similarity

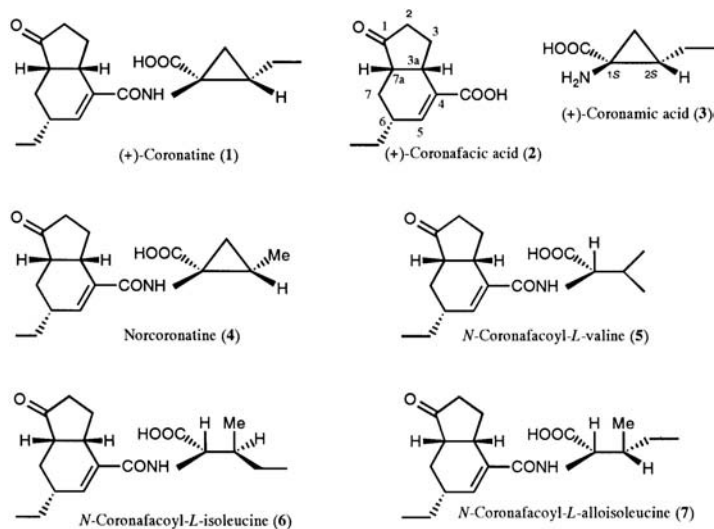


FIGURE 8.1

Structures of (+)-coronatine and the related compounds.

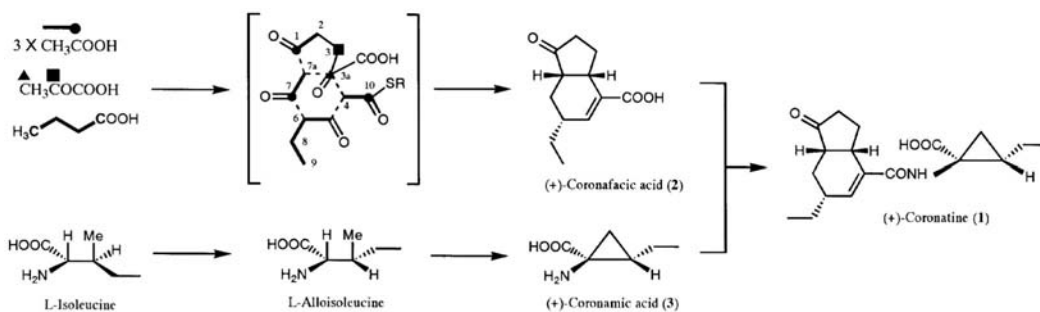
of the structure of coronamic acid with that of 1-amino-1-cyclopropanecarboxylic acid (ACC), the direct precursor of the plant hormone ethylene, made it possible to develop inhibitors of ethylene biosynthesis.

8.2 Structures of Coronatine and Analogs

The structure and stereochemistry of coronatine (1) were determined by spectroscopic methods and x-ray analyses of coronafacic acid (2) and (–)-*N*-acetylcoronamic acid.^{2–4} The structure of coronatine (1) is quite unique in that it consists of coronafacic acid (2) and coronamic acid (3) (Figure 8.1). After the discovery of 1, it was found that several *P. syringae* pathovars (pv. *maculicola*,⁵ pv. *glycinea*,⁶ pv. *morsprunorum*⁷ and pv. *tomato*⁸) produce 1. Besides coronatine, *Pseudomonas syringae* pv. *glycinea* produces several coronatine analogues, norcoronatine (4), *N*-coronafacoyl-L-valine (5), *N*-coronafacoyl-L-isoleucine (6), and *N*-coronafacoyl-L-alloisoleucine (7) which are all biologically active.^{9,10} The absolute configuration of norcoronatine (4) was confirmed by the comparison of physicochemical data with those of natural and synthetic specimens.¹¹ Furthermore, *Pseudomonas syringae* pv. *tomato* produces *N*-coronafacoyl-L-valine (5)¹² and *Xanthomonas campestris* pv. *phormiicola* produces *N*-coronafacoyl-L-valine (5) and *N*-coronafacoyl-L-isoleucine produces (6).¹³ It also was proved that *Xanthomonas campestris* pv. *phormiicola* produces coronatine (1) itself.⁴

8.3 Biosynthesis of Coronatine

The two components of coronatine (1) are biosynthesized from two different pathways respectively (Scheme 8.1). Coronafacic acid (2) was shown to be a novel polyketide derived

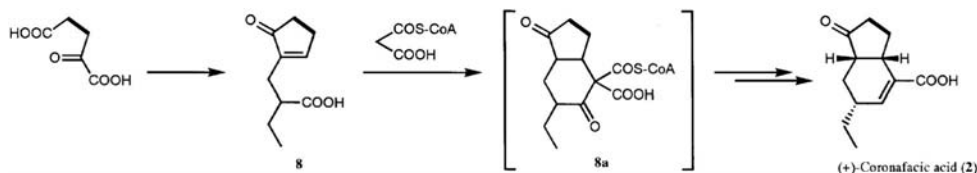


SCHEME 8.1

Biosynthesis of (+)-coronatine.

from three acetate units, one butyrate unit, and one unit of pyruvate.¹⁵ Recently, it was found that the labeled pyruvate was carboxylated to oxaloacetate which was further converted into α -ketoglutarate through the TCA cycle. Further support of the intermediacy of α -ketoglutarate was provided by incorporation experiments of a labeled precursor, [3,4- $^{13}\text{C}_2$]-DL-glutamate to give a labeled **2** at C-2 and C-3. Interestingly, Mitchell et al.¹⁶ have described the isolation of the cyclopentenone derivative **8** from *Pseudomonas syringae* pv. *tomato* which is able to produce coronatine as a major component. Feeding experiments of the [3,4- $^{13}\text{C}_2$]-DL-glutamate into this bacterium gave a labeled cyclopentenone **8** which exhibits ^{13}C - ^{13}C coupling between C-2 and C-3 with an enrichment at each carbon of ca. 0.89%. The coupling pattern is completely consistent with that observed in coronafacic acid (**2**). The results also are compatible with the previous biosynthetic studies of coronafacic acid, and the cyclopentenone **8** would be a plausible intermediate that would be transformed by Michael's addition of malonyl CoA to an indanone derivative **8a** and successive decarboxylation, reduction of the C-5 carbonyl, and dehydration to give coronafacic acid (**2**) (Scheme 8.2).

The biosynthetic studies of coronamic acid (**3**) showed that both L-isoleucine and L-alloisoleucine are specifically incorporated into the amino acid (**3**), the latter L-alloisoleucine is a much more efficient precursor. Since the absolute configuration of coronamic acid (**3**) is consistent with that of L-alloisoleucine, the incorporation efficiency is easily understandable, though the cyclization mechanism has been completely elucidated.¹⁷ Previously, coronatine analogues, *N*-coronafacoyl-L-isoleucine (**6**) and *N*-coronafacoyl-L-alloisoleucine, were regarded as promising intermediates in the biosynthesis of coronatine (**1**). However, this is not the case. Since biosynthetic condensation of coronafacic acid (**2**) and coronamic acid (**3**) has been made by the incorporation of ^{14}C -coronamic acid (**3**) into coronatine (**1**) in *Pseudomonas syringae* pv. *glycinea* PG 4180.¹⁸ Extensive genetic studies have been done with the same strain and, using genetic complementation, substrate feeding, and co-culturing experiments, regions involved in the biosynthesis of major intermediates, coronafacic acid (**2**) and coronamic acid (**3**) and the ligase of these components were identified in the coronatine production gene cluster.¹⁹



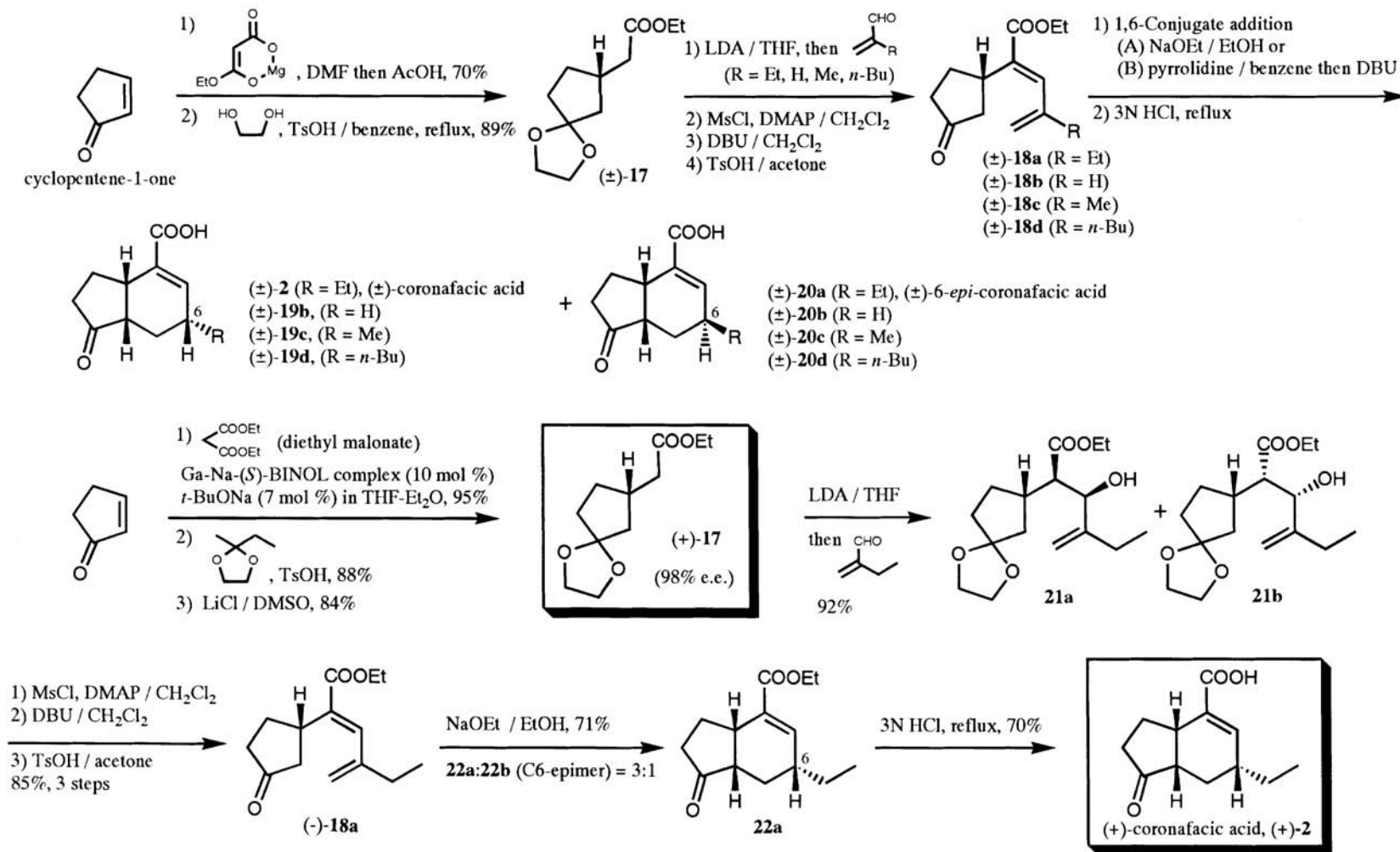
SCHEME 8.2

Plausible biosynthesis of (+)-coronafacic acid.

removal of the Boc group under usual acidic conditions. Furthermore, esterification of **12** with diazomethane and subsequent hydrogenolysis gave another half-acid **14** which could be converted to (+)-allocoronamic acid [(+)-**16**]. Antipodes, (–)-coronamic acid [(–)-**3**] and (–)-allocoronamic acid [(–)-**16**], were synthesized starting from (S)-malic acid. In this way, we could develop a practical route for all four stereoisomers of coronamic acid in high chemical yields and high optical purities without the optical resolution step. Several grams of the four isomers were synthesized by our new route.

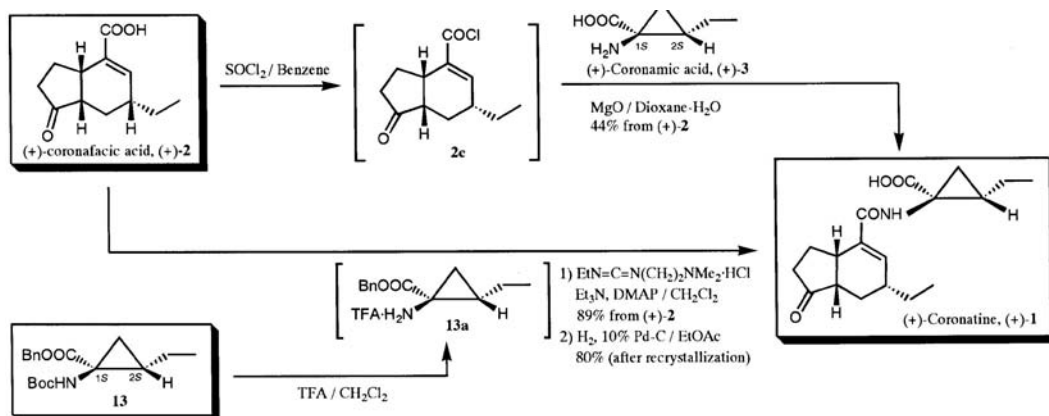
We have already developed a new approach using intramolecular 1,6-conjugate addition for construction of a 1-hydrindane framework such as **2** on a racemic substrate (Scheme 8.4).²¹ By this methodology, the C₆-non, C₆-methyl, and C₆-butyl substituted analogs [(±)-**19b**, **-19c**, **-19d**, **-20a**, **-20b**, **-20c**, and **-20d**] of **2**, could be synthesized from (±)-**18a-d** (Scheme 8.4).²² In principle, this method would be applied to synthesize the optically active form.^{23,24} For our synthesis of (+)-**2**, the efficient preparation of the optically active ester [(+)-**17**] is required. This problem was solved using the catalytic asymmetric Michael reaction promoted by the heterobimetallic BINOL complex developed by Shibasaki and co-workers.²⁵ Michael reaction between 2-cyclopenten-1-one and diethyl malonate was carried out in the presence of Ga-Na-(S)-BINOL complex (10 mol%) and sodium *tert*-butoxide (7 mol%) to give a keto-diester in 95% yield. Acetalization and subsequent decarboxylation gave desired the ester (+)-**17** in 74% yield. The optical purity of (+)-**17** was determined as 95% *e.e.* by an HPLC analysis using a chiral column which was sufficiently pure for subsequent reactions. The total synthesis of (+)-**2** was carried out according to the synthetic route of racemic **2**. Aldol condensation between the lithium enolate of (+)-**17** and 2-ethylacrolein proceeded stereospecifically in the newly formed stereogenic centers to give a ca. 1:1 mixture of **21a** and **21b** which possesses a *syn*-relationship between the ethoxycarbonyl and hydroxyl groups. Mesylation of the mixture of **21a** and **21b** and subsequent β-elimination with DBU gave α, β, δ, γ-unsaturated ester possessing (*E*)-geometry as the sole product. Further deacetalization with *p*-TsOH in acetone gave (–)-**18a**, the precursor of intramolecular 1,6-conjugate addition in 78% yield [four steps from (+)-**17**]. Treatment of (–)-**18a** with NaOEt (3 eq.) in EtOH gave **22a** corresponding to the ethyl ester of coronafacic acid and its C₆-epimer **22b** in 71% yield (diastereoselectivity; ca. 3:1). Isomerization of **22b** with DBA also was possible to give a 1:1 mixture of **22a** and **22b**. The optical purity of **22a** was determined as 98% *e.e.* by an HPLC analysis using a chiral column. This result demonstrates that the optical purity of (+)-**17** was completely retained in **22a** without any loss through the sequence of reactions including intramolecular 1,6-conjugate addition. The effectiveness of our new approach, on both racemic and optically active forms, was proven. Acidic hydrolysis of **22a** gave (+)-**2** in 70% yield after recrystallization; mp 142–143°C; [α]_D²³ +122° (c 1.00, MeOH) [lit. mp 141–142°C; [α]_D²⁰ +119° (c 3.30, MeOH)], whose spectral data were identical with those of natural **2** in all respects. The overall yield via our new route was 24% in nine steps from 2-cyclopenten-1-one. The optical purity of (+)-**2** is estimated to be at least >98% *e.e.* based on 98% *e.e.* of **22a**. In practice, judging from the specific rotation, (+)-**2** can be regarded as optically pure.

After deprotection of the Boc group of **13** with TFA, the resulting amine TFA salt **13a** (evaporated to dryness and used without purification) was coupled with (+)-**2** in the presence of a water-soluble carbodiimide to give coronatine benzyl ester (**1a**) in 89% yield (Scheme 8.5). In a preliminary experiment, coupling between racemic **2** and optically active **13a** gave a mixture of **1a** and its diastereomer (inseparable on TLC), whose ¹H-NMR spectrum gave partially separated signals based on the respective diastereomers (ratio ca. 1:1). However, in the case of coupling (+)-**2**, the ¹H-NMR spectrum of **1a** was observed as the single diastereomer. This result means that the synthetic (+)-**2** has practical enantiomeric purity and **1a** can be regarded as optically pure. Deprotection of **1a** by hydrogenolysis in the presence of 10% Pd-C in ethyl acetate provided (+)-**1** in 80% yield; mp 162–164°C [α]_D²² +76.6°



SCHEME 8.4

Syntheses of coronafac acid and its analogs.

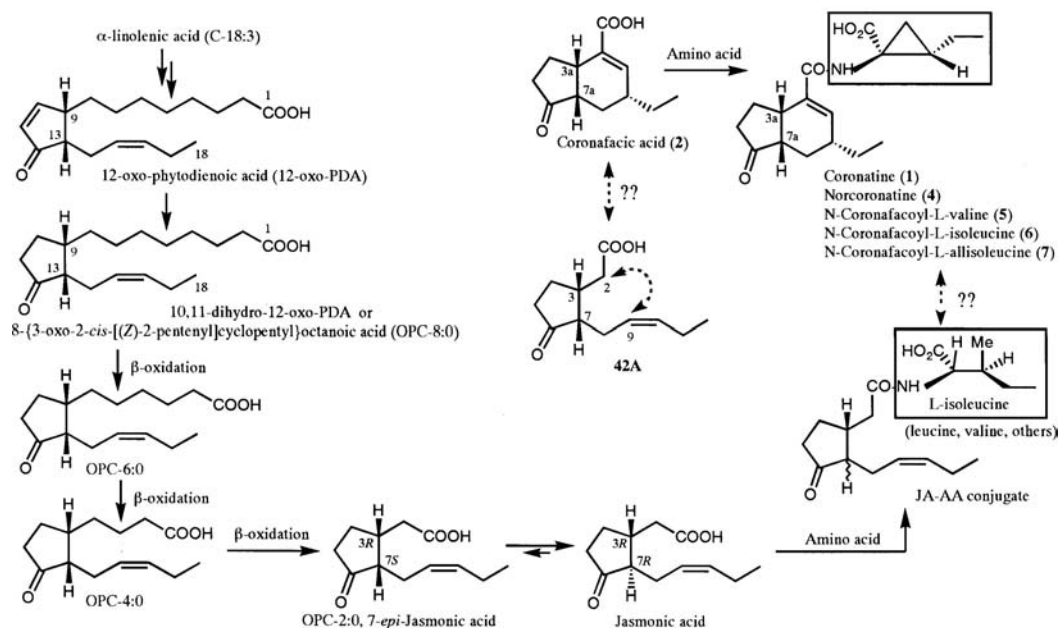


SCHEME 8.5
Total synthesis of coronatine.

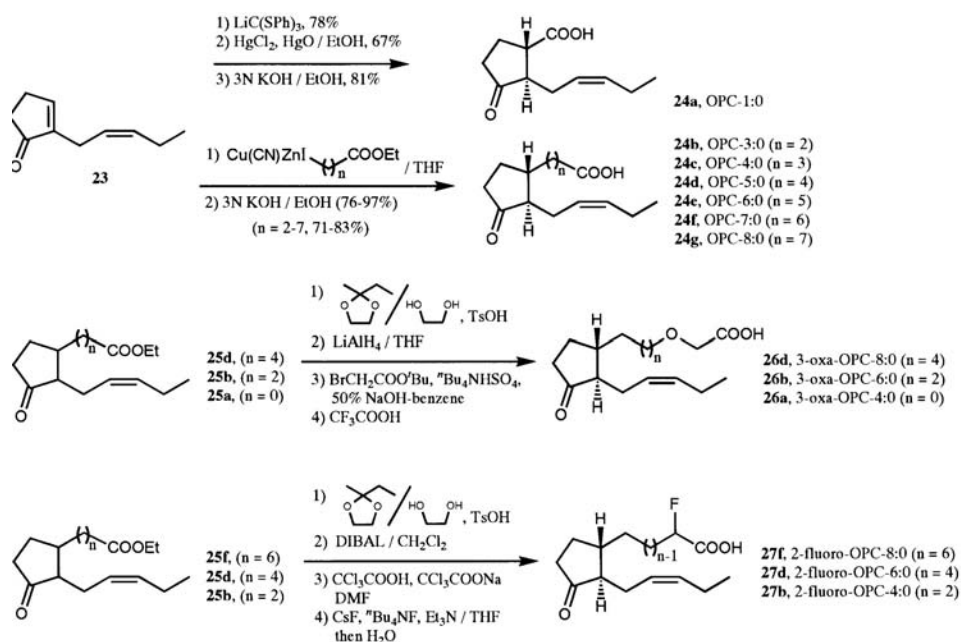
(*c* 2.20, MeOH) [lit. mp 161-163°C; $[\alpha]_D^{20} +68.4^\circ$ (*c* 2.20, MeOH)]. The spectral data of synthetic (+)-1 were identical with those of natural 1 in all respects including the specific rotation. In this way, the first asymmetric total synthesis of (+)-1 has been accomplished. The yield was remarkably improved in contrast to the previous synthesis of (+)-1 from natural 2.

8.4.2 Syntheses of Analogs of Jasmonic Acid and its Biosynthetic Intermediates

Even-numbered OPC homologs (OPC-8:0, -6:0, and -4:0) as biosynthetic precursors of jasmonic acid (JA) (Scheme 8.6) and odd-numbered OPC homologues (OPC-7:0, -5:0, -3:0, and



SCHEME 8.6
Biosynthesis of jasmonic acid and structural similarity to coronatine.



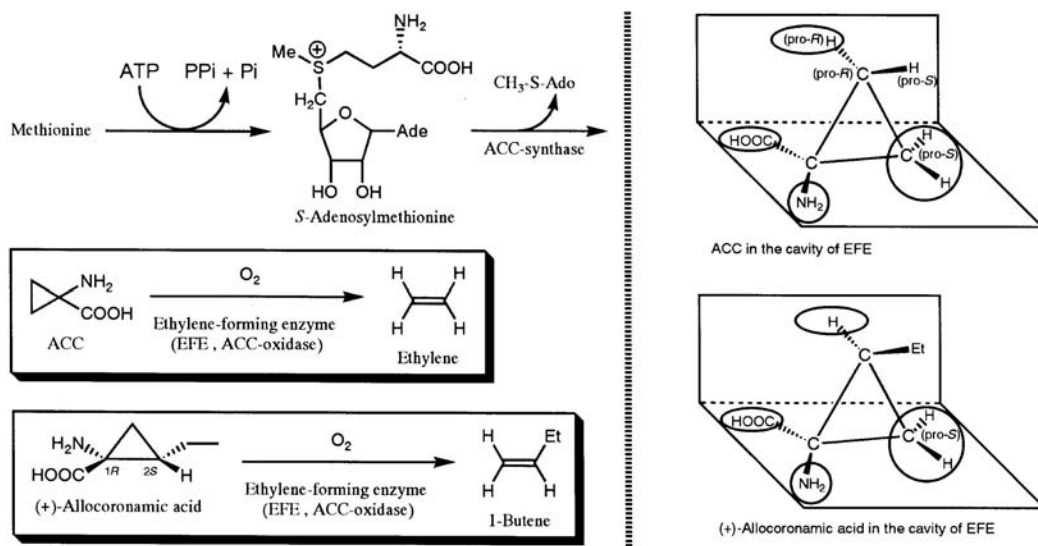
SCHEME 8.7

Syntheses of OPC-homologous series, 3-oxa-OPC- and 2-fluoro-OPC-analogs.

-1:0) were synthesized from 2-[(Z)-2-pentenyl]cyclopenten-1-one (**23**) as the common starting material in short steps and with high yields via conjugate addition (Scheme 8.7).²⁶ Only in the case of synthesizing OPC-1:0, *tris*(phenylthio)methyl lithium as a C_1 -synthon was used for the conjugate addition to give the orthothioester which was converted to OPC-1:0 (**24a**) via ethanolysis and subsequent hydrolysis. In the other cases, zinc-copper reagents which were prepared from iodo-esters possessing the requisite carbon chain, were applied for the conjugate addition. The resulting esters were hydrolyzed to OPC homologues [OPC-3:0 (**24b**), -4:0 (**24c**); -5:0 (**24d**), -6:0 (**24e**), -7:0 (**24f**), and -8:0 (**24g**)]. Each acid was observed as a *trans*-rich mixture in the ^{13}C -NMR spectra.

Esters of odd-numbered OPC homologues (**25a**, **25b**, **25d**, and **25f**) are convenient substrates for synthesizing analogs which are not structurally subject to β -oxidation.²⁷ Protection of the carbonyl group and subsequent LAH reduction gave an alcohol which was treated with *tert*-butyl bromoacetate to give a 3-oxa-OPC ester. Acidic hydrolysis gave 3-Oxa-OPC-8:0 (**26d**), -6:0 (**26b**), and -4:0 (**26a**). Instead of LAH reduction, DIBAL reduction gave an aldehyde which was treated with trichloroacetic acid/sodium trichloroacetate to give a trichloromethylcarbinol. Fluorination and hydrolysis gave 2-fluoro-OPC-8:0 (**27f**), -6:0 (**27d**), and -4:0 (**27b**). Each acid also was observed as *trans*-rich mixture in the ^{13}C -NMR spectra.

N-(Jasmonyl)amino acid conjugates (JA-AAs) were also synthesized from JA (racemic and *trans*-rich) and the appropriate amino acids.²⁸ In addition to naturally occurring JA-Leu, JA-Ile, JA-Val (derived from aliphatic amino acids), JA-Phe, JA-Tyr, and JA-Trp (derived from aromatic amino acids), not naturally occurring JA-Gly, JA- β -Ala, JA-ACC, JA-(+)-coronamic acid were also synthesized. Among them, JA-Leu, JA-Ile, JA-Val, JA-ACC, and JA-(+)-coronamic acid are structurally similar to coronatine.



SCHEME 8.8

Biosynthesis of ethylene and stereospecific recognition of ACC/(+)-allocoronamic acid by EFE.

8.5 Biological Activities of Coronatine

8.5.1 Allocoronamic Acid as Inhibitors of Ethylene Biosynthesis

Although naturally occurring coronamic acid [(+)-**3**] has the (1*S*, 2*S*)-configuration, three other possible stereoisomers [(−)-**3**, (+)-**16**, and (−)-**16**, see Scheme 8.3] are considered and have been prepared via optical resolution or asymmetric synthesis as described above.^{3,20,29} Coronamic acids are regarded as analogs of ACC and correspond to substituted ACC with an ethyl group. ACC is well known as the direct precursor of a plant hormone, ethylene. Therefore, coronamic acids were applied to elucidating stereospecific recognition of ACC by the ethylene-forming enzyme (EFE).³⁰ One stereoisomer, (1*R*, 2*S*)-allocoronamic acid [(+)-**16**] has been found to be converted to 1-butene at a 50 to 250 times higher rate than any of the other stereoisomers (Scheme 8.8). Since 1-butene does not exhibit ethylene-like actions, (+)-**16** would be used as a competitive inhibitor of ethylene biosynthesis. Therefore, we examined the inhibitory effect of coronamic acids on senescence in cut carnation flowers (*Dianthus caryophyllus* L. cv. Lightpink Barbara) (Table 8.1).³¹ Although both racemic and optically active allocoronamic acids exhibited antisenescence activity, (+)-**16** exhibited the strongest activity at 10 mM (longevity, 12 to 13 days). The longevity was slightly shorter than that of silver thiosulfate complex (STS) used as a positive control. In the case of using (−)-**16**, the stems changed to yellow and broke in extreme cases. In addition, we measured the relative EFE activity using a tomato suspension-cell culture. The relative EFE activity was decreased by both (+)-**16** (78.2%) and (−)-**16** (85.8%). α -aminoisobutyric acid (AIB) which is a known competitive inhibitor of EFE, also decreased the relative EFE activity with similar intensity (84.7%) to those of allocoronamic acids. Thus, it was proven that allocoronamic acids acted as inhibitors of ethylene biosynthesis.

TABLE 8.1

Inhibitory Effect of Coronamic Acids on Senescence in Cut Carnation Flowers (*Dianthus caryophyllus* L. cv. Lightpink Barbara)

Compound ^a	Concentration (mM)	Longevity (Days)
Control (H ₂ O)	–	6
STS (Koto fresh K-20)	1/1000 ^c	15
(±)-CA	10	6
(+)-(1S,2S)-CA	5	6–7
(–)-(1R,2R)-CA	5	6
(±)-AlloCA	10	8–10
(±)-AlloCA ^b	10	10–11
(+)-(1R,2S)-AlloCA	10	12–13
	5	10–11
	1.67	9
(–)-(1S,2R)-AlloCA	10	8–9
	5	9–10
	1.67	10–11

^a Three flowers were used for each compound tested.

^b After treating the flowers in the reagent for 24 h, the solution was changed to distilled water.

^c The original solution of a commercially available STS reagent was used after diluting 1000 times.

8.5.2 Similar Biological Activities of Coronatine to Those of Jasmonic Acid

A chlorosis-inducing phytotoxin, **1**, was isolated with the guidance of the potato cell expansion-inducing activity, and the hormone-like activities of **1** already have been studied. Recently, there has been strong interest in **1** which has been shown to exhibit various biological activities similar to those of JA, known as an endogenous plant-growth regulator and signal transmitter.³² JA and JA-Me (or as jasmonoid) are recognized as a kind of phytohormone and known to exhibit various biological activities in higher plants: senescence-promoting activity, growth-inhibitory activity, potato tuber-inducing activity, potato cell expansion-inducing activity, etc. These biological activities also are induced by **1** and the latter two activities were the first discovery in the similarity of the biological activities of coronatine to those of JA. Furthermore, the potent activities of **1** are 100 to 10,000 times higher than those of JA.^{33–35} There are other examples that revealed that **1** and JA (or JA-Me) exhibited similar biological activities. Both **1** and JA-Me caused similar growth-inhibitory effects on *Arabidopsis* (seedling and root), stimulated anthocyanin accumulation, and increased the level of two proteins (31 and 29 kD). *Arabidopsis* mutants selected for resistance to **1** are male sterile are also insensitive to JA-Me and resistant to bacterial pathogens.³⁶ It was proven with a tomato suspension culture that both **1** and JA played a central role in regulating EFE activity in ethylene biosynthesis.^{33,34} Both **1** and JA stimulated the biosynthesis and emission of volatiles (terpenes, acetogenins, and aromatics) in many plant species.³⁷ Antitumor taxane-type diterpenes were increased ca. 10 times by treating *Taxus baccata* with **1** and JA-Me.³⁸ In the tendril coiling assay of *Bryonia dioica*, **1**, the 12-oxo-PDA methyl ester, the OPC-8:0 methyl ester, and JA-Me exhibited activity that decreased in that order.³⁹ This result suggested the importance of not only JA, but octadecanoid precursors as signaling molecules of higher plants. In our recent study (Figures 8.2 and 8.3), the following results were obtained: (1) JA-amino acid conjugate (JA-Leu, JA-Ile, JA-Val) exhibited weaker

potato cell expansion-inducing activity than that of JA; (2) OPC-8:0, -6:0, and -4:0 exhibited both potato cell expansion-inducing and tuber-inducing activity at a similar concentration to JA; (3) not naturally occurring OPC-7:0 and OPC-5:0 also exhibited tuber-inducing activity; (4) the C6-alkyl-substituted analogs of **2** exhibited weak tuber-inducing activity;²² (5) a unique activity, decreasing the weight of potato tissue by 2-fluoro-OPC-8:0 in the cell expansion-inducing assay was first detected; and (6) 3-oxa-OPC-8:0, -6:0, and -4:0 also exhibited weak tuber-inducing activity. The last result suggests that β -oxidation is not required and that the corresponding biosynthetic precursor of JA, OPC-8:0, -6:0, and -4:0 in themselves exhibit tuber-inducing activity. In all the examined results of comparative bioassays of **1**, JA, biosynthetic precursors and analogs, **1** was always the most active compound. Since *in vivo* coronatine has not been isolated as a secondary product, 12-oxo-PDA and/or OPC-homologs would play an important role as signaling compounds in higher plants.⁴⁰⁻⁴⁴

8.6 Perspective

From the first isolation of coronatine as a phytotoxin, about 20 years have already past. During the period, coronatine was applied to various disciplinary fields and played important roles. There has been strong interest in coronatine again with respect to jasmonic acid-like activities. At the present stage, coronatine is the most promising probe for investigating plant physiological actions relating to JA (jasmonoid and octadecanoid). The elucidation of the JA-(jasmonoid- and octadecanoid-) receptor family is the remaining biggest problem, and coronatine would play an important role in solving the problem. Our asymmetric synthesis is able to provide a certain amount of coronatine; however, further synthetic studies on more structurally simple and biologically active analogs of coronatine should continue, and might result in developing a new plant-regulatory agent. The synthetic approach becomes increasingly important to obtain a practical, marketable product.

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Biochemical Interactions of the Microbial Phytotoxin Phosphinothricin and Analogs with Plants and Microbes

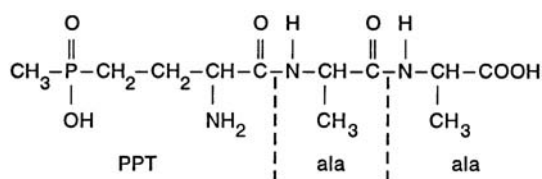
Robert E. Hoagland

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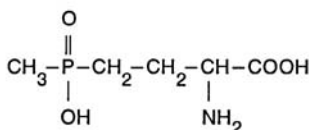
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9.1 Introduction

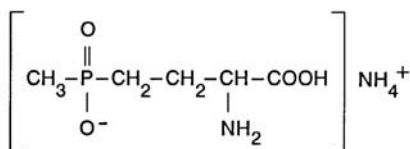
During 1971, two independent research groups discovered a naturally occurring compound with an unusual structure. The compound was identified as bialaphos, a tripeptide containing a unique amino acid, L-2-amino-4-[hydroxy(methyl)phosphinyl]butyric acid (called phosphinothricin or PPT) linked to two L-alanyl moieties (Figure 9.1). Bayer et al.¹



Bialaphos



Phosphinothricin



Glufosinate-ammonium

FIGURE 9.1

Chemical structures of bialaphos, phosphinothricin, and glufosinate-ammonium.

obtained this compound from *Streptomyces viridochromogenes*, while Kondo et al.² identified it in cultures of *Streptomyces hygroscopicus*. Degradative^{1,3} and synthetic^{1,4} studies provided proof of structure. The natural form of PPT is the L-isomer (L-PPT), and it was the first reported naturally occurring amino acid with a phosphinic group.

Initial biological testing showed that bialaphos had some antifungal (*Botrytis cinerea*) and antibacterial (Gram-negative and Gram-positive) activity,^{1,5} thought to be attributed to L-PPT. Glutamine was found to reverse growth inhibition by bialaphos in cultures of *Bacillus subtilis*, and it also was found that PPT was a potent inhibitor of glutamine synthetase [E.C. 6.3.1.2; GS] activity in *Escherichia coli*.¹ Examination of L-PPT for phytotoxicity by Hoechst AG showed that this new compound had strong herbicidal activity and summation of these data culminated in a patent.⁶ Synthesis of the DL-PPT ammonium salt (common name: glufosinate) resulted in the commercial herbicidal formulation of this active ingredient. The free acid and the ammonium salt were initially the numbered compounds HOE-35956 and HOE-391866, respectively. IUPAC nomenclature designates DL-PPT as DL-homo-alanin-4yl-methylphosphinic acid. Glufosinate is presently produced by AgrEvo USA Co. and Hoechst Schering AgrEvo GmbH and marketed globally under various trade names; e.g., Basta®, Buster®, Challenge®, Finale®, Harvest®, Ignite®, Liberty®, and Rely®. The tripeptide bialaphos also exhibits herbicidal activity when release of L-PPT occurs via hydrolytic cleavage of the alanine moieties. Bialaphos has been patented as a herbicide by Meiji Seika Kaisha,⁷ and is marketed in Japan as Herbiace®.⁸

Over the past 25 years, numerous published data on a variety of aspects of this unique natural product (PPT) and the development of its synthetic ammonium salt (glufosinate) as a herbicide have appeared. This paper will present an overview of some of the important

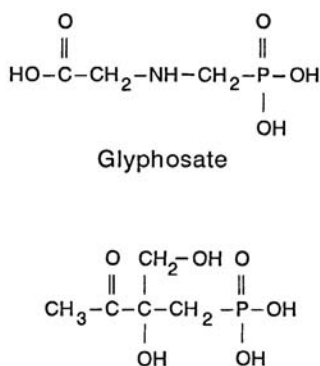


FIGURE 9.2
Chemical structures of glyphosate and phosphonothrixin.

properties, characteristics, and developments concerning the chemistry and biological activity of PPT, glufosinate, and some analogs. Throughout this chapter the terms PPT and glufosinate are used interchangeably and refer to the active ingredient, PPT.

9.2 Naturally Occurring C-P Bond Compounds Used in Agriculture

The natural occurrence of L-PPT is somewhat unique. Phosphorus compounds are produced abundantly by living organisms, but phosphonates [carbon-phosphorus (C-P) linkages] are only rarely produced in nature. In fact, naturally occurring phosphonate compounds were only discovered in the late 1950s. Some of these natural products possess biological activity. Closely related to the phosphonates are the phosphinates (C-P-C linkage) which were first discovered in nature over 25 years ago (e.g., PPT). Some phosphinates were synthesized even before PPT had been discovered.⁹ The discovery and use of C-P compounds (synthetic and natural) in agriculture, as well as aspects of enzyme inhibition by various phosphonate and phosphinates, have been reviewed and discussed.^{10,11} The synthetic herbicide glyphosate (Figure 9.2) is also a C-P compound that is structurally related to, but not a direct analog of, glufosinate.

Phosphonothrixin (Figure 9.2) is a newly discovered, herbicidally active, low molecular weight compound containing a C-P bond isolated from a bacterium, *Saccharathrix* sp. ST-888.¹²⁻¹³ Its structure has been verified by total synthesis, accomplished in six steps.¹⁴ Although phosphonothrixin is a C-P bond compound, it differs structurally from PPT and glyphosate in that it lacks N atoms. The structures of glyphosate and phosphonothrixin are compared in Figure 9.2.

Two oligopeptides containing phosphinothricin accumulated in cultures of a bialaphos producer, *Streptomyces hygroscopicus* SF1293, when large amounts of bialaphos were added to the cultures.¹⁵ One oligopeptide was a new compound, a bialaphos dimer (phosphinothricyl-ala-ala-phosphinothricyl-ala-ala), and the other was a previously known metabolite (phosphinothricyl-ala-ala-phosphinothricin). Other herbicidal peptide analogs of bialaphos have been isolated: phosalacine (PPT-ala-leu) from *Kitasatosporia phosalscinea* KA-338^{16,17} and trialaphos (PPT-ala-ala-ala) from *Streptomyces hygroscopicus* sp. KSA-1285.¹⁸ Peptidases can cleave the amino residues from both these molecules yielding PPT. Phosalacine has been patented for use as a defoliant for hops (*Humulus lupulus*).¹⁹

9.3 Chemical Properties of PPT, Glufosinate, and Bialaphos

PPT and glufosinate are whitish crystalline powders with relatively low molecular weights of 181.13 and 198.16, respectively.²⁰ These compounds are highly stable molecules with extremely high water solubility (>1350 g/l at pH 7.0), but have substantially lower solubility in common organic solvents. Procedures for the stereoselective synthesis of L-glufosinate have recently been developed.²¹ Bialaphos is produced as the sodium salt and is a white powder with a molecular weight of 345.26.²² This tripeptide is very soluble in water, soluble in methanol, but insoluble in other organic solvents such as acetone, benzene, and chloroform. An excellent review of various chemical synthetic methods used initially and subsequently for the preparation of PPT and some analogs has recently been published.²³ Glufosinate can form coordination complexes with metal ions, including Ca^{2+} , Fe^{2+} , Fe^{3+} , Mg^{2+} , and Ni^{2+} , but the toxicological effects of such complexes on plants or animals are unknown.²⁴ The synthetic herbicide glyphosate also is known to be a metal ion chelator and forms similar complexes.²⁵ Lowering polyvalent cation concentration in carrier water increased glyphosate phytotoxicity,²⁶ presumably due to the decreased chelation by metal ions. However, the phytotoxicity of HOE-00661, a formulated glufosinate product, was not affected by carrier water quality.²⁶ This suggests that the metal ion complexes formed with PPT may not be as strong as those formed with glyphosate or that glufosinate-metal ion complexes retain phytotoxicity.

9.4 Toxicity to Nontarget Species

Many studies have been conducted on glufosinate to determine its toxicological effects on various mammals, other animals, and insects. Some of this information has been summarized.²³ This compound has been demonstrated to have low toxicity in a variety of tests. In rats, the oral LD_{50} values are 2000 mg/kg for males and 1620 mg/kg for females. Dermal LD_{50} values are two-fold higher in both male and female rats.²⁷ For wildlife species, representative values are: oral LD_{50} > 2000 mg/kg for Japanese quail, LC_{50} > 320 mg/l for rainbow trout (96 h), and it is nontoxic to honeybees.²⁰ Generally, the data indicate no genotoxic, carcinogenic, or teratogenic potential, or other specific toxicological hazards.

9.5 Herbicidal Use and Efficacy

Glufosinate is a nonselective, postemergence herbicide used for weed control in orchards and vineyards, in chemical fallow situations, as a preharvest desiccant, as a burn-down herbicide of cover crops and/or weeds prior to no-till planting,²⁸ and for weed control in transgenic crops resistant to the herbicide.²⁹ Dicots are generally more sensitive than grasses and both annual and perennial weeds are controlled. Plant age and developmental stage of weeds influence the herbicide application rates required for control,³⁰ as has been shown for many herbicides. Rainfall within 2 to 7 h after glufosinate application can substantially reduce efficacy; e.g., foxtail (*Setaria* sp.).³¹

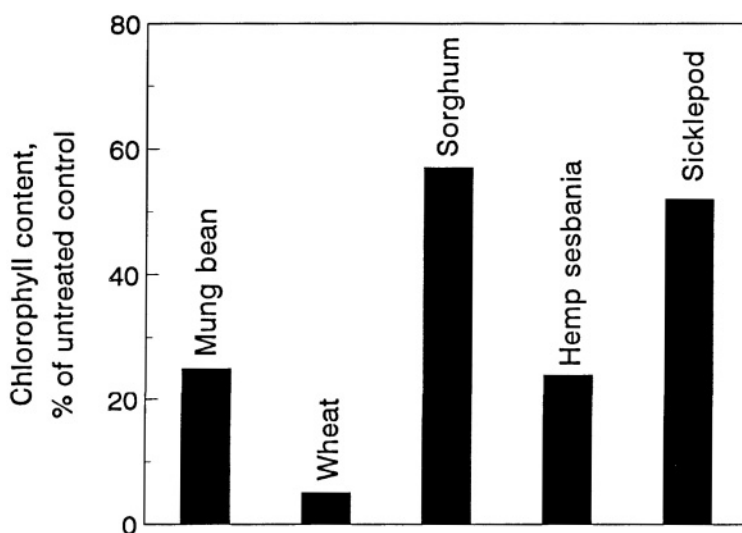


FIGURE 9.3

PPT (0.1 mM) effects on greening of excised etiolated seedling cotyledons or coleoptiles, 48 h after exposure to chemical and light (ca. 100 μ E). Chlorophyll from the tissues of three crop [mung bean (*Vigna radiata*), wheat (*Triticum aestivum*), and sorghum (*Sorghum bicolor*)] and two weed [hemp sesbania (*Sesbania exaltata*) and sicklepod (*Senna obtusifolia*)] species was extracted using dimethylsulfoxide and quantified spectrophotometrically.

Studies of annual weeds showed that glufosinate at 420 or 560 g ai/ha provided most effective control when weeds were 10 cm tall, rather than at 5 or 15 cm tall. Common lamb-quarters (*Chenopodium album* L.) was the most tolerant of one grass and three broadleaf weeds tested.³² In another study, a greater than 70-fold difference in susceptibility of seven plant species to glufosinate was found, possibly due in part to different ratios of the two GS isoenzymes in these species.³³ Comparison of factors affecting glufosinate herbicidal activity in barley (*Hordeum vulgare* L.) and green foxtail (*Setaria viridis* L. Beauv.) showed a strong correlation between species sensitive to glufosinate and the quantity absorbed and translocated, but there was no evidence of significant metabolic transformation.³⁴

The symptomology of glufosinate action in plants is the development of chlorosis and wilting 3 to 5 days after application.²⁰ Symptom development can be accelerated by high light intensity, high humidity, and high soil moisture. Seedlings are not injured prior to emergence. In greening tests of several species of etiolated seedlings, PPT (0.1 mM) and low light (ca. 100 μ E) reduced the chlorophyll content which accumulated in excised cotyledons or coleoptiles by varying degrees, 48 h after treatment (Hoagland, previously unpublished) (Figure 9.3).

9.6 Uptake, Translocation, and Metabolism of Bialaphos and PPT in Plants

Bialaphos controls a wide range of weeds, including perennials when applied to foliage.²² It is generally used in the same situations as PPT and exhibits the same symptomology. Bialaphos is absorbed through plant leaves and some translocation (of bialaphos or its

metabolites) has been shown to occur.²² After absorption, it is rapidly metabolized *in planta* to yield the active herbicidal ingredient, PPT. PPT also can be rapidly absorbed by plant tissues, but translocation to other plant parts is minimal. In cogongrass (*Imperata cylindrica*) and sour paspalum (*Paspalum conjugatum*), half the applied ¹⁴C-PPT was absorbed within 4 h, but little or no translocation was found.³⁵ Studies of PPT uptake in duckweed (*Lemna gibba*) showed a linear absorption phase over 10 min, followed by a second, slower uptake phase between 30 and 120 min.³⁶ L-Glutamic acid and L-alanine competitively blocked PPT uptake. Three days after application of ¹⁴C-glufosinate to five plant species, ¹⁴C was transported and detected in untreated plant tissues at levels of 2 to 4% of the initially applied dose.³⁷

Some studies suggested that differential sensitivity to foliar-applied glufosinate is due to uptake and translocation differences. For example, differences in uptake and translocation were suggested as the cause of toxicity differences in barley vs. green foxtail.³⁴ There is controversy concerning the mobility of glufosinate in phloem.^{38,39} One study concluded that glufosinate is mobile in both the xylem and phloem,⁴⁰ but its phloem mobility is modest compared to that of other compounds.^{40,41} In a study of several weed species, differences in sensitivity were attributed to various concentrations of active ingredient transported to roots.³⁷

Several reports indicate that PPT is not metabolized or degraded in plants.^{20,34,39} Other studies suggest there is some biodegradation of PPT by plants.^{37,42} In these latter experiments, whole plants were used under nonsterile conditions, but the plants were cultured hydroponically in the greenhouse and ¹⁴C-glufosinate applied to specific leaf areas. Cell cultures of several plant species [soybean (*Glycine max*), corn (*Zea mays*), and wheat (*Triticum aestivum*)] were shown to metabolize PPT.⁴³ The metabolic rates in these cultures (as percentage of applied radioactivity) were 12.5 (maize variety 2), 4.2 (maize variety 1), 2.4 (soybean), and 1.1 (wheat). These cell cultures absorbed different amounts of ¹⁴C-PPT and produced different numbers of identified metabolites: maize, 50% absorbed with 4 metabolites; soybean, 10% absorbed with one metabolite; wheat, 6% absorbed with 2 metabolites. The overall metabolic profile for PPT biodegradation in plants is presented in Figure 9.4. (Metabolism of PPT in genetically transformed plants will be discussed later in this review.) Although the C-P bond-containing herbicide glyphosate also is not metabolized (or metabolized to only a slight degree) in whole plant studies, plant cell cultures (without possible bacterial contribution) were able to metabolize ¹⁴C-glyphosate to aminomethyl phosphonic acid and incorporate ¹⁴C into bound residues at a high rate.⁴⁴ Thus, it appears that plants do not metabolize some C-P bond compounds (e.g., glyphosate and glufosinate) to a great degree, thereby allowing a prolonged period for herbicidal action *in planta*. These factors also contribute to the nonselective action of these herbicides.

9.7 Mode of Action

9.7.1 Glutamine Synthetase (GS) Reaction

The formation of glutamine from glutamate involves coupled cleavage of ATP, catalyzed by GS (Figure 9.5). Formation of γ -glutamyl phosphate is an intermediate in this reaction. This key reaction is catalyzed by GS in microorganisms and in plants, and plays a pivotal role in the assimilation of reduced nitrogen. Accumulated evidence suggests the coupled reaction of GS and glutamate synthase is the main pathway for ammonia assimilation in

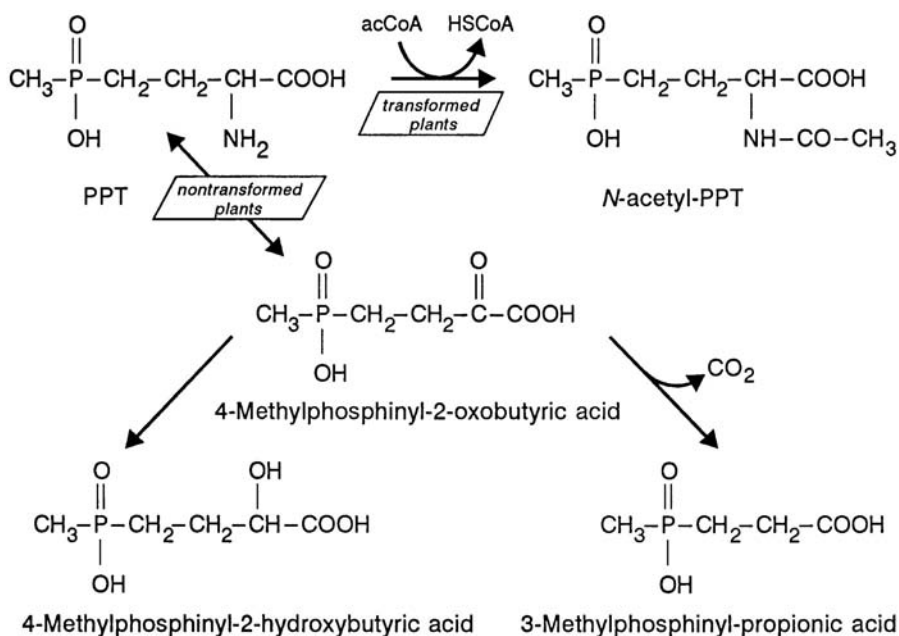


FIGURE 9.4

Summary of phosphinothricin metabolism in transformed and nontransformed plants. (From Dröge-Laser et al., *Plant Physiol.*, 105: 159-166, 1994. With permission.)

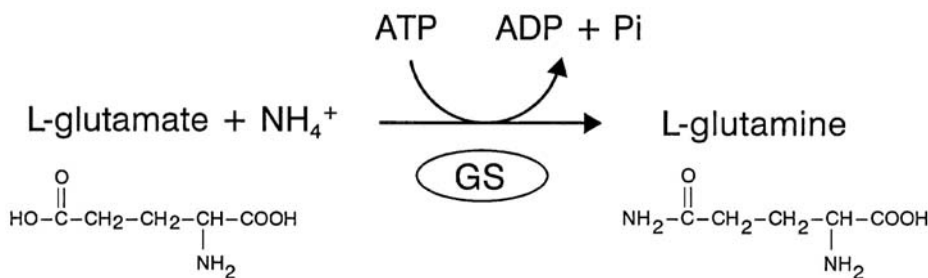


FIGURE 9.5

The formation of L-glutamine from L-glutamic acid catalyzed by glutamine synthetase (GS).

plants.⁴⁵ In the 1950s, a multitude of compounds were found to inhibit GS in microorganisms.⁴⁶⁻⁴⁸ Some of these compounds could inhibit bacterial growth and/or inhibit GS activity in cell-free bacterial preparations. Furthermore, many of these compounds were structurally related to glutamate, and some contained phosphonic or phosphinic acid moieties. It was not until the early 1980s that inhibitors of GS began to be widely tested in plants. For example, L-PPT was found to inhibit GS in the pea (*Pisum sativum*), thus providing evidence that the action of this compound was the same in plants and bacteria.⁴⁹ Many other GS-inhibiting compounds have been tested in plants.⁵⁰ Some of these compounds will be presented and discussed later in this chapter.

GS activity generally occurs in two isozyme forms in plants. GS₁ is the cytoplasmic form and GS₂ is chloroplastic. In nonphotosynthetic tissue, the major portion of GS activity is GS₁,⁵¹ with only small amounts occurring in plastids.⁵² Examination of GS isozyme content of a diverse range of plants showed that four groups could be discerned, including one group that possessed only GS₂ and another where GS₁ predominated.⁵³

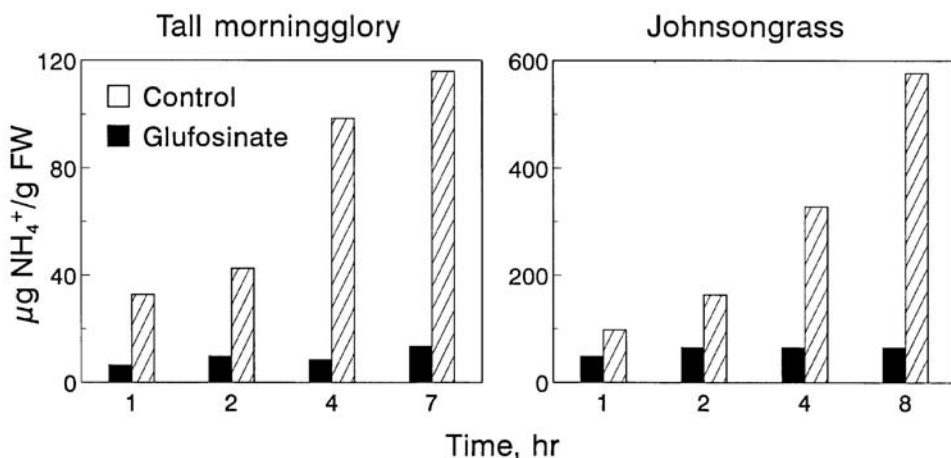


FIGURE 9.6

Ammonia accumulation in tall morningglory (*Ipomoea purpurea*) and johnsongrass (*Sorghum halapense*) following treatment with phosphinothricin. (Adapted from Köcher, H. and Löttsch, K., *Proc. Asian-Pacific Weed Sci. Soc.*, 10, 193, 1985. With permission.)

9.7.2 GS as a Site of Herbicidal Action

Although phosphinothricin was initially found to be a potent inhibitor of GS in *E. coli*,¹ the kinetic properties of GS inhibition on a wide variety of plants showed that K_i values varied by a factor of five.^{33,54} Furthermore, whole plant susceptibility to glufosinate ranged over two orders of magnitude,³³ but the reason for these susceptibility differences is not fully understood.^{50,55,56}

GS is inhibited and ammonia levels rise soon after treatment with PPT. Although the subsequent cascade of events leading to plant death has not been totally elucidated, it has been explained in several ways. One explanation is that the lack of amino donors causes disruption of photorespiration, and then photosynthesis, minutes after herbicide treatment.⁵⁷ An alternative proposal is a decoupling of photophosphorylation by excess ammonia.⁵⁸ Substantial increases in ammonia production have been shown to occur very early after PPT application in a number of species; for example, tall morningglory (*Ipomoea purpurea*) and johnsongrass (*Sorghum halapense*) (Figure 9.6). Although GS inhibition and accumulation of ammonia are generally thought to be pivotal to glufosinate mode of action, as substantiated by GS gene amplification for glufosinate tolerance,⁵⁹ other molecular target sites have been suggested. These include nitrate reductase (NR) inhibition,⁵⁸ membrane depolarization,³⁶ and membrane transport processes.⁶⁰ Following treatment of maize leaves with PPT, NR activity was initially reduced (i.e., at 120 to 180 min after treatment), but recovered to levels equal to the untreated controls by 240 min after treatment.⁶¹ Loss of NR activity also occurs in PPT-treated cyanobacteria (*Anacystis nidulans*)⁵⁸ and duckweed (*Lemna gibba*).⁶⁰ In both cases, this loss of activity has been explained by decreased nitrate uptake. However, when etiolated soybean seedlings were fed glufosinate in nitrate-free aqueous solutions, NR activity was reduced in both light- and dark-grown plants 24 h after treatment (Hoagland, unpublished). NR activity also was reduced in PPT-treated alfalfa (*Medicago sativa*) seedlings, but not until 24 h after treatment.⁶² Recently, it was found that glufosinate action in algae required an induction process, possibly indicating *de-novo* synthesis of an amino acid membrane carrier.⁶³ These authors also showed that one algal species could incorporate nitrogen into glutamate during GS inhibition via glutamate dehydrogenase (GDH) action. Although the effects of PPT on these parameters (membrane interactions, NR activity,

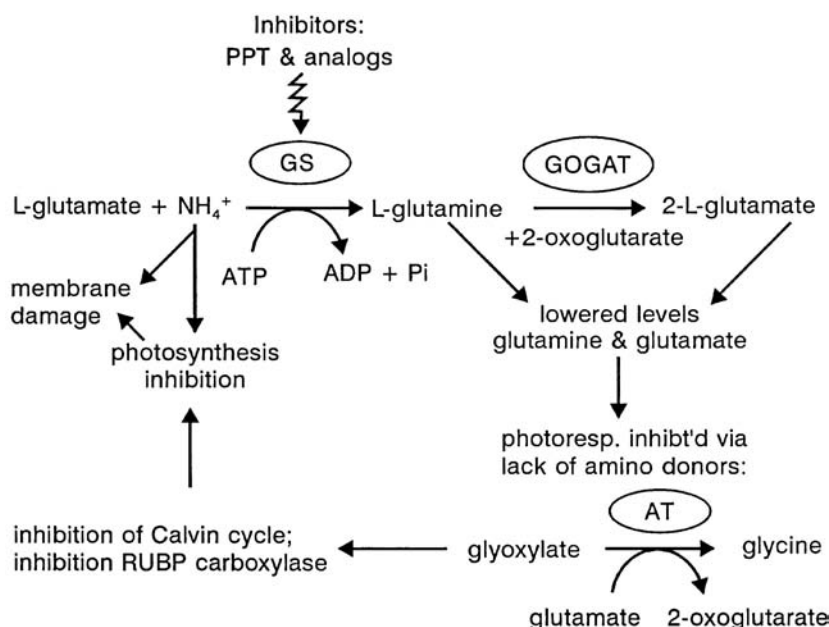


FIGURE 9.7

Overall events involved in the mode of action of PPT and other inhibitors of glutamine synthetase (GS). (Adapted from Wild, A. and Wendler, C., *Z. Naturforsch.*, 48c, 369, 1993. With permission.)

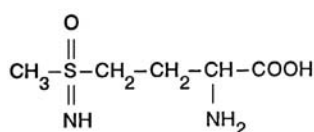
and GDH) might explain the wide range of sensitivities to this herbicide among some species, these effects are generally considered secondary to its mode of action.

PPT caused a more rapid inhibition of photosynthesis in C_3 than in C_4 plants under normal atmospheric conditions, but under nonphotorespiratory conditions, photosynthesis was not inhibited.⁶⁴ Also, ammonia accumulation was lower in C_4 plants. The addition of several amino acids such as glutamine or glutamate resulted in a significant alleviation of photosynthetic inhibition, even though ammonia levels were greatly increased. This suggests that ammonia accumulation may not be the primary cause of photosynthesis inhibition by glufosinate. Transamination of glyoxylate to glycine in photorespiration was inhibited due to lack of amino donors which could cause glyoxylate accumulation, thereby inhibiting ribulose-1,5-bisphosphate carboxylase and, consequently, CO_2 fixation.⁶⁴ The most complete understanding of the events occurring in plants after treatment with PPT (or other GS inhibitors) is complex (Figure 9.7), and has been discussed elsewhere.⁶⁵

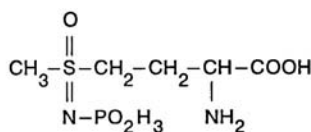
Since PPT inhibits GS, it was important to determine its effects on different isoforms and sources of this enzyme. Several isozymes of GS occur in different plant organs.⁵³ Multiple forms of GS sometimes occur in the same organ. For example, in leaves one isozyme resides in the cytosol and the other in chloroplasts. All isozymic forms of GS are inhibited by PPT, with K_i values of 5 to 10 μM .³³ Bialaphos does not inhibit GS, but is rapidly metabolized by peptidases in plant tissues yielding PPT.^{66,67} The D-isomer of PPT does not inhibit GS,⁶⁸ is nonherbicidal,²⁹ and is not degraded in transgenic plants.⁶⁹

9.7.3 Inhibition of GS by Other Natural and Synthetic Compounds

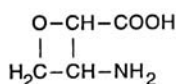
Other naturally occurring GS inhibitors that are phytotoxic have been discovered. Some of these compounds have structural similarity to PPT (Figure 9.8). L-Methionine sulfoximine (MSO), a close analog of PPT, was first crystallized as a toxic constituent from zein.⁷⁰ MSO was initially synthesized,^{71,72} and then found to occur as a natural product in the bark of a



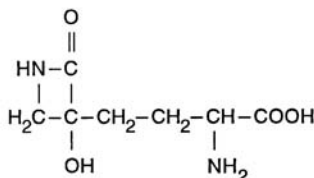
L-Methionine sulfoximine



L-(N⁵-Phosphono)methionine-S-sulfoximine



Oxetin



Tabtoxinine-β-lactam

FIGURE 9.8

Comparative chemical structures of additional natural products that inhibit glutamine synthetase (GS).

tree, *Cnestis glabra*.⁷³ MSO was the first reported inhibitor of GS.^{74,75} Another PPT analog, L-(N⁵-phosphono)methionine-S-sulfoximine (PMSO) (see Figure 9.8) is a potent GS inhibitor and a metabolite of L-(N⁵-phosphono)methionine-S-sulfoximiny-L-ala-L-ala (PMSO-Ala-Ala)⁷⁶ isolated from a *Streptomyces* species.⁷⁷ PMSO and MSO can result from the action of peptidase on PMSO-Ala-Ala or phosphatase action on PMSO, respectively.^{78,79} In a comparative study, MSO and PPT caused elevated ammonia levels and decreased free glutamine in several plants.⁵⁸ PPT caused ammonia evolution in both nitrogen-fixing and nitrate-reducing cyanobacterial cultures, indicating cellular uptake and *in vivo* GS inhibition.⁵⁸ Although MSO has been patented as a herbicide,⁸⁰ its inhibitory activity on GS is much less than that of PPT.^{49,81} In whole plant tests, PPT was generally 5- to 10-fold more phytotoxic than MSO.⁵⁵ Although numerous GS inhibitors that are analogs of PPT have been synthesized, none have been found to be more herbicidal than PPT.^{9,47}

Tabtoxin is a dipeptide produced by several pathovars of *Pseudomonas syringae*. Hydrolysis of tabtoxin produces tabtoxinine-β-lactam (Figure 9.8) which is an active GS inhibitor and the toxin responsible for causing wildfire disease.⁸² Oat varieties that are resistant to the *P. syringae* pathogen were found to have GS that was less sensitive to tabtoxinine-β-lactam.⁸³ Tabtoxinine-β-lactam increases nitrogen fixation of legume root nodules via selective inhibition of one GS isoenzyme.⁸⁴ Oxetin (Figure 9.8), produced by a *Streptomyces* sp., is a noncompetitive (with respect to glutamate) inhibitor of GS₂ in spinach and also possesses herbicidal activity.⁸⁵ Its inhibitory activity is much less than that of PPT.

Because L-γ-hydroxyglutamic acids can act as substrates of GS, the synthesis of γ-oxygenated analogs of PPT as possible inhibitors was examined.⁸⁶ A new inhibitor, DL-γ-hydroxyphosphinothricin (GHPPT) and its derivatives inhibited GS and caused *in vivo* phytotoxicity in several species.⁸⁶ Tests with α- and γ-substituted analogs of PPT showed a range of effectiveness on *E. coli* GS inhibition.⁸⁷ γ-Methyl-PPT, α-ethyl-PPT, and cyclohexane-PPT were only weakly phytotoxic and had low inhibitory action on GS.⁸⁸ The 2-oxo-PPT analog exhibits herbicidal activity similar to PPT, but lacks *in vitro* GS inhibitory action. It is probably transaminated in plants to form PPT.⁵⁵ Although numerous synthetic analogs of PPT have been examined, none has been found to rival the herbicidal potency of PPT.

9.8 Behavior of PPT, Glufosinate, and Bialaphos in Soils

9.8.1 Dissipation and Metabolism in Soils

The tripeptide bialaphos is rapidly degraded in soil to PPT.²² The principal metabolic pathway of glufosinate or PPT in soils is degradation to 3-methylphosphinyl-propionic acid (MPPA) (see Figure 9.4) and 2-methylphosphinyl-acetic acid (MPAA), and eventual CO₂ release. Half-lives between 4 and 7 days were found in two different nonsterile soils under laboratory conditions.⁸⁹ The only volatile metabolite from these glufosinate-treated soils was ¹⁴CO₂. In other soils, half-life values of 3 to 7 days at 20°C and 8 to 11 days at 10°C were reported.⁹⁰ Of 300 bacterial isolates from soil, all strains could degrade L-PPT to the 2-oxo analog of PPT via transamination.⁹¹ Only one strain (*Rhodococcus* sp.) utilized L-PPT as a sole source of nitrogen, and formed 2-oxo-PPT by oxidative deamination. D-PPT was not metabolized by any of these strains.

Generally, the degradation rate of PPT and glufosinate is dependent on soil characteristics and environmental conditions. In a forest soil environment (i.e., high organic matter), the half-life and maximum leaching depth of glufosinate were 4.3 days and 10 cm, respectively.⁹² In addition, the two main metabolites of glufosinate, MPPA and MPAA, were not found below 10 cm. Only 10 to 20% of the parent compound and metabolites were present in the soil 32 days after application, and during the following season (295 days), residue levels were nearly undetectable.

Several other studies in various soils have shown that glufosinate does not leach below a soil depth of 15 cm.^{90,93,94} The overall short half-lives and shallow leaching indicate that this compound is not persistent and, therefore, is not problematic with respect to herbicide carry-over that could affect future rotational crops or contaminate groundwater.

An algal strain of *Chlamydomonas reinhardtii*, which used PPT or its analog MSO as sole nitrogen sources, has been isolated and characterized.⁹⁵ This mutant strain also possessed an α -ketoglutarate aminotransferase activity not found in wild-type cells. Microbes (including algae) are important agents in the biotransformation of herbicides and/or other xenobiotics and natural products in the environment.

9.8.2 Effects of PPT on Soil Microbes

Soils are inhabited by numerous microbes, including pathogenic and nonpathogenic bacteria and fungi. Some of the more important of these species are pathogenic or plant-growth-promoting microbes in the rhizosphere zone. *Trichoderma* are fungal species that are key members of suppressive microflora due to their aggressive colonization in the rhizosphere and their antagonism against various phytopathogens.^{96,97} Some of these fungi are used as biofungicides.^{98,99}

As previously mentioned, PPT has long been known to possess strong antibiotic activity.¹ Recently, PPT was evaluated for its influence on interactions between phytopathogens and their antagonists.¹⁰⁰ PPT reduced the activity of certain antagonistic soil microbes, including *Bacillus subtilis*, *Pseudomonas fluorescens*, and many *Trichoderma* species. PPT also was inhibitory to some phytopathogenic fungi (e.g., *Rhizoctonia solani* and *Sclerotinia sclerotiorum*) whereas others (*Fusarium oxysporum* and *Pythium aphanidermatum*) were highly resistant to the herbicide. Biological control of *Fusarium oxysporum* by *Trichoderma* was improved by PPT exposure.¹⁰¹

The effects of glufosinate-ammonium on soil microbial populations and soil enzyme activities have recently been assessed in clay and sandy loam soils.¹⁰² Although glufosinate-ammonium at 100 ppm significantly decreased fungal and bacterial populations after a 1-day incubation in both soils, populations recovered after 7 days. Soil phosphatase activity also was reduced by glufosinate treatment, but recovered after 7 days. Urease activity was not affected in clay loam soil during a 1-day incubation, but increased in glufosinate-treated (100 ppm) soil from day 7 through day 28. Urease activity declined during the first 7 days in sandy loam treated with glufosinate, but then increased through the 28-day experiment.

In studies on the interaction of PPT with soil microflora, 15 agricultural and nonagricultural soils were examined.¹⁰³ PPT at 1 mM reduced fungal populations by 20% and bacteria by 40%. In forest soils, bacterial populations were reduced by 20%. Soil isolates grown in the presence of 1 mM PPT exhibited a broad range of tolerance to PPT concentrations up to 50 mM. Of the fungal isolates, *Verticillium albo-atrum* was among the most resistant to PPT, while the mycoparasitic species *Trichoderma harzianum* and *T. longipilus* were among the most sensitive. The survival of a PPT-resistant strain of *Pseudomonas putida* with a recombinant plasmid in PPT-treated soils also has been evaluated.¹⁰⁴

Soils treated with PPT for three consecutive seasons showed almost a complete lack of PPT-sensitive bacteria.⁹¹ Sites with no PPT history possessed numerous bacteria and fungi that were either sensitive or resistant to 1 mM PPT.¹⁰³ In laboratory tests, glufosinate applied to soils generally had little effect on microbial respiration.¹⁰⁵ However, fungi isolated from these soils showed some sensitivity to glufosinate, depending on herbicide concentration and growth conditions.¹⁰⁵ PPT is reported to inhibit GS in fungi and thereby diminishes the coupled operation of the key components of ammonia assimilation, GS and NADPH-dependent glutamate dehydrogenase.¹⁰¹

Soils contain many different *Rhizobium* species that are important in nodulation and nitrogen fixation in a number of leguminous plants. The nodule-inducing bacterium *Rhizobium meliloti* was found sensitive to low concentrations of PPT and bialaphos.¹⁰⁶ Growth of *R. meliloti* was more strongly reduced in sterile synthetic media than in sterile soil, but in nonsterile soil only a transient growth reduction was noted. Sensitivity was found in five of eight microbial species tested and spontaneous resistance to PPT occurred in all the sensitive strains. Nodulation also was significantly reduced by bialaphos and PPT in PPT-resistant alfalfa seedlings under sterile (except for the presence of *R. meliloti*) conditions. The use of PPT for cover crop removal and in transgenic crops resistant to PPT could influence populations of beneficial and phytopathogenic microorganisms that affect crop growth and yield.

9.9 Biochemistry of Bialaphos and Development of Transgenic Plants Resistant to Glufosinate

9.9.1 Biochemistry and Biotechnology of Bialaphos Production

The biosynthetic pathway of bialaphos has been elucidated using precursors, metabolic inhibitors, blocked mutants, and ¹³C- and ³¹P-NMR spectroscopy for identification of products accumulated and metabolized by a series of nonproducing mutants of *S. hygroscopicus*.¹⁰⁷ Starting with precursors containing three carbons, bialaphos is produced in a complex series of over a dozen steps. This biochemistry has been summarized elsewhere.¹⁰⁷ One step

involves an acetyl CoA-dependent reaction that modifies either demethyl-PPT or PPT. The gene (*bar*) responsible for encoding this step confers resistance to bialaphos in the organism. The gene has been isolated and characterized and found to encode acetyl transferase activity that converts PPT to an acetylated metabolite that is nonphytotoxic.¹⁰⁸

Because of certain similarities in phosphinomethylmalic acid (PMM) synthase and citrate synthase from several sources, these enzymes were compared in *S. hygroscopicus*. PMM synthase catalyzes a condensation reaction between phosphinopyruvic acid and acetyl-CoA to form PMM, an intermediate of bialaphos formation in *Streptomyces hygroscopicus* SF-1293.¹⁰⁹ PMM synthase uses a phosphonic acid analog of oxalacetic acid as substrate in a reaction comparable to that of citrate synthase. The enzymes differed with respect to several properties, including amino acid composition, N-terminal amino acid sequence, and stereo-chemical reaction mechanism.

9.9.2 Development of Transgenic Plants Resistant to Bialaphos

Over the past few years, many vegetable and cereal crop species have been transformed with either the *bar* gene of *Streptomyces viridochromogenes* or the *pat* gene of *S. hygroscopicus* to impart resistance to PPT. Cloning of a PPT-resistant gene (*bar*) from *Streptomyces hygroscopicus*¹¹⁰ and the transformation of PPT-resistant plants has been accomplished.¹¹¹ A similar gene (*pat*) from *S. viridochromogenes* Tü 494 with the same function was simultaneously isolated and introduced into various plant species.^{69,112,113} Presently there are more than 20 crop plant species transformed for resistance to PPT (Table 9.1). Some of these genetically transformed plants are resistant to PPT at rates as high as 4 kg ai/ha, which is about 10 times the lowest normal field application rate (0.35 to 1.7 kg ai/ha).¹¹⁰ The use of transgenic crops and the increased use of minimum tillage practices (in which cover crops

TABLE 9.1

Phosphinothricin-Resistant Crops

Botanical Name	Common Name	Reference
<i>Avena sativa</i>	Oats	114
<i>Beta vulgaris</i>	Beet root	115
<i>Brassica napus</i>	Oilseed rape	116
<i>B. oleracea</i>	Wild cabbage	116
<i>Capsicum</i> spp.	Red pepper	117
<i>Cucumis sativus</i>	Cucumber	117
<i>Daucus carota</i>	Carrot	69
<i>Festuca arundinacea</i>	Tall fescue	118
<i>Glycine max</i>	Soybean	119
<i>Gossypium hirsutum</i>	Cotton	120
<i>Hordeum vulgare</i>	Barley	121
<i>Lycopersicon esculentum</i>	Tomato	111, 116
<i>Medicago sativa</i>	Alfalfa	122, 123
<i>Nicotiana tabacum</i>	Tobacco	111
<i>Oryza sativa</i>	Rice	124, 125
<i>Populus</i> spp. hybrids	Poplar	126-128
<i>Secale cereale</i>	Rye	129
<i>Solanum tuberosum</i>	Potato	111
<i>Sorghum bicolor</i>	Sorghum	130
<i>Triticum aestivum</i>	Wheat	131-133
<i>Zea mays</i>	Maize	134, 135

(Adapted from Vasil, I.K., in *Herbicide-Resistant Crops*. Duke, S.O., (Ed.), CRC Press LLC, Boca Raton, FL, 1996, 85.)

are used for weed control and to reduce erosion) are leading to the increased use of herbicides. It is reasonable to believe this trend will continue. PPT is currently used as a temporary nonselective postemergence herbicide to control broadleaf and grass weeds.⁸⁹ The metabolism of PPT was compared in transgenic PPT-resistant tobacco (*Nicotiana tabacum*), alfalfa, and carrot (*Daucus carota*) expressing the PPT-*N*-acetyltransferase gene *pat*.¹³⁶ Transgenic plants converted PPT to *N*-acetyl-PPT, and untransformed and transformed plants expressing low acetylating ability produced 4-methylphosphinyl-2-oxobutyrate, 3-methylphosphinylpropionate, and 4-methylphosphinyl-2-hydroxybutyrate (see Figure 9.4).

9.9.3 Effects of Bialaphos and Glufosinate on Control of Pathogens in PPT-Resistant Crops

The antifungal activity of bialaphos and glufosinate was recently assessed on three pathogens *in vitro* and *in vivo* on PPT-resistant transgenic creeping bentgrass (*Agrostis palustris*), an important turf grass.¹³⁷ Results show that bialaphos may simultaneously control weeds and fungal pathogens when this transgenic grass is grown in the field.

Bialaphos has antibiotic activity against *Rhizoctonia solani* Kühn that causes rice sheath blight,¹³⁸ and *Magnaporthe grisea* (Herbert) Barr² that causes rice blast disease. Substantial suppression of sheath blight symptoms was reported when bialaphos was applied to transgenic plants that had been infected with *R. solani* 2 days before herbicide treatment.¹³⁹ Inoculated transgenic rice plants [bialaphos-resistant (*bar*) gene] exhibited a reduction in lesions and symptoms of rice blast disease after treatment with bialaphos.¹⁴⁰ Thus, it also may be possible to control these serious diseases by using *bar*-transgenic rice cultivars and bialaphos for weed and disease control.

An improved transformation protocol, utilizing selection for resistance to bialaphos, has been developed for the plant pathogen *Cercospora kikuchii*.¹⁴¹ Previously, transformation conferring resistance to bialaphos was developed for other fungi, e.g., *Neurospora crassa*¹⁴² and *Cochliobolus heterostrophus*.¹⁴³ These systems may allow the elucidation of biosynthetic pathways, e.g., those important in the development of resistance in soybean cultivars against cercosporin produced by *C. kikuchii*.

9.9.4 Effects of Glufosinate on Weed Control in Transgenic Crop Plants

The use of bialaphos as a herbicide in rice may have some effect on pollen sterility. Bialaphos induced a high level of sterility in rice when applied (0.1 mM) at meiosis.¹⁴⁴ Bialaphos (0.1 to 3.0 mM) did not inhibit GS activity in rice shoot extracts, but the metabolite of bialaphos, PPT, completely inhibited GS at 1 mM. The sterility in rice anthers was attributed to alteration of free amino acid levels and ammonia accumulation, since excess ammonia nitrogen is known to inhibit rice pollination.¹⁴⁵

Evaluation of glufosinate control of the weed red rice (*Oryza sativa*, a conspecific weed of cultivated rice) in *bar*-transformed rice indicated that this crop was not injured by glufosinate application.¹⁴⁶ Although red rice was controlled, flooding or partial submersion of red rice significantly reduced its control. Thus, sequential glufosinate applications are required for consistent red rice control in flooded and nonflooded situations.¹⁴⁶

Mixtures of glufosinate alone and glufosinate plus several other herbicides (pendimethalin [*N*-(1-ethylpropyl)-3,4-dimethyl-2,6-dinitrobenzenamine]; thiobencarb [*S*-[(4-chlorophenyl)methyl]-diethylcarbamothioate]; quinclorac (3,7-dichloro-8-quinolinecarboxylic acid); bensulfuron [methyl 2-[[[(4,6-dimethoxy-2-pyrimidinyl)amino]carbonyl]amino]sulfonyl]methyl]benzoate]; or bentazon [3-(1-methylethyl)-(1*H*)-2,1,3-benzothiadiazin-4(3*H*)-one

2,2-dioxide]) caused no more injury to *bar*-transformed rice than did glufosinate alone. However, glufosinate plus the herbicides propanil [*N*-(3,4-dichlorophenyl)propanamide]; acifluorfen (5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitro-benzoic acid); or triclopyr ([3,5,6-trichloro-2-pyridinyl)oxy]acetic acid) caused increased injury to this transgenic rice variety.¹⁴⁷

9.10 Concluding Remarks

It has been about 26 years since the discovery of PPT and just over 20 years since its herbicidal properties were patented. Since that time, a multitude of research papers have been published on various aspects of PPT and related compounds. Industry undoubtedly has performed numerous unpublished experiments and made major advances in its successful global marketing of this chemistry as a herbicide. Field trials have confirmed superior postemergence weed control and excellent crop safety with the use of glufosinate in four major transgenic (*pat* gene) crops: canola (*Brassica rapa*), corn, soybean, and sugar beet (*Beta vulgaris*).¹¹⁹ Major North American seed companies are developing glufosinate-tolerant varieties of a number of crops.¹⁴⁸ Although glufosinate has been used extensively on a global basis, no weed resistance to this herbicide has been reported. However, it has been recently hypothesized that due to the low level of control of *Lolium rigidum* by economical application rates of glufosinate and the ability of *L. rigidum* to become resistant to other herbicides, it is probable that this grass will become resistant when this compound is used in transgenic cropping areas.¹⁴⁹

The increasing use of transgenic crops resistant to PPT will magnify the influence of this compound on soil microorganisms (i.e., more applications per season, higher application rates). Thus, the impact of frequent and long-term use of PPT on microbes associated with phytopathogenicity and those associated with plant growth promotion and soil fertility will need to be studied in greater detail. The use of several herbicides has been correlated with increased disease in some crops.¹⁵⁰ Therefore, the effects of PPT on soil bacteria and fungi make microbial ecology studies imperative.

Bialaphos and PPT are unique among commercial herbicides in that they have both antibiotic and herbicidal properties. Their antibiotic properties have already proven useful for disease control in PPT-resistant crops, such as rice.^{139,140} These are the first studies to demonstrate the successful use of a herbicide to control both weeds and diseases in a transgenic crop. This dual strategy will no doubt be utilized more widely with the increasing availability of PPT-resistant crops.

The discovery of the natural product PPT has led to the development of several major commercial herbicides and many transgenic crops, and has provided tools for controlling diseases in transgenic crops. Glufosinate and bialaphos have potent herbicidal activity on a variety of weed species. Yet their tendency to be rapidly metabolized, coupled with their low toxicity and persistence in soils and water, result in minimal environmental and human impact. This is truly a remarkable success story for an agricultural product developed from a naturally occurring compound.

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Sequestration of Phytotoxins by Plants: Implications for Biosynthetic Production

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10.1 Introduction

The function of most secondary plant products or phytochemicals is unknown. Clearly, the chemical composition of plant species varies considerably and this variation is largely the result of selection pressure from many environmental and biotic factors. Understanding the biological function of a phytochemical in the producing plant provides a strong clue as to how humans may benefit from the products of secondary plant metabolism.

Each plant species has co-evolved with a large number of other plant species, insects, plant pathogens, soil microbes, nematodes, grazing animals, birds, and other organisms. The response of the plant to evolutionary pressures caused by these interactions is sometimes expressed as the production of specific phytochemicals that influence the interaction in favor of the plant. For example, synthesis of certain phytoalexins has evolved in response to selection pressures caused by particular plant pathogens. The selection pressures that forced the evolution of secondary biochemical pathways that produce potent chemical defenses,^{1,2} also resulted in the evolution of mechanisms and structures, such as secretory glands, to localize and sequester the compounds associated with these defenses. Sequestration can be advantageous in delivery of the phytochemical at the proper time, place, and event, as well as in protecting the producing plant from cytotoxicity of the sequestered compound.

The tissue and/or cellular localization of phytochemicals can be useful in predicting function and potential biological activity. Furthermore, this information can be useful in discovery of human uses of these compounds and in planning production of the compounds by

biosynthesis.³ We provide several examples of such relationships of the localization or sequestration of plant secondary products to their biological activities.

10.2 Examples

Only two types of sequestration will be discussed in this chapter, glandular and subepidermal localization — the cases with which we are most familiar. Secondary products can be sequestered by other mechanisms and structures, including idioblasts (anomalous, lone cells located in an otherwise homogeneous tissue), lactifers, nectaries, resin canals, specialized cell layers other than subepidermal layers, or secretion into cell walls.

10.2.1 Glandular Localization

The aerial surfaces of 20 to 30% of vascular plant species contain glandular trichomes.^{4,5} A common type of glandular trichome, the peltate gland, is characterized by the swollen, globular appearance of the cuticle which has split from the secretory cell walls as the subcuticular space engorges with secretory product^{e.g.,6,7} (Figure 10.1). The secretory product often includes highly concentrated secondary metabolites with biological activities of interest to the pesticide, pharmaceutical, and flavor and fragrance industries.

In some cases, plants have been grown to produce such compounds (e.g., the pyrethroids of *Chrysanthemum* species⁸ or essential oils of mints and other culinary herbs⁹). Attempts to produce compounds found in glandular trichomes in undifferentiated plant cell or tissue cultures have not been very successful.^{3,10,11} This may be because many of the compounds

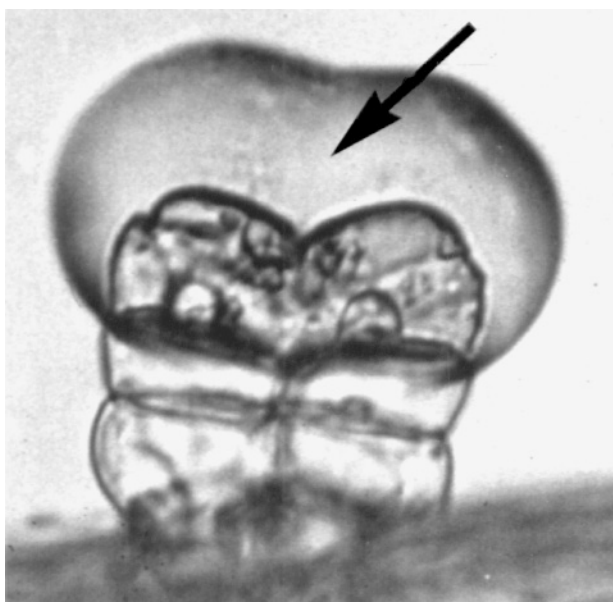


FIGURE 10.1

Light micrograph of an *Artemisia annua* trichome with the subcuticular space (→) filled with essential oils. (From Ferreira, J.F.S. and Janick, J., *Intl. J. Plant Sci.*, 156, 801, 1995. With permission.)

found in glandular trichomes are autotoxic to the producing plant if they are not sequestered in the subcuticular space of the peltate glands.

The general cytotoxicity of many of these compounds necessitated concomitant evolution of secretory mechanisms and/or sequestration to avoid autotoxicity and to deliver the compounds to the proper site for their ecological function. One can speculate that peltate glandular trichomes are largely the result of this evolutionary interaction between chemistry and anatomical structure, in response to biotic selection pressures.

The functions of glandular trichomes and the compounds contained within them are often unclear. There is no evidence to suggest that these structures and their contents help the plant to grow faster or provide a direct advantage in plant–plant competition. Nor do they give it a direct reproductive advantage. Unlike other types of trichomes, the relatively sparse coverage of mature plant surfaces by glandular trichomes precludes a direct function such as a physical hindrance to insects or as a means of reducing solar heating, evaporative loss, or damaging ultraviolet irradiation.⁴ However, some glandular trichomes produce sticky materials that entrap insects, and others can secrete sufficient resinous material to coat the plant surface, particularly in immature tissue, thus indirectly performing these functions.^{4,12} Considering the biological activities of many of the compounds sequestered in or exuded from these structures, the selection pressures that favored evolution of secreting glandular trichomes in many species were likely those of interspecies interactions. Such interactions could occur with other plant species, microbes, or insects and other herbivores.

Secretion of phytotoxins by glandular trichomes could be of secondary benefit to the producing plant. For example, one of the first compounds demonstrated to play a role in plant–plant allelopathy was 1,8-cineole.¹³ This highly phytotoxic monoterpene is probably entirely localized in the glandular trichomes of *Salvia* species,^{14,15} and together with other terpenoids, inhibits growth and development of surrounding plants.^{13,16} Such compounds apparently reach competitors through volatilization and/or leaching from ground litter. This delivery system for phytotoxins does not appear to be very efficient.

Glandular trichomes often contain potent phytotoxins that play no apparent role in plant–plant allelopathy. For example, artemisinin, a sesquiterpenoid antimalarial drug,¹⁷ is highly phytotoxic to most plants, including the producing species, *Artemisia annua* L.^{18–21} Nevertheless, to our knowledge, *A. annua* has not been reported to be allelopathic toward other plant species. The efforts to devise weed management methods for commercial monocultures of this drug plant species indicates that it is not a good competitor.²²

Artemisinin is found only in the subcuticular space of the glands of *A. annua*,^{23,24} thus, sequestering it to avoid autotoxicity. These glands clearly play no essential role for the plant, because glandless biotypes are similar to glanded biotypes in most respects.²³ We think that the most likely function of the glandular trichomes of *A. annua* and many other species is as repositories of chemicals that defend against insects, herbivores, and microbial pathogens. Many of the compounds stored in these glands are often suggested as allelochemicals involved in plant–plant interactions without rigorous proof of such a role.

Developing and very young leaves are particularly vulnerable to attack by insects, herbivores, and pathogens. Glandular trichomes develop rapidly on leaf primordia (Figure 10.2A). As trichomes mature and become more sparsely distributed over the expanding leaf surface (Figure 10.2B), they may spread their contents on the leaf surface by leakage through the cuticle^{e.g., 4,25} or by rupturing of the cuticle.^{e.g., 6,24} Some of the compounds may play roles in plant–insect interactions other than protective functions, such as attraction of pollinators or other beneficial insects.

Another property of many of the compounds produced by glandular trichomes, particularly the terpenoids, is their antimicrobial activity.^{15,26} Essential oils containing these compounds have been used for many years as preservatives and disinfectants. It follows that

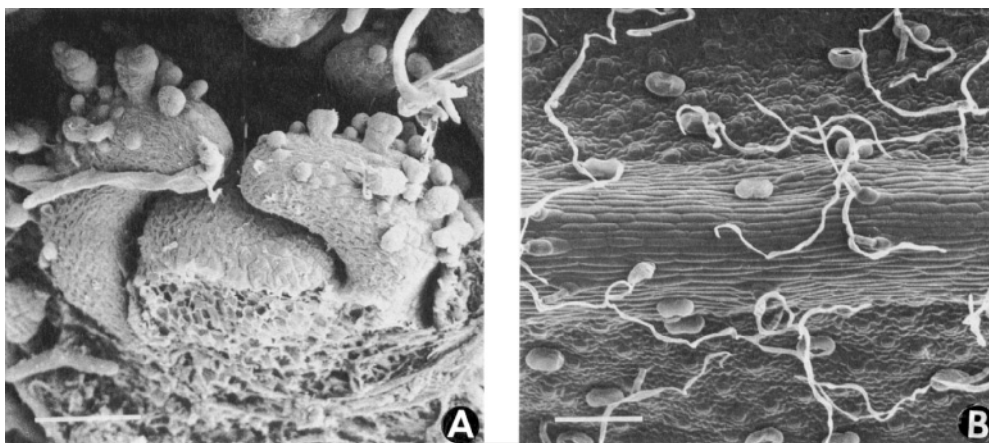


FIGURE 10.2

Scanning electron micrographs of trichomes on (A) leaf primordia and (B) mature leaves of *Artemisia annua*. (From Duke, S.O. and Paul, R.N., *Intl. J. Plant Sci.*, 154, 107, 1993. With permission.)

the antifungal and antibacterial properties of these compounds must have some role in plant defenses against pathogens. However, constitutive defenses of plants against pathogens have not received as much study as inducible defenses.

Plant species that produce glandular trichomes generally produce relatively large amounts of bioactive secondary products. The glandular trichomes are the primary production sites of many of these compounds and perhaps the only site of synthesis of some. There are a few cases, such as that of certain tobacco diterpenes,²⁷ in which the trichome has conclusively been demonstrated to be the exclusive site of biosynthesis.

Phenolic compounds from the shikimate pathway are common constituents of secretory glands. Many phenolics found in glands have biological activities. However, there has been relatively little interest in commercial exploitation of these compounds. In some *Solanum* species, phenolic compounds are released by breakage of specialized glands by insects which results in rapid oxidation of the phenolics to quinones by polyphenol oxidase (PPO) released from gland cell plastids.²⁸ The phenolic quinones polymerize rapidly enough to “glue” insects to the leaf surface or to render their mouth parts unusable. Other secondary compounds of glandular trichomes, such as sugar esters of fatty acids,¹² also play a role in combating plant pests.

Extracting secondary compounds from producing plants has been the traditional method of obtaining these compounds. This has apparently been done with little or no consideration of the biochemistry or anatomy of the plant. An example of this is the pharmaceutical industry’s approach to artemisinin production from plants.²⁹ Artemisinin is an effective antimalarial drug that is desperately needed to fight *Plasmodium falciparum* strains that have become highly resistant to drugs currently in use.^{17,30} The plant has been selected and bred for the most productive biotypes and varieties.³¹ Effects of agricultural chemicals, soil types, and cultural methods of farming on artemisinin production have been determined.³² The relative artemisinin yields of different plant parts and the same plant parts at different developmental stages have been studied. This has evidently been done with no consideration of the functioning of the secretory glands, in which all of the artemisinin appears to be synthesized. Furthermore, development of extraction methods for the compound has been conducted without regard to this knowledge.^{e.g.,33,34} Pharmaceutical chemists have apparently assumed that all of the plant cells must be extracted. However, maceration of the entire tissues breaks cellular compartments, blending oxidative enzymes

with secondary compound substrates, often leading to enzymatic “browning”. Part of the browning process is caused by the generation of quinones from hydroxylated phenolic compounds. The highly reactive quinones react with each other and other compounds to generate brown, polymeric compounds.

We found that all of the drug and one of its precursors, artemisitene, could be extracted by a brief dip in organic solvent to extract only the subcuticular space of the gland.²³ This procedure minimizes the possibility of reactions of these relatively unstable compounds with degrading enzymes or other plant constituents.

Our studies with fresh leaf tissue of *Artemisia annua* support this view. Leaf tissues (stems excluded), were cut from 8-week-old potted plants and weighed. A half portion of the fresh plant material was extracted by brief immersion in CHCl_3 and the other half was extracted by homogenizing in CHCl_3 . Extraction by immersion was done by steeping 5 g of leaves in 100 ml CHCl_3 for 20 sec. The extract was filtered and the process repeated two more times. The extracts were pooled and dried in a vacuum. Complete extraction of artemisinin was ascertained by homogenizing the dipped leaf tissue in CHCl_3 (3x) using the same volume as in dipping. Pooled extracts were vacuum dried. Homogenization of fresh leaves was done in the same way as that of the dipped leaves, keeping the ratio of 100 ml CHCl_3 per 5 g of fresh leaves.

Quantitation of artemisinin followed the procedure of Zhao and Yeng³⁵ with minor modification. The dried extracts were dissolved in MeOH to a concentration of 10 mg/ml. A 50 μl aliquot was taken and 420 μl of 0.2% NaOH was added. The mixture was heated in a heating block at 50°C for 30 min, then 30 μl of 20% methanolic acetic acid was added. This sample was used for HPLC analysis under the following conditions: column was a C18 Waters $\mu\text{Bondapak}^{\text{TM}}$ 3.9 \times 300 mm; mobile phase was 0.01M NaH_2PO_4 buffer (pH 7.00):MeOH eluted in gradient from 59:41 to 55:45 over a period of 25 min; flow rate was 1 ml/min; injection volume was 100 μl ; detection γ was at 260 nm. The retention time of artemisinin was 9.7 min.

With fresh tissue, homogenization of the tissues yielded only 73.2% (SE = 17.6%) of the artemisinin that was extracted by dipping, and only 66.4% (SE = 15.5%) of that artemisinin produced by dipping plus homogenization. Dipping alone extracted 90.7% (SE = 4.4%) of the amount produced by dipping plus homogenization. This improved extraction efficiency could be found with fresh tissues only, suggesting that intact glands may be important in order for effective exploitation of this method. Nevertheless, with a high-value product such as artemisinin, adoption of a method that improves yield by as much as 35% would be cost-effective, even if the method is some what more involved.

Glands are not always as conspicuous and well-differentiated as the peltate glands. For example, the generally cytotoxic hypericin is localized in undifferentiated glands found on the aerial organs of several species of *Hypericum* (St. John’s wort).³⁶ Hypericin appears to be the active compound in the antidepressant properties of St. John’s wort preparations.³⁷ It also has been found to have activity as an antiviral agent,^{38,39} as a pharmaceutical to prevent macular degeneration,⁴⁰ and as an anticancer drug.⁴¹ Hypericin is a red, polycyclic naphthodianthrone dye that generates singlet oxygen in the presence of light and molecular oxygen. Singlet oxygen is very cytotoxic, causing rapid membrane lipid peroxidation. This highly damaging process can cause cellular death. Hypericin is both herbicidal and insecticidal in light.^{42,43} The potential value of such a cytotoxic compound in defense of the plant is apparent. But how does the plant protect itself? We do not know the entire mechanism of protection, but we have sufficient information for informed speculation.

The hypericin-containing glands appear to the eye as almost black dots on stems, leaf margins, and flower petal margins (Figure 10.3). There appear to be two general types of glandular structures that produce the compound. *H. perforatum* L. has more flattened,

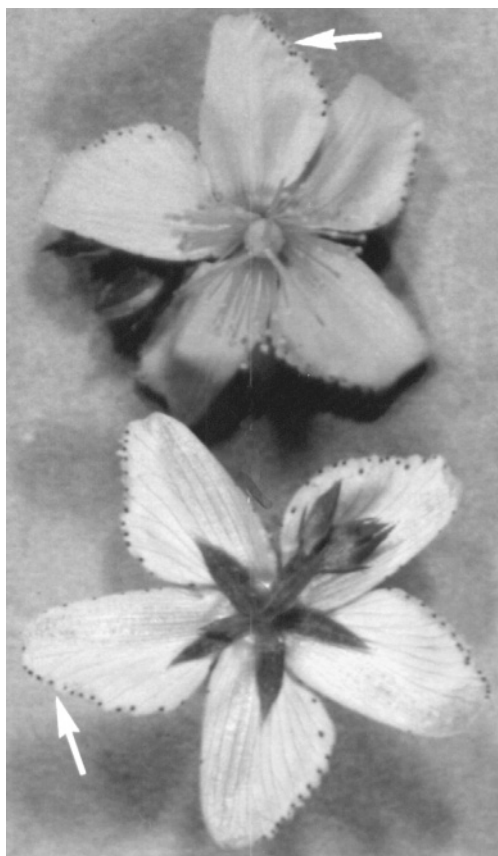


FIGURE 10.3

Leaf of *Hypericum perforatum* L. showing hypericin-containing glandular structures.

“nodular” structures,⁴⁴ whereas other species such as *H. hirsutum* L.⁴³ and *H. punctatum* Lam. display stalked nodules. We have found the pigment to be localized in spherical vacuolar inclusions of a group of relatively undifferentiated gland cells that are surrounded by more flattened, unpigmented cells (unpublished data). Isolated vacuoles from the pigment-containing cells are filled with hypericin. We speculate that hypericin is found entirely within these “droplets” and that the oxygen concentration within the droplet is insufficient for significant levels of singlet oxygen to be formed. One might expect oxidative bleaching of the pigment if singlet oxygen were formed at the rate expected at atmospheric oxygen levels.

Hypericin is a very expensive pharmaceutical at this time. We have not compared different extraction methods for hypericin. However, tissue and cellular localization information could be helpful in devising methods that produce higher yields.

10.2.2 Subepidermal Localization

In some cases, secondary products may be localized within a single cell layer within a leaf. For example, the mung bean leaf has a subepidermal layer of cells with a higher concentration of phenolic compounds in the vacuoles than in the vacuoles of other cell layers. These

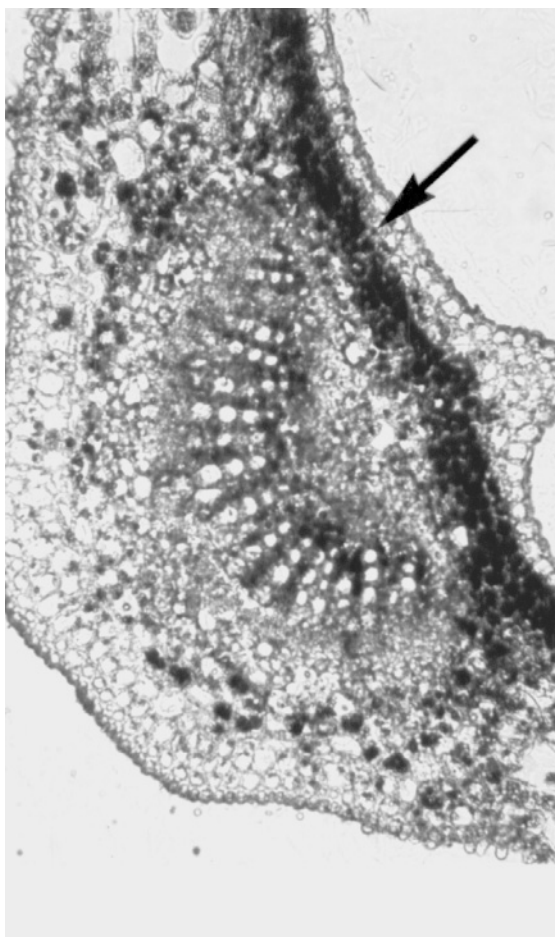


FIGURE 10.4

Cross section of *Erythroxylum coca* var. *coca* leaf stained with Dragendorff's solution. Note intensely stained subepidermal cells (→). (From Ferreira, J.F.S., Duke, S.O., and Vaughn, K.C., *Intl. J. Plant Sci.*, 159, 492, 1999. With permission.)

compounds are paired with high polyphenol oxidase (PPO) activities compartmentalized in the plastids^{45,46} (K. C. Vaughn, unpublished). When the cell is broken, the vacuolar phenolic compounds mix with the plastidic PPO, and highly reactive quinones are rapidly produced. These quinones could retard the activity of herbivores or pathogens.

A further example is that of the foliar localization of certain alkaloids. We have found tropane alkaloids to be localized primarily in the adaxial subepidermal layer (palisade parenchyma), as well as spongy mesophyll and vascular parenchyma cells of the leaves of both *Erythroxylum coca* var. *coca* and *E. novogranatense* var. *novogranatense* (Figure 10.4).⁴⁷ Other plant species also concentrate alkaloids in similar subepidermal layers of the foliage.^{e.g., 48} Furthermore, in *E. coca*, the alkaloids were found by immunocytochemistry to be localized within the vacuoles of these subepidermal cells.⁴⁷ The alkaloids appear to be aggregated around a core of phenolic compounds, making them perhaps immobile. Tropane alkaloids could be extracted easily by dipping fresh, young leaves in chloroform with up to 75% of the alkaloids of young leaves extracted with an 80 sec dip. This technique did not work with dried leaves.

What is the functional significance of the tissue and cellular location of these compounds? Several functions are plausible. Subepidermal localization may be a good location for plant defense by chemical toxicants. Plant pathogens and small herbivorous insects would quickly come in contact with the subepidermal layer during infection or grazing. But why are these compounds not found in all cell layers? We speculate that producing these compounds in all cells of the leaf would reduce the productivity of the plant due to some level of phytotoxicity and/or the high amount of resources required to produce the compounds. Their vacuolar localization within the cells indicates that they play no role in normal cellular functions. Further, localization may result in higher concentrations of toxicants at strategic sites and, thus, a more effective deterrent. Secondary compounds so localized probably have biological activity against one or more organisms with which the plant species has coevolved.

10.3 Conclusions

If phytotoxic compounds produced by higher plants are autotoxic, they must be sequestered to avoid self-poisoning. Conversely, if a compound has been sequestered, one might suspect that it could be a phytotoxin. Thus, the secondary compound contents of glands and specialized cell layers should be the most lucrative sources of phytotoxic allelochemicals. Plant-derived phytotoxins have been the sources of two commercial herbicides: a 1,8-cineole-derived herbicide, cinmethylin,⁴⁹ and the newly commercialized triketones,⁵⁰ a group of compounds derived from leptospermone, a constituent of the bottlebrush plant, *Calistemon* spp. As discussed above, the glandular localization of 1,8-cineole provided a clue to its activity. We are not aware of the tissue or cellular localization of leptospermone.

We have not discussed every mechanism or structure by which a plant can sequester a secondary compound and only a few examples are provided of the discussed sequestration mechanisms. Some highly potent phytotoxins, such as sorgoleone⁵¹ and polyacetylenes,⁵² are secreted or exuded by roots of producing plants rather than being sequestered. How these plants avoid autotoxicity is not known. However, their molecular target site as a herbicide may not reside in the roots and they may not be translocated to potential foliar sites of action.

A generally positive correlation between the degree of sequestration and the phytotoxicity of sequestered compounds might be expected. If so, one would expect compounds from peltate glands to generally be more phytotoxic than those from subepidermal layers. However, this and other aspects of the coevolution of anatomical features and secondary products will require considerably more study. Our understanding of the complex interactions between the selection pressures of biotic stress and autotoxicity in the shaping of anatomical structures and physiological mechanisms that aid in the safe and benign delivery of cytotoxic compounds to appropriate sites for plant defense is quite limited. A more complete understanding of these interactions will be useful in discovery of new uses for natural products and in production of these compounds by biosynthesis.

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Potent Mosquito Repellents from the Leaves of *Eucalyptus* and *Vitex* Plants

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CONTENT

- 11.1 Mosquito Repellent from *Eucalyptus* Oils
 - 11.2 Mosquito Repellent from *E. citriodora* Oil
 - 11.3 Mosquito Repellent from *E. camaldulensis* Oil
 - 11.4 New Natural Mosquito Repellent from *Vitex rotundifolia*
- References

ABSTRACT *N,N*-Diethyl-*m*-toluamide (DEET), which is a commercially available repellent against mosquitoes, has many disadvantages such as an unpleasant odor, skin penetration, and carcinogenicity. We have explored alternative repellents without such drawbacks. New repellents, *p*-menthane-3,8-diols (*cis* and *trans*), were isolated from the leaves of *Eucalyptus citriodora*. Especially the pentanoyl (C₅) and caproyl (C₆) esters of the diols exhibited much higher activity than DEET in terms of repellency and repellent durability against mosquitoes, *Aedes albopictus* and *Culex pipiens*. In addition, (+)-eucamalol, (1*R*, 6*R*)-(+)-3-formyl-6-isopropyl-2-cyclohexene-1-ol, which is a new mosquito repellent, was isolated from *E. camaldulensis* leaves. The absolute configuration was determined by synthesizing from (*S*)-(-)-perillaldehyde. While, from information of the traditional usage of *Vitex rotundifolia* for repelling mosquitoes at the southern part of Japan, we tried to isolate a mosquito repellent from the leaves. As a result, a cyclopentene dialdehyde, named rotundial, was identified.

11.1 Mosquito Repellent from *Eucalyptus* Oils

As a postdoctoral fellow at the University of California, Berkeley (1975 to 1977), I became interested in research on *Eucalyptus* oil (Nishimura, 1995). One day, I noticed that mosquitoes never appeared at the staff barbecues held near the *Eucalyptus* groves surrounding the campus. While enjoying the barbecues, I could smell *Eucalyptus* volatiles in the air and I observed that the volatile chemicals from *Eucalyptus* leaves repelled mosquitoes quite a distance from the groves.

N,N-Diethyl-*m*-toluamide (DEET) has been used as a repellent against blood-sucking insects such as mosquitoes all over the world. However, DEET has many drawbacks such

as an unpleasant odor and its skin penetration (Moody et al., 1986). Furthermore, DEET also reacts with certain plastics and synthetic rubber, resulting in considerable damage to eyeglasses and watchbands, pens, and other plastic items. Therefore, a search for new repellents lacking these undesirable properties has been undertaken.

A bioassay of repellent activity against mosquitoes was carried out according to the following protocol. Pupae of *Aedes albopictus* (Hatoyama race) were obtained from Laboratory of Parasitology at Teikyo University and incubated at 25°C for 3 weeks. The hatched adults were released into a cage (25 × 25 × 25 cm) made of stainless steel and nylon gauze, and the bioassay was performed in the cage. Female Wistar mice (Nippon SLC Ltd.), 6 to 7 weeks old were used. Test samples were diluted with acetone at concentrations of 1.0, 5.0, or 10 mg/ml. The acetone solution of each test sample was applied to a wire gauze bag (7 cm I.D. × 12 cm) at rate of 50 ml/m² (50, 250, and 500 mg/m²) and the bag was air-dried at room temperature. A mouse was then put into the bag and the bag placed in the cage of mosquitoes. Each test was run using 20 female mosquitoes which were 7 days old following emergence from pupae. The total number of mosquitoes landing on the mouse was counted immediately. The mouse was then taken out of the cage after 10 min, and the mosquitoes killed in a drying oven at 160°C. Each dead mosquito was crushed and the mosquitoes that had sucked blood were counted. Repellency (%) was calculated as

$$\frac{\text{Total mosquitoes} - \text{attracted mosquitoes}}{\text{Total mosquitoes}} \times 100\%$$

and feed inhibition as

$$\frac{\text{Total mosquitoes} - \text{bloodsucking mosquitoes}}{\text{Total mosquitoes}} \times 100\%$$

Leaves of several species of *Eucalyptus* which had been collected in Australia were cut into small pieces and extracted with acetone in a glass bottle at ambient temperature. Acetone extracts were steam-distilled to obtain essential oils. The repellent activities of the essential oils against mosquito (*Aedes albopictus*) are shown in Table 11.1. The larger number indicates the greater repellency. From this result, the essential oils from *E.citriodora* (lemon-scented gum tree) and *E.camaldulensis* (river red gum tree) had relatively high activities against mosquitoes.

TABLE 11.1
Repellent Activities of Several *Eucalyptus* Oils
Against Mosquito, *Aedes albopictus*

Essential oils	Repellency (%)	
	2.5 0	0.25 g/m ² Mouse Skin Area
<i>Eucalyptus radiata</i>	54	24
<i>Eucalyptus citriodora</i>	90	43
<i>Eucalyptus viminalis</i>	27	—
<i>Eucalyptus camaldulensis</i>	93	60
<i>Eucalyptus pulverulenta</i>	16	—
<i>Eucalyptus globulus</i>	—	30
<i>Cinnamomum camphora</i>	0	0

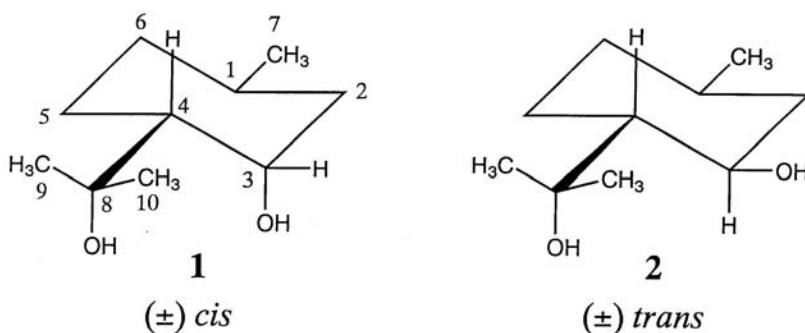


FIGURE 11.1

Chemical structures of *p*-menthane-3,8-diols (*cis* and *trans*) from *E. citriodora* leaves.

11.2 Mosquito Repellent from *E.citriodora* Oil

Silica gel column chromatography of the *E.citriodora* essential oil was guided by the bioassay of repellent activity against mosquitoes. Subsequently, two active crystalline compounds were isolated.

The spectral interpretation of the chemicals isolated from *E. citriodora* oil gave rise to the identification of *p*-menthane-3,8-diols (*cis* type 1; 4.5 mg/g fresh weight leaves and *trans* type 2; 2.2 mg/g fresh weight leaves) as shown in Figure 11.1 (Nishimura et al., 1986; Nishimura and Mizutani, 1989). The physicochemical data were as follows:

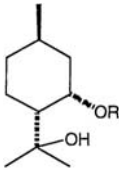
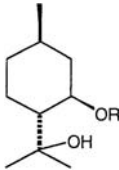
p-Menthane-3,8-*cis*-diol (1). Mp 81.0-82.5° (crystallized from Et₂O-hexane), $[\alpha]_D^{23} \pm 0^\circ$ (CHCl₃; c = 0.2), IR $\nu_{\text{max}}^{\text{KBr}}$ cm⁻¹ 3240, 2930, 2900, 1450, 1420, 1250, 1160, 930. High resolution FIMS m/z (rel.int.): 173.1532 [M+H]⁺ (16), 157 [M-CH₃]⁺ (37), 154 [M-H₂O]⁺ (100), 114 (9), 96 [M-OH-hydroxyisopropyl]⁺ (83), 77 (41), 59 (85). EIMS (probe) 70 eV, (rel.int.): no. M⁺ peak, 157 [M-CH₃]⁺ (1), 154 [M-H₂O]⁺ (2), 139 [M-H₂O-CH₃]⁺ (3), 121 (2), 111 (2), 96 (40), 81 [M-OH-hydroxyisopropyl-CH₃]⁺ (100), 68 (6), 59 (79), 55 (21), 54 (21), 43 (42), 41 (34). ¹H NMR (200 MHz, CDCl₃, TMS): 0.87 (3H, d, J = 6.4 Hz, H-7), 1.22 (3H, s, H-9), 1.36 (3H, s, H-10), 4.41 (1H, q, J = 2.4 Hz, H-3). ¹³C NMR (50 MHz, CDCl₃, TMS): δ 20.4 (*t*, C-5), 22.3 (*q*, C-7), 25.7 (*d*, C-1), 28.8 (*q*, C-9), 29.0 (*q*, C-10), 35.0 (*t*, C-6), 42.6 (*t*, C-2), 48.4 (*d*, C-4), 68.1 (*d*, C-3), 73.3 (*s*, C-8).

p-Menthane-3,8-*trans*-diol (2). Mp 77.3-78.3° (from Et₂O-hexane), $[\alpha]_D^{23} \pm 0^\circ$ (CHCl₃; c = 0.1). IR $\nu_{\text{max}}^{\text{KBr}}$ cm⁻¹ 3250, 2960, 2920, 1450, 1420, 1220, 1180, 1000, 910, 870. High resolution FIMS m/z (rel.int.): 173.1546 [M+H]⁺ (47), 157 [M-CH₃]⁺ (11), 154 [M-H₂O]⁺ (30), 114 (10), 113 (15), 96 [M-OH-hydroxyisopropyl]⁺ (54), 77 (87), 59 (100). EIMS (probe) 70 eV, (rel.int.): no. M⁺ peak, 157 [M-CH₃]⁺ (1), 154 [M-H₂O]⁺ (1), 139 [M-H₂O-CH₃]⁺ (3), 121 (2), 111 (1), 96 (38), 81 (100), 68 (10), 59 (90), 55 (13), 54 (20), 43 (34), 41 (19). ¹H NMR (200 MHz, CDCl₃, TMS): δ 0.92 (3H, d, J = 6.4 Hz, H-7), 1.22 (6H, s, H-9 and H-10), 3.72 (1H, dt, J = 10.4, 4.3 Hz, H-3). ¹³C NMR (50 MHz, CDCl₃, TMS): δ 22.0 (*q*, C-9), 23.8 (*q*, C-10), 27.1 (*t*, C-5), 30.1 (*q*, C-7), 31.4 (*d*, C-1), 34.6 (*t*, C-6), 44.7 (*t*, C-2), 53.5 (*C*-4), 72.9 (*d*, C-3), 75.0 (*s*, C-8).

To explore repellents with higher activity against mosquitoes, several esters of *p*-menthane-3,8-diols were prepared. The repellent activities of the esters are shown in Table 11.2. From this result, the pentanoyl (C₅) and caproyl (C₆) esters of *p*-menthane-3,8-*cis*-diol had

TABLE 11.2

Repellent Activities of *p*-Menthane-3,8-diols and their Derivatives Against Mosquito, *Aedes albopictus*

		
<i>cis</i>		<i>trans</i>
Repellency (%)	R	Repellency (%)
59	H	52
22	COCH ₃	15
38	COCH ₂ CH ₃	26
30	CO(CH ₂) ₂ CH ₃	—
72	CO(CH ₂) ₃ CH ₃	—
67	CO(CH ₂) ₄ CH ₃	—
65	DEET	

Note: Sample concentration: 30 mg/m² mouse skin area.

higher activity than DEET which is a commercially available repellent. Interestingly enough, the caproyl ester had higher repellent durability than DEET (unpublished data).

11.3 Mosquito Repellent from *E. camaldulensis* Oil

As shown in Table 11.1, the essential oil from *E. camaldulensis* leaves had significant repellent activity against *Aedes albopictus* (Nishimura et al., 1986). These observations prompted us to purify the mosquito repellent in the *E. camaldulensis* essential oil.

The leaves of *E. camaldulensis* (1.5 kg) were collected in Matsudo, Chiba prefecture, Japan. They were cut into small pieces and steam-distilled to yield 1.6 g of the essential oil. The oil was purified by successive preparative TLC to give two repellents against mosquito.

Bioassay of repellent activity against mosquitoes was carried out according to the following methods: A chick whose abdominal feathers were removed with a haircutter was fixed on a wood board (7 × 15 cm). The shaved abdominal skin of the chick (2.5 × 4 cm) was exposed and an ethanol solution of a test compound was applied to the skin (1.5 g/m²). About 500 adult mosquitoes which were 6 to 8 days old after emergence (*Aedes aegypti*, approximately equal numbers of females and males) were released in a cage (21 × 21 × 30 cm) made of stainless steel and nylon gauze. Two chicks (one was treated and the other untreated) were put in the cage for 2 min. Then, the total number of landed mosquitoes on each chick was counted. Repellency (%) was calculated according to following equation.

$$\text{Repellency (\%)} = 1 - \frac{\text{Number of landed mosquitoes on treated chicks}}{\text{Number of landed mosquitoes on untreated chicks}} \times 100$$

The repellency was evaluated every hour after treatment until the repellency was reduced to less than 80%.

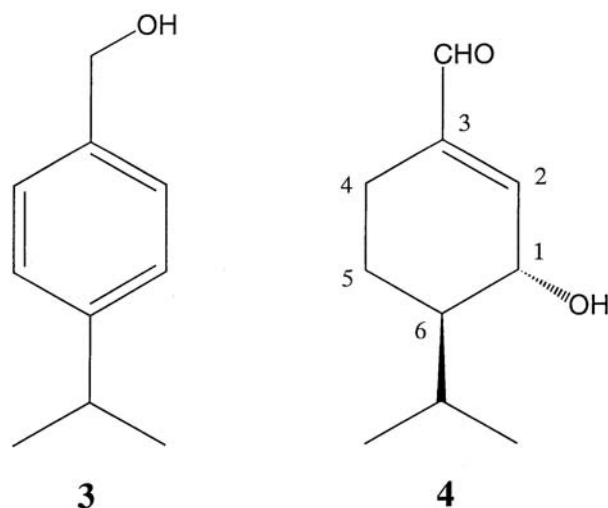


FIGURE 11.2

Chemical structures of 4-isopropylbenzyl alcohol (**3**) and (+)-eucamalol (**4**) from *E. camaldulensis* leaves.

TABLE 11.3

Repellent Activities of 4-Isopropylbenzyl alcohol (**3**), (+)-Eucamalol (**4**) and DEET against the Yellow Fever Mosquito, *Aedes aegypti*

Compounds	Repellency (%)			
	0 h ^a	1 h	2 h	3 h
3	100	23	–	–
4	100	92	87	75
DEET	100	84	55	–

Note: Sample concentration: 1.5 g/m² chick skin area.

^a Immediately after treatment.

The bioassay-guided chromatography of the essential oil from *E. camaldulensis* leaves gave rise to the isolation of two active principles. From the spectral interpretation, two repellents were identified as 4-isopropylbenzyl alcohol (**3**) and a new compound, (+)-eucamalol (**4**) (3-formyl-6-isopropyl-2-cyclohexen-1-ol) (Watanabe et al., 1993) as shown in Figure 11.2.

The mosquito-repelling activity of 4-isopropylbenzyl alcohol (**3**) and eucamalol (**4**) were examined against *A. aegypti* (Table 11.3) in comparison with that of DEET. All tested compounds exhibited potent mosquito repelling activities against *A. aegypti* immediately after the application. In 1 h after treatment, the effectiveness of **3** was lost. Although the duration of the effectiveness for DEET was within 2 h after the treatment by our test method, eucamalol (**4**) showed 75% repellency even 3 h after the treatment. This result indicates that the repellency of eucamalol against *A. aegypti* is superior to that of DEET.

4-Isopropylbenzyl alcohol(**3**):MS,m/z 150 [M]⁺, 132(M-H₂O)⁺(base peak), 107 [M-C₃H₇]⁺;
¹H NMRδ_{CDCl₄}^{TMS} 7.05(2H,d,J = 7.0 Hz), 6.95(2H,d,J = 7.0 Hz), 4.00(2H,s,CH₂OH),
 2.85(1H,m,CH), 1.00 (6H, d,J = 6.8Hz).

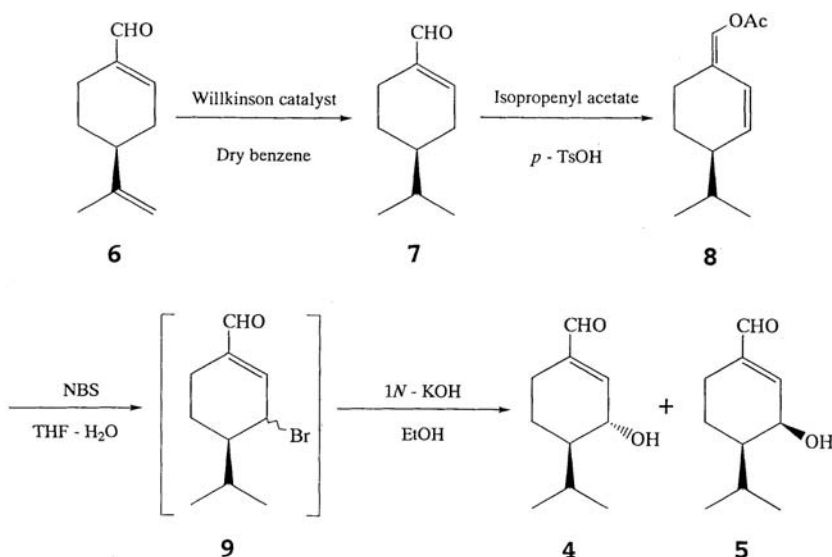


FIGURE 11.3

Synthesis of (+)-eucamalol (**4**) and its 1-epimer (**5**) from (S)-(-)-perillaldehyde (**6**).

(+)-Eucamalol(**4**): $[\alpha]_D^{25} +13.5^\circ$ ($c = 0.80$, CH_3OH); HRMS m/z 168.1151 ($\text{C}_{10}\text{H}_{16}\text{O}_2$, Calcd. 168.1150); MS, m/z 168 $[\text{M}]^+$, 139 $[\text{M}-\text{CHO}]^+$, 125 $[\text{M}-\text{C}_3\text{H}_7]^+$, 69(base peak); $\text{IR}_{\text{max}}^{\text{KBr}}$ 3400(OH group), 2980, 1680(conjugate aldehyde) cm^{-1} ; $^1\text{H NMR}_{\text{CDCl}_3}^{\text{MS}}$ 9.45(1H,s), 6.63(1H,d, $J = 2.2\text{Hz}$), 4.28(1H,dd, $J = 2.2\&9.3\text{Hz}$), 2.37(1H,m), 2.08(1H,m), 2.04(1H,m), 1.78(1H, m), 1.39(1H,m), 1.24(1H,m), 0.98(1H,d, $J = 6.8\text{Hz}$), 0.84(1H,d, $J = 6.8\text{Hz}$).

However, it was difficult to determine the absolute configuration of (+)-eucamalol from these results. Synthesis of (+)-eucamalol (**4**) and its 1-epimer (**5**) from (S)-(-)-perillaldehyde (**6**) was carried out to determine the absolute configuration and compare their repellent activities against *Aedes albopictus*. The synthetic scheme is shown in Figure 11.3.

(S)-(-)-Perillaldehyde (**6**) was converted to 8,9-dihydro-perillaldehyde (**7**) by homogeneous hydrogenation with *tris*(triphenylphosphine)rhodium chloride as a catalyst in a 73% yield. Conversion of **7** to 3-bromo-8,9-dihydroperillaldehyde (**9**) was performed by the procedure of Ishihara et al. (1990). Enol acetylation of **7** with isopropenyl acetate gave an enol acetate (**8**) in a 38% yield. This enol acetate (**8**) was brominated by *N*-bromo-succinimide. Since 3-bromo-8,9-dihydro-perillaldehyde (**9**) was unstable, nucleophilic substitution of bromide (**9**) was subsequently carried out by treating with potassium hydroxide to give two alcohols, (+)-eucamalol (**4**) and (-)-1-epi-eucamalol (**5**) in yields of 7.7 and 8.4%, respectively (Satoh et al., 1995).

The $J_{1,6}$ value (9.2Hz) of synthetic (+)-eucamalol (**4**) shows *axial-axial* coupling, while the smaller $J_{1,6}$ value (<2.0 Hz) of synthetic (-)-1-epi-eucamalol (**5**) shows *axial-equatorial* coupling. Thus, the $J_{1,6}$ value of synthetic (+)-eucamalol (**4**) indicates that the relative configuration at C-1 and C-6 was, like that of natural (+)-eucamalol, of *trans*-form. The optical rotation of synthetic (+)-eucamalol was $+14.1^\circ$ in methanol, and was very close to the optical rotation of natural eucamalol, $[\alpha] = +13.5^\circ$ ($c = 0.80$, MeOH) (Watanabe et al., 1993). Consequently, the absolute configuration of (+)-eucamalol was determined to be (1*R*,6*R*)-(+)-3-formyl-6-isopropyl-2-cyclohexen-1-ol (Satoh et al., 1995).

The repellent activity of synthetic eucamalol and its epimer were evaluated by using *Aedes albopictus* as the test mosquito strain (Table 11.4). (+)-Eucamalol and its epimer had

TABLE 11.4

Repellent and Feeding Inhibition Activities of (+)-Eucamalol and its (–)-1-Epimer Against *Aedes albopictus*

Repellent Activity (RA)

Compounds	RA(%)		
	500	250	50 g/m ² Mouse Skin Area
(+)-Eucamalol	100.0	100.0	84.2
(–)-epi-Eucamalol	100.0	100.0	75.0
DEET	100.0	100.0	80.0

$$RA = \frac{\text{Total mosquitoes} - \text{attracted mosquitoes}}{\text{Total mosquitoes}} \times 100\%$$

Feeding Inhibitory Activity (FIA)

Compounds	FIA(%)		
	500	250	50 g/m ² Mouse Skin Area
(+)-Eucamalol	100.0	100.0	74.5
(–)-epi-Eucamalol	100.0	100.0	65.0
DEET	100.0	100.0	85.0

$$FIA = \frac{\text{Total mosquitoes} - \text{bloodsucking mosquitoes}}{\text{Total mosquitoes}} \times 100\%$$

repellent and feeding inhibitory activities against *A. albopictus* to the same degree as DEET. In addition, both the repellent and feeding inhibitory activities of (+)-eucamalol were the same as that of its epimer. Structure–activity relationships will be presented elsewhere.

11.4 New Natural Mosquito Repellent from *Vitex rotundifolia*

Vitex rotundifolia has long been used as a medicinal plant for a headache and a cold, and various compounds such as flavonoid, iridoid glycosides, diterpenoids, and sesquiterpenoids have been identified in this plant (Kimura et al., 1967; Asaka et al., 1973; Tada and Yasuda, 1984). It also has been reported that the leaves and twigs of this plant can be used for repelling mosquitoes (Okuda, 1967). However, the principle responsible for its activity has not been previously studied. Hence, we investigated the mosquito-repelling principle of *V. rotundifolia* and isolated a new natural cyclopentene dialdehyde named rotundial (**10**) as shown in Figure 11.4. In this report, we describe the identification and mosquito repelling activity of rotundial.

The volatile constituents of fresh leaves (5 kg) from *V. rotundifolia* were subjected to silica gel column and preparative thin-layer chromatography, eluting with hexane-EtOAc (2:1, v/v), to afford 250 mg of rotundial (**10**, 0.005%) as a colorless and odorless oil; C₆H₁₂O₂ [high resolution MS (HRMS), M⁺, m/z 152.0780, [calcd. as 152.0837], [α]_D²⁵+39.3°] (c = 1.0, CHCl₃). The existence of both a simple aldehyde and an α,β-unsaturated aldehyde in **10** was confirmed by UV (λ_{max} 250nm, ε = 11,000), IR (ν_{max} 1720 and 1665 cm^{–1}) and NMR spectral

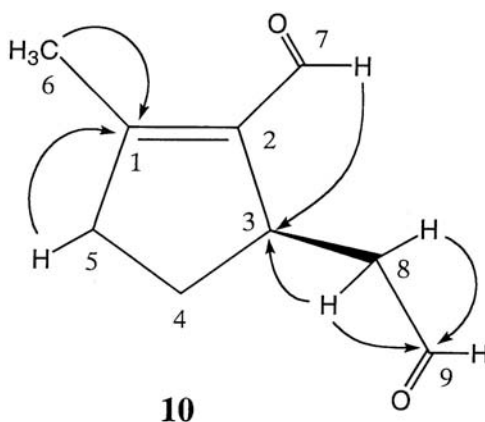
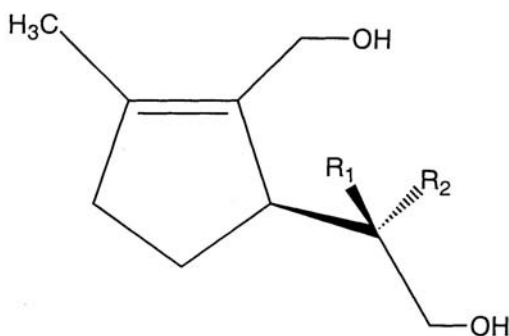


FIGURE 11.4

Chemical structure of rotundial (**10**) and correlations of its COLOC spectrum (\rightarrow).

data [$^1\text{H}\delta$: 9.93(1H,*s*) and 9.70(1H,*dd*, $J = 2.0$ and 2.0 Hz); $^{13}\text{C}\delta$: 201.9(*d*) and 187.9(*d*)]. The ^1H -NMR spectrum of **10** showed a broad singlet at $\delta 2.15$ (3H, $-\text{C}=\text{C}-\text{CH}_3$), two double double doublets at $\delta 2.88$ (1H, $J = 2.0, 4.4$ and 17.0 Hz) and 2.32 (1H, $J = 2.0, 9.1$ and 17.0 Hz) assignable to the methylene protons adjacent to the simple aldehyde (CH_2CHO). The ^{13}C -NMR signals at $\delta 164.2$ (*s*) and 139.0 (*s*) indicated full substitution of the double bond in **10**. The ^1H -NMR signals indicated the existence of a $\text{CH}_2\text{CH}_2\text{CH}$ moiety which formed a cyclopentene ring. The long-range correlations from its COLOC spectrum (Kessler et al., 1984) established the structure of rotundial as **10** as shown in Figure 11.4 (Watanabe, 1995).

The absolute stereochemistry of **10** was then determined. The LiAlH_4 reduction of **10** gave rotundiol (**11**; $[\alpha]_D^{25} -16.6^\circ$). The optical rotation data ($[\alpha]_D$) for its methylated (at C-8) derivatives, (3*R*,8*S*)-isodehydroiridodiol (**12**) and (3*R*,8*R*)-isodehydroiridodiol (**13**) (Figure 11.5) were -20.7° and -15.3° , respectively, suggesting the 3*R* configuration of **11** (Sakai et al., 1980). Accordingly, the configuration of **10** at C-3 was deduced to be *R*. This is



- 11:** $\text{R}_1=\text{R}_2=\text{H}$ Rotundiol
12: $\text{R}_1=\text{CH}_3$, $\text{R}_2=\text{H}$ (3*R*,8*S*)-dehydroiridodiol
13: $\text{R}_1=\text{H}$, $\text{R}_2=\text{CH}_3$ (3*R*,8*R*)-isodehydroiridodiol

FIGURE 11.5

Chemical structures of rotundiol (**11**), (3*R*,8*S*)-dehydroiridodiol (**12**) and (3*R*,8*R*)-isodehydroiridodiol (**13**).

TABLE 11.5

Repellent Activities of Rotundial (**10**) and DEET
Against the Yellow Fever Mosquito, *Aedes aegypti*

Compounds	Repellency (%)			
	0h ^a	1h	2h	3h
Rotundial	100	90	85	70
DEET	100	90	50	35

Note: Sample concentration: 1.5 g/m² chick skin area.

^a Immediately after treatment.

the first isolation of rotundial (**10**) from a natural source, compared with enzymatic or acid hydrolysis of the aucubin-terated iridoid giving compound **10** (Bianco et al., 1977).

The mosquito repelling activity of rotundial (**10**) was examined against *Aedes aegypti* in comparison with that of DEET (Table 11.5). Both compounds had potent repellent activity against *A. aegypti* immediately after the application, while 1 h after treatment, the effectiveness of **10** was almost equivalent to that of DEET. Two h after treatment, the repellency of **10** became higher than that of DEET, indicating that the mosquito repelling activity of rotundial (**10**) was superior to that of DEET in respect to its long-lasting effectiveness.

A monoterpene dialdehyde, chrysomedial, has been isolated from larvae of the chrysomelid beetle, *Plagiodra versicolora*, as its defensive substance (Meinwald et al., 1977; Meinwald and Jones, 1978). The structural similarity between **10** and chrysomedial leads us to speculate that rotundial (**10**) might serve the plant as a defensive principle against insect attack which will be the subject of further investigation.

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Arthropod Semiochemicals as Multifunctional Natural Products

Murray S. Blum

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12.1 Introduction

The number of arthropod species (insects, millipedes, spiders, mites, crabs, and related groups) is truly fulsome, conservatively numbering between 1,000,000 and 5,000,000 species, and constituting about 80% of all kinds of animals.¹ The beetles alone (~500,000 species) dominate the insects¹ and the vertebrates as well; there are at least 10 times more beetle species than vertebrate species,² and new species of beetles are being described daily.

Significantly, a large variety of unique compounds has already been identified as arthropod natural products,³ notwithstanding the fact that relatively few species have been subject to analytical scrutiny. Clearly, for arthropod natural products the best is yet to come and considering what has already been established, the future would appear to be remarkably bright both in terms of structural chemistry and functionality.

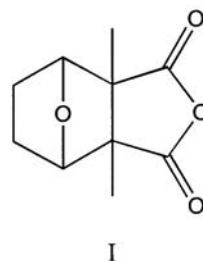
In this brief review, the ability of arthropods to biosynthesize a variety of novel compounds and to utilize these glandular products multifunctionally will be emphasized as critical developments that have enhanced the competitiveness of the arthropods. Particular emphasis will be placed on semiochemicals, signaling agents utilized in intra- and inter-specific contexts.

12.2 Multifunctional Semiochemicals

Arthropods are the paramount producers of semiochemicals in the animal kingdom and these compounds have been adapted to subserve a variety of important functions for selected groups of these invertebrates. For example, the virtuosity of arthropods as producers of alkaloids chiefly reflects the biosynthetic prowess of ants which generate most of these compounds as poison gland products that often possess diverse functions⁴ both inside and outside of the colonial milieu. In short, the success of many arthropod species is clearly identified with both the synthesis and adaptive utilization of semiochemical natural products. In the discussion that follows, representative semiochemicals, each of which has been demonstrated to possess a considerable diversity of important biological roles, will be examined as examples of what has been referred to as *pheromonal parsimony*.⁵

12.2.1 Cantharidin

Cantharidin, the anhydride of cantharidic acid (I), is produced by beetles in the family Meloidae and a few related families and has been referred to as Spanish fly for hundreds of years.⁶ These insects are sometimes described as blister beetles because, when disturbed, they discharge cantharidin-fortified blood from their legs and this exudate can cause severe dermal lesions in vertebrates and also can repel invertebrates.⁷ As we shall see later, the powerful vesicatory properties of cantharidin have been indirectly responsible for its widespread use as a putative sexual stimulant.



Cantharidin possesses potent antifungal activities; the female beetle is reported to coat her eggs with this compound, a potent growth inhibitor for *Microsporum* and *Trichophyton* species.⁸ Since the eggs of the beetles are incubated in warm and moist environments which favor the growth of invasive fungi, the main function of this terpenoid anhydride is probably to protect developing blister beetle embryos from insect-attacking fungi.⁹ Consistent with this suggestion is the fact that during copulation male beetles actually transfer large amounts of cantharidin to the females as a copulatory "bonus."¹⁰ The transferred cantharidin is used to coat the eggs in what is almost certainly an antifungal strategy.

In addition to its function as both a vesicant and a fungicide, cantharidin has been tested as a powerful anthropogenic agent in a wide variety of human systems, dedicated to either treating pathological conditions or attempting to enhance sexual ambitions.

Complete remission of epidermal cancer in pigs has been effected by topical treatment with cantharidin.¹¹ In addition, this vesicatory anhydride when applied in low doses, resulted in the total remission of dermal cancer in human beings.¹² Cantharidin, which is structurally similar to a well-known herbicide, is highly active as a selective herbicide.¹³

Spanish fly has a long history in medicine, having been used by Hippocrates as a cure for dropsy nearly 2000 years ago.¹⁴ This compound was considered to be of great medicinal value and was a major drug in the treatment of bladder and kidney infections and stones, strangury, dropsy, and certain venereal diseases.¹⁵ Painful conditions such as pleurisy and sciatica were routinely treated with cantharidin as further testimony to the high esteem with which this drug was held in medical circles. However, it is the long-standing reputa-

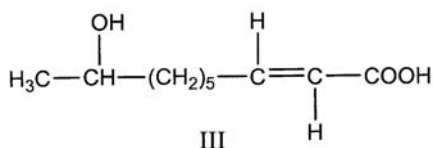
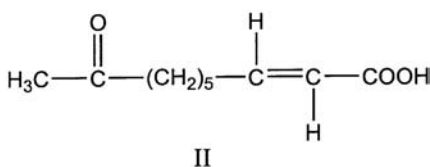
tion of this compound as a putative aphrodisiac that has led to its use as a sexual stimulant and in some cases, an abortifacient.

Although cantharidin in minute doses can cause bladder and kidney irritation and painful voiding of urine with a sensation of burning, these symptoms hardly justify its reputation as a powerful aphrodisiac. Many women have died of cantharidin poisoning. Indeed, the Marquis de Sade was beheaded in absentia for feeding aniseed sweets treated with cantharidin to two prostitutes who identified de Sade before they died.¹⁶ The corrosive action of the anhydride, first seen in skin lesions, is particularly devastating when it is ingested, causing corrosion of tissues in the mouth, especially the palate. Curiously, cantharidin has a completely unexpected effect on the human male.

In two instances French troops in North Africa ate the legs of frogs that had eaten copious numbers of blister beetles. The frogs legs contained cantharidin that had been absorbed from the ingested beetles. In both cases the soldiers experienced a potpourri of minor medical problems but the troops were particularly inconvenienced by a painful priapism.¹⁷⁻¹⁸ The erections incapacitated the Zouaves as effectively as any enemy action. "The Beetle of Aphrodite" had struck again!

12.2.2 Multifunctional Queen Pheromones

Pheromonal parsimony may reach its highest expression in the honey bee, *Apis mellifera*, in terms of social communication in the arthropods. The mandibular glands of the queen bee biosynthesize a complex mixture of acids and esters which is dominated by a few novel C₁₀ compounds. The activities of one of these glandular constituents, the queen substance or (*E*)-9-oxo-2-decenoic acid (9-ODA) (II), appears to be synergized by other compounds produced in the mandibular glands.¹⁹ One of these, (*E*)-9-hydroxy-2-decenoic acid (9-HDA) (III), also possesses several pheromonal roles of its own, further demonstrating the elegant control of sociality achieved with the queen's mandibular gland products. The utilization of a limited series of very characteristic natural products as behavioral regulators for *both* workers and drones has enabled queen bees to "fine tune" — and control — the social structure of populous honey bee colonies.



9-ODA possesses two critical primer activities that are synergized by other compounds such as 9-HDA; neither compound is active alone. Primer pheromones do not produce an immediate response but rather, exhibit a delayed response which may occur after 24 hours or more and frequently involves the reproductive system. For example, ovarian develop-

ment of the nonreproductive workers is inhibited by 9-ODA, but the workers must have physical contact with the queen in order for the the pheromonal activity to be expressed.²⁰ Another primer activity results in inhibiting worker activity leading to the construction of queen cells, thus enabling the queen to pheromonally suppress the rearing of potential competitors.²¹ In addition to these primer functions that occur in the milieu of the hive, remarkable multifunctionality characterizes the roles of 9-ODA (plus synergists) both in and out of the hive. In the hive, workers are attracted to the queen by 9-ODA and form characteristic retinues around her that can result in food and pheromonal exchange.²² 9-ODA also possesses a major releaser role for the workers, but this is expressed outside the hive. The swarming of honey bees resulting in the selection of new nesting sites is controlled by a medley of pheromones. The formation and movement of swarms is regulated by worker (scout) pheromones that are secreted from an abdominal structure, the Nasanov gland. The Nasanov secretion is dominated by a mixture of terpenes (e.g., geraniol, citral, farnesol) that is ideally suited to attract other scout bees to a potential nesting cavity that has been marked with the secretion.²³ Oriented swarm movement is realized if the queen secretes 9-ODA which acts as a short-range attractant for workers. In the absence of the Nasanov terpenes, 9-ODA is unattractive to the swarming bees. 9-ODA has another releaser function, but in this case it is with drone bees rather than with workers. This acid is a powerful sex attractant for male bees, attracting large numbers of drones at altitudes of at least 200 m.²⁴ Only 9-ODA is required for powerful sex attractancy, in contrast to the need for glandular synergists when this compound functions as a pheromone for workers. 9-ODA is a highly specific sex pheromone, closely related compounds exhibiting no activity whatsoever.²⁵

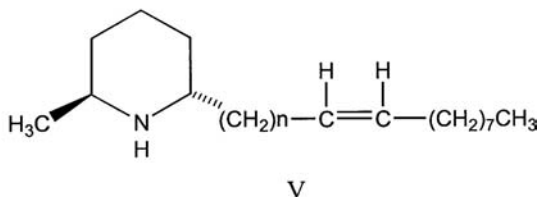
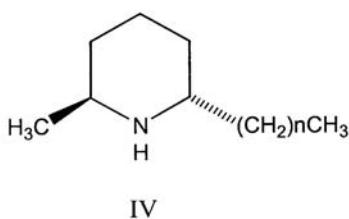
12.2.3 Venomous Alkaloids

Many groups of ants in the subfamily Myrmicinae characteristically produce poison gland secretions that are dominated by alkaloids, rather than the proteins typical of the ants' relatives, bees and wasps. A large variety of alkaloids are produced by ants and characteristic compounds are identified with different genera.²⁶ However, while a variety of pharmacologically active compounds are biosynthesized by these insects, many are not injected into their assailants. Diverse alkaloid-producing ants do not have a functional sting, and the externalized venom is smeared on adversaries often packing a topical wallop. On the other hand, a variety of ant species attack their enemies by introducing venom by hypodermic injection and the deterrent effects can be considerable. Nevertheless, notwithstanding their method of introduction to an adversary, venomous alkaloids have been demonstrated to possess great multifunctionality as defensive compounds.

12.2.4 2-Alkyl-6-Methylpiperidines

Mixtures of *cis*- and *trans*-2-alkyl-6-methylpiperidines have been identified as poison gland products of workers and queens of ants in the genus *Solenopsis*, the *trans*-isomers generally predominating in the venom of workers. The known dialkylpiperidines in the genus *Solenopsis* possess long *n*-alkyl groups that are either saturated (IV) or contain a carbon-carbon double bond at the ninth carbon from the terminal methyl group (V).²⁷

Members of the subgenus *Solenopsis* are known as fire ants because of the great pain that is associated with the worker sting. A concatenation of toxicological and pharmacological events occurs when *Solenopsis* venom is introduced subdermally into a human. An examination of the diverse activities of the venom alkaloids emphasizes their multifunctionality as agents of deterrence against vertebrates.



Stings of *Solenopsis* workers produce pronounced dermal necrosis followed by the formation of pruritic and sterile pustules.²⁸ The alkaloids liberate histamine from mast cells resulting in considerable algogenicity, a reaction that intensifies the deterrent effectiveness of the alkaloids.²⁹ In addition to these reactions, the dialkylpiperidines possess powerful lytic activity, instantly hemolyzing mammalian erythrocytes.³⁰ In addition to these toxicological effects, the alkaloids demonstrate their not inconsiderable multifunctionality by exhibiting a great range of pharmacological activities that are unleashed against a variety of biochemical systems. These compounds are strong inhibitors of ATPases³¹ and in addition, they reduce mitochondrial respiration and uncouple oxidative phosphorylation.³²

The ability of the dialkylpiperidines to also block neuromuscular junctions³³ further identifies these compounds as very versatile defensive agents. However, in addition to their activities against pharmacological targets, these alkaloids exhibit considerable semiochemical parsimony in a variety of ecological contexts. The poison gland products possess a wide range of antimicrobial properties and, in addition, they are phytotoxic as well, further identifying these compounds as versatile agents of semiochemical multifunctionality.³⁴ The 2,6-dialkylpiperidines possess powerful antibacterial activity against a variety of species³⁵ and in addition, these compounds are potent growth inhibitors of diverse fungal species.³⁶ These alkaloids also exhibit considerable activity as insecticides which compares to that of commercial insecticides.³⁷ Furthermore, the ability of these compounds to exhibit insecticidal activity when applied either topically or by injection, enables fire ants to be both effective aggressors and predators.

The fire ant's offensive arsenal is further expanded by the well-developed repellency to ants exhibited by the 2,6-dialkylpiperidines,³⁸ which should allow these ants to successfully compete with other ants for critical resources. In some cases repellency of different ant species is achieved by dispersing venom through the air (gaster flagging),³⁹ but in the milieu of the nest dispersion of venom by workers is utilized to treat vulnerable fire ant larvae with the antibiotic alkaloids.⁴⁰ Similarly, the queen fire ant treats her freshly laid eggs with her poison gland contents, and the concentration of alkaloids is high enough to inhibit the growth of entomopathogenic fungi.⁴¹ The chemical ecology of the fire ants vis-à-vis the 2,6-dialkylpiperidines constitutes a highly adaptive system for both exploiting the acquisition of food and protecting the immatures from intrusive microorganisms.

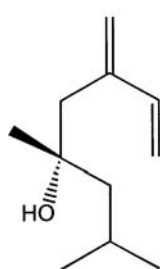
12.2.5 Pheromones as Interspecific Inhibitors

Many species of bark beetles in the family Scolytidae have evolved a unique strategy for attacking coniferous trees and utilizing these often formidable plants as sites for mating, feeding, and reproduction. Optimal utilization of the tree as a resource requires that the bark beetles develop a large enough population to overcome the mostly chemical defenses of the target tree. In addition, maximum utilization of the trees' not inconsiderable resources also requires the establishment of large numbers of immature and adult beetles to convert the tree into an attractive target for additional bark beetles of the *same* species. This scenario presupposes that successful colonization of a pine tree will be achieved by a single species of bark beetle. Since bark beetles utilize aggregation pheromones to attract members of their own species, these volatile information-bearing compounds would seem to constitute ideal agents for guaranteeing the specific integrity of the "chosen" tree. And indeed it has been established that the pheromonal attractants of selected species of bark beetles possess critical multifunctional value as inhibitors of the attraction of competitive beetle species.⁴²

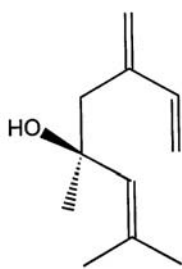
Aggregative pheromones are frequently synthesized by bark beetles from monoterpenes derived from the host's oleoresin. Novel mixtures of oxygenated monoterpenes have been identified as aggregative pheromones in species in the genera *Ips* and *Dendroctonus* and these compounds have been demonstrated to function, under field conditions, as both intra- and interspecific agents of mutual interruption.⁴² For example, colonizing beetles of the species *Ips paraconfusus* produce an aggregation pheromone that is dominated by the monoterpenes ipsenol (VI), ipsdienol (VII), and *cis*-verbenol (VIII).⁴³ These compounds, which are mainly produced by male beetles, constitute a true synergistic pheromone, all three compounds being required for attraction to the host, ponderosa pine. On the other hand, the attraction of another *Ips* species, *I. latidens*, which also attacks ponderosa pine, is inhibited by the ternary mixture employed by *I. paraconfusus* as an aggregation pheromone.⁴⁴ Nevertheless, *I. latidens* responds to both ipsenol alone and to ipsenol plus *cis*-verbenol. However, when the complete *I. paraconfusus* aggregation pheromone containing ipsdienol, ipsenol, and *cis*-verbenol is presented to *Ips latidens*, the attraction of adults of *I. latidens* is completely inhibited. Whereas adults of *I. paraconfusus* convert myrcene to ipsdienol,⁴⁵ an important aggregation pheromone for this species,⁴³ adults of *I. latidens* do not produce this oxygenated monoterpene and it functions as a powerful inhibitor of aggregation when they encounter it.

Mutual interruption of attraction can occur when species in the same genus inhabit the same host tree. For example, males of *I. pini* and *I. paraconfusus*, boring side by side in logs of ponderosa pine, attract fewer beetles of both species than logs containing males of a single species. Furthermore, the response of *I. pini* is reduced in the presence of either (–)-ipsenol or (+)-ipsdienol,^{46,47} the latter compound being the enantiomer of (–)-ipsdienol, the aggregation pheromone of *I. pini*. Since both ipsdienol and ipsenol are part of the aggregation pheromone of *I. paraconfusus*,⁴³ it is probable that these terpenes are primarily responsible for inhibiting the attraction of *I. pini*.

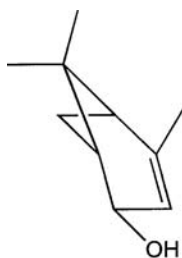
Mutual interruption also can occur between species in different genera that inhabit the same tree. The western pine beetle, *Dendroctonus brevicomis*, competes with *Ips paraconfusus* for ponderosa pine, and these species frequently inhabit the same host tree. Verbenone (IX), one of the aggregation pheromones produced by males and females of *D. brevicomis*, effectively interrupts the attraction of *I. paraconfusus* to host trees.⁴⁸ However, mutual interruption between these species also has been observed,⁴⁹ and it would appear that individuals of both species generate cross-specific attractant inhibitors that probably ensure that a newly invaded host tree will not be catastrophically overwhelmed by beetles of either species.



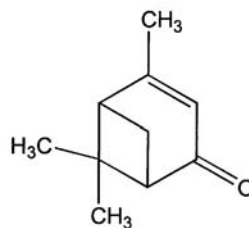
VI



VII



VIII



IX

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Tobacco as a Biochemical Resource: Past, Present, and Future

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T. A. Bartholomew, and H. E. Swaisgood

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ABSTRACT For over 50 years, researchers have been examining the potential to use tobacco (*Nicotiana tabacum*, L.) for the production of numerous biochemical products. As part of this effort, research at North Carolina State University has been conducted on the selection of cultivars for optimal biomass and protein content, the genetic engineering of tobacco to produce foreign proteins, agronomic production methods for bioprocessed tobacco, upstream processing, and downstream purification procedures required to yield such products. This chapter will present an overview of the progress that has been made in tobacco bioprocessing since its inception. Studies conducted at NCSU will be used to illustrate the possibilities and pitfalls of bioprocessing tobacco. Based upon these studies, the feasibility of using field grown tobacco as a “bioreactor” for production of fine biochemicals will be discussed.

13.1 Introduction

When Columbus first arrived on the shores of North America, he found Native Americans growing and using a plant unknown to Europeans. This plant held great spiritual significance

to Native Americans. Scientists who followed in the footsteps of the early North American explorers would later name this plant tobacco. Tobacco (*Nicotiana tabacum*, L.) farming began in the early 1600s near the Jamestown colony in Virginia. As the use of tobacco products for smoking, chewing, and snuff was promoted in Europe, tobacco became a leading item of commerce between the colonies and England. Notably, George Washington and Thomas Jefferson both farmed tobacco. Thus, the history of America is inextricably linked with the history of tobacco production.

Today, the production of tobacco in the Southeastern U.S. continues to be an important contributor to the economy of that region. Tobacco income has allowed many small family farms to remain self-sufficient. In North Carolina, tobacco accounts for over one billion dollars/year in cash receipts at the farm gate.⁴³ At a profit of roughly \$1000 to \$2000/acre, tobacco is the most profitable row crop grown in the U.S. In contrast, a good farmer can expect a profit of \$60, \$100, or \$200/acre for soybeans, corn, or cotton, respectively.

Despite its economic significance, the tobacco industry is in turmoil. The proposed legal settlement between the states Attorneys General and the tobacco industry,¹ the widespread recognition of the health hazards of tobacco and tobacco smoke, and a decline in tobacco consumption have led farmers to question the future of the crop. These farmers are seeking alternatives that might take the place of tobacco. Unfortunately, no other crop is likely to be able to provide the level of income on an acre-for-acre basis. Truck crops such as tomatoes, strawberries, and peaches yield profits similar to those of tobacco. Nevertheless, the potential acreage in North Carolina from these crops is at least an order of magnitude less than that of the current tobacco acreage. Substantial increases in the acreage of any of these truck crops would lead to market saturation and a collapse of prices. If North Carolina farmers are to diversify, what is required is the identification of a panoply of alternative crops. Perhaps surprisingly, a growing number of researchers believe that one such crop is tobacco grown for nontraditional use as a renewable biochemical resource.

This chapter describes past and current research on biochemical products from bioprocessed tobacco. These efforts date back at least to the early 1940s. Where possible, research conducted at NCSU will be used to illustrate the problems and potential for tobacco as an industrial crop. We also will discuss the biological and technological hurdles that must be overcome to make such an industry possible.

13.2 Bioprocessing of Tobacco — The Past

Over 50 years ago, researchers at the USDA Eastern Regional Laboratory began to explore the use of tobacco for the production of biochemical commodities. The regional labs were established to study alternative uses for excess farm commodities. Among the products examined for their commercial potential were nicotine, nicotinic acid, beta-carotene, cellulose, waxes, chlorophyll, and citrate.^{10,21,36,37} This research resulted in the development of a commercial nicotine recovery process, an effort that was later abandoned as nicotine sulfate was supplanted by the first generation of synthetic insecticides.

Interest in extended uses of tobacco arose once again in the early 1970s when Kawashima and Wildman^{25,26} demonstrated that it was possible to isolate Ribulose bis phosphate Carboxylase-Oxygenase (RuBisCO, "Fraction 1 Protein", or F-1-p) from tobacco using relatively simple process technology.^{61,62} RuBisCO catalyzes the reaction of atmospheric CO₂ with ribulose biphosphate, a key step in the Calvin cycle. Because RuBisCO is found in

abundance in all photosynthetic organisms, it holds the distinction of being the single most abundant protein in the world.

Considerable work on the isolation of crude soluble leaf protein from green plants preceded the work by Wildman on tobacco. These "leaf protein concentrates" are used in both animal and human diets.^{44,47,52,54} Significantly, however, tobacco and a few closely related species have been shown to be unique in their ability to produce a tasteless and odorless high-grade crystalline Fraction 1 protein.^{61,62} Because it can be isolated in high purity, has high nutritive value,^{17,29} and unique physiochemical properties,^{4,49-51,57} tobacco-derived RuBisCO has potential as a new protein for utilization in the food, medical, and cosmetics industries. F-1-p has exceptional nutritional and functional properties including an excellent amino acid balance, neutral taste and odor, hydrophilicity, jelling properties, texture, and structural stability.²⁷ These properties compare favorably with those of casein, i.e., milk protein. A driving factor for use of plant proteins is an increasing preference by consumers for such proteins in place of animal-based sources. RuBisCO protein is in demand by the food industry as a means of modifying existing products or fabricating new products with improved quality and nutritional value.

High-grade proteins such as casein and F-1-p also are important for non-food uses including their use as emulsifiers for personal care products.⁵⁵ While casein has been a traditional source of such proteins, increased demand coupled with tight supplies have led to demand in the marketplace for casein substitutes. In addition, high purity, easily digested proteins also are of interest in medicine. Patients with severe impairment of renal function must restrict their intake of sodium and potassium.¹⁷ Crystalline F-1-p is low in these ions and could serve as a source of protein for such patients. In addition, F-1-p could also be incorporated into low-residue, nutritionally complete diets for patients suffering from a range of gastrointestinal diseases.

Wildman recognized the potential for F-1-p and began development of a process for the large-scale isolation of Fraction 1 and residual plant proteins (Fraction 2 proteins, F-2-p) from tobacco. Leaf Protein International was formed by Wildman and associates to further this effort and a pilot plant was built near Wilson, NC. Working with Ray Long and coworkers at NCSU, considerable progress was made towards the goal of developing agronomic methods for the production of tobacco biomass and for producing crystalline Fraction 1 protein using the pilot scale facility.

This work resulted in a prototype system for the agronomic production of green tobacco biomass and subsequent recovery of F-1 and F-2 proteins. Agronomically, the system consists of growing tobacco in fumigated beds at high plant densities. The plants are then harvested up to four times during the growing season. During the development of these procedures a number of variables including plant variety, planting density, harvest schedule, fertility regime, and pest control methods were examined.^{5,34} The system consists of direct seeding of tobacco into raised beds to produce a total seasonal yield of 180,000 lb/acre of fresh biomass. This is approximately equivalent to 18,000 lb/acre of dry biomass of which approximately 1800 lb is plant protein. Fertilization consists of an initial application of 12-6-6 N/P/K at a rate of 75 lb/100 linear feet of bed, followed by re-fertilization of the beds with 100 lb/100 linear feet of 15-0-14 after each harvest. Insect, weed, and disease control are accomplished using standard cultural practices used in traditional tobacco production. A significant problem that has been encountered in this system is the development of hollow stalk rot [*Erwinia carotovora* (Jones) Holland], following each harvest. Additional work is needed to examine control methods for this organism. The green tobacco biomass is harvested when the tobacco has reached a height of approximately 60 cm. A forage harvester is used to collect the plant material which is placed into wooden crates for transport

to the processing facility. During this phase, it is important that the tobacco lamina maintain its turgidity. This is facilitated by close coupling of harvesting and processing. Prior to processing, the tobacco can be cooled by percolating water through the tobacco biomass.

Tobacco bioprocessing (Figure 13.1) is initiated by chopping the green biomass followed by immediate homogenization using a tissue disintegrator. Aqueous sodium meta-bisulfite is added as a reductant prior to tissue disruption. The resulting green pulp is then passed through a screw press, separating the solid biomass residue from the green juice that contains plant proteins, starch, pigments, and other materials. Complete extraction and maximum recovery of protein requires re-extraction of the biomass residue. The resulting green juice from this secondary recovery process is then added to the initial liquid stream.

The green extract is then passed through a heat exchanger and brought to a temperature of approximately 48°C for several minutes.^{61,62} This step coagulates the green, lipophilic material in this fraction and is a key step in successful protein isolation. The resulting mixture is centrifuged and the coagulated green “sludge” and starch are separated from the aqueous stream. This stream exits the centrifuge as a clear amber liquid, similar in appearance (but not taste!) to a dark ale. If necessary, further polishing of this fraction can be achieved through an additional step such as bed filtration.

The dark amber liquid is transferred to a holding tank chilled to 5 to 10°C. If necessary, the pH of the solution is adjusted to 5.5. The crystalline F-1-protein slowly precipitates and settles over approximately 24 h at which time the protein is collected using an industrial decanter. The aqueous F-2-p stream resulting from this step is typically pale amber — similar in appearance to a good Pilsner beer. This F-2-p fraction contains native plant enzymes as well as salts, soluble carbohydrates, and other water solubles. The F-1-protein concentrate can be re-solubilized by adjusting to pH 8.0. Repeated solubilization and precipitation can be used to polish this fraction and remove residual foreign materials. F-1-p is obtained in solid form by filtration of the acidic mixture or spray-drying of the basic solution.

Fraction-2-proteins are recovered by adjusting the pH of the pale amber solution to 4.5 followed by cooling to 5–10°C for 24 h. As in the case of F-1-p, this leads to precipitation of F-2-p. Recovery of the precipitated protein mixture is identical to that described for F-1-p. The “Fraction 2” proteins which consist of a mixture of the residual plant proteins (enzymes) after RuBisCO recovery also are of commercial significance. F-2-p leaf proteins (and total leaf protein concentrates) can be used commercially as animal feed supplements.⁴⁵

Among the naturally occurring tobacco constituents found in F-2-p are protease inhibitors. These macromolecules are especially prevalent in chlorotic tobacco leaf tissues.³⁰ *In planta*, protease inhibitors are believed to function as a mechanism of defense against herbivores.²⁰ Significantly, protease inhibitors are of interest in the medical community as anticarcinogens and radioprotectants.⁵⁶ A collaborative research project between faculty at NCSU and Bowman-Gray Medical School demonstrated that enriched protease inhibitor fractions could be obtained from senesced tobacco (Figure 13.2). The initial process technology used was as described above. The crude F-2-p fraction was precipitated using ammonium sulfate and heating at 80°C for 10 min at pH 4.5. This was followed by Sephadex G-75 size exclusion chromatography and, ultimately, agarose-chymotrypsin affinity chromatography to obtain pure tobacco Chymotrypsin Inhibitor-1 (CH-1) (St. Clair and Danehower, unpublished data). Bioassays of CH-1 indicated significant activity in the suppression of radiation-induced transformation of a C3H/10T1/2 cell line. Results were comparable to those for the soybean-derived “Bowman Burke Inhibitor” that has been widely studied for its anticarcinogenic potential.

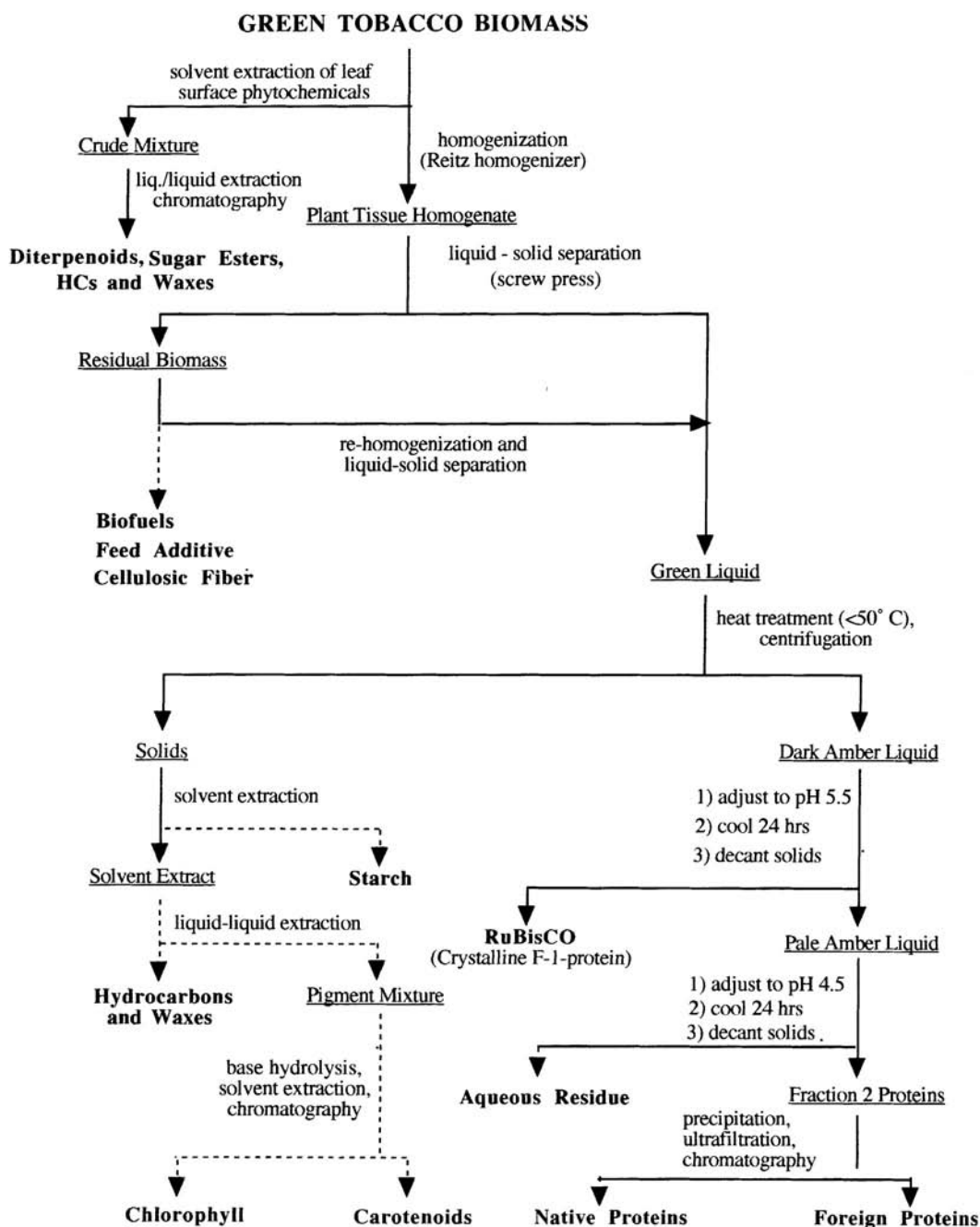


FIGURE 13.1

Process flow and tobacco bioproducts resulting from the bioprocessing of *Nicotiana tabacum*. (Process flowchart: solid lines = process development to date, dashed lines = future process development. Bioproducts: underlined = intermediate fractions, bold = final products.)

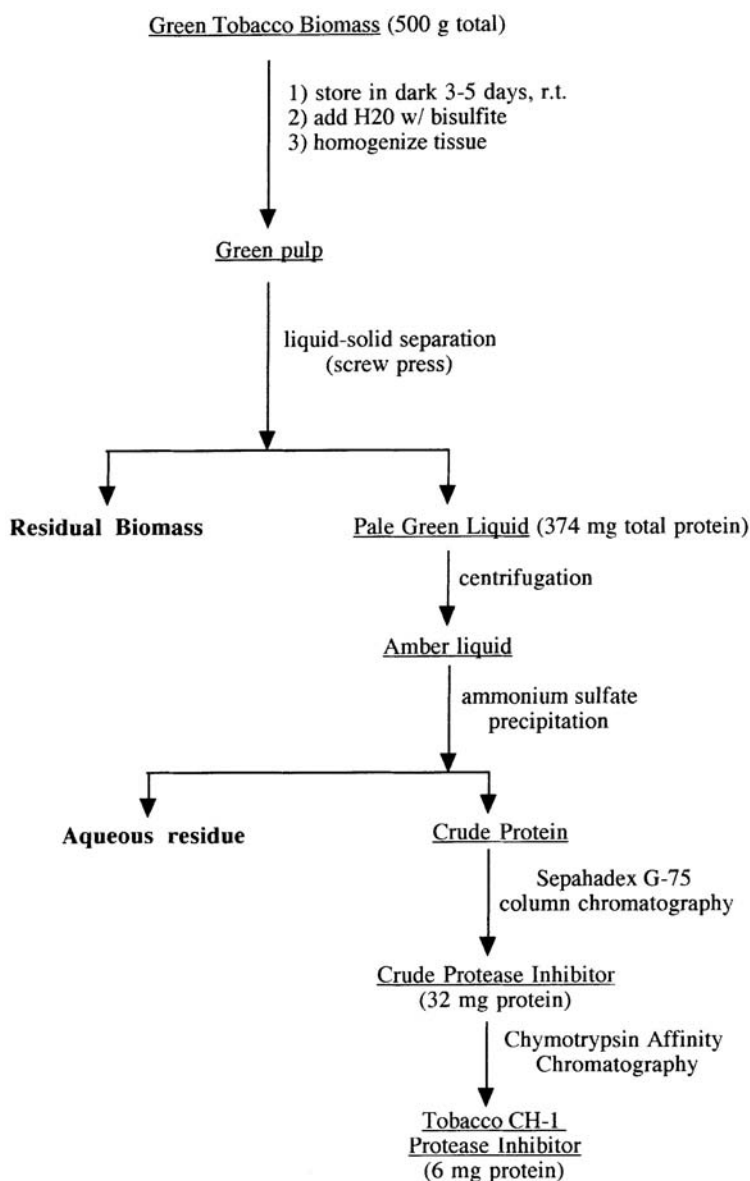


FIGURE 13.2

Isolation of protease inhibitor-I from bioprocessed tobacco. (From St. Clair, W. and Danehower, D.A. Unpublished data.)

13.3 Tobacco Bioprocessing — The Present

The F-2-protein mixture also has been shown to contain foreign proteins expressed by transgenic tobaccos.⁶⁰ Because of tobacco's ability to be transformed reliably using a number of molecular biology techniques, numerous foreign proteins and, to a lesser extent,

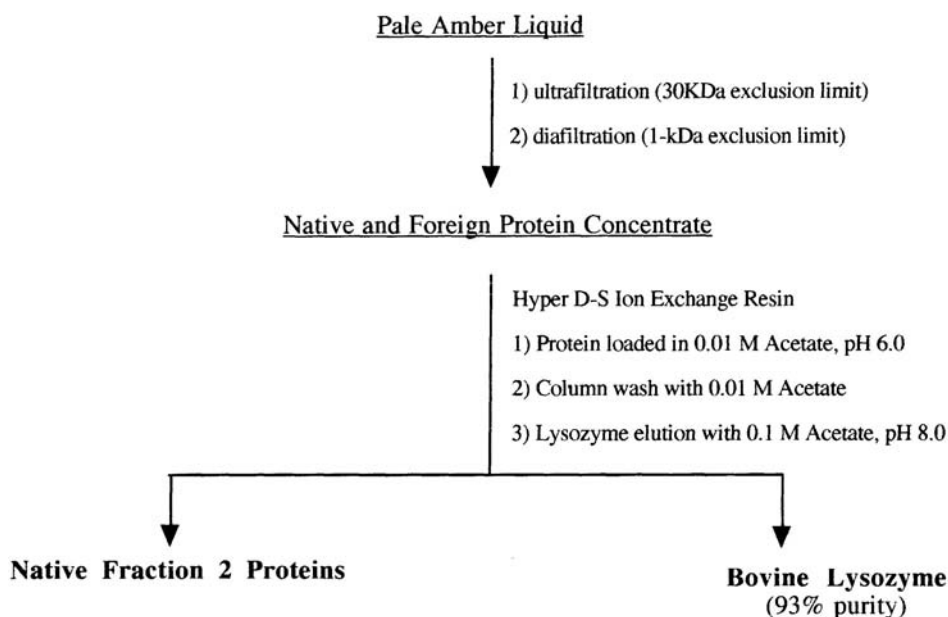


FIGURE 13.3
Downstream bioprocessing of bovine stomach lysozyme from tobacco.

their resultant products have been expressed in this crop and many have been subsequently isolated in significant quantity (see Owen and Pen⁴⁶ and Goddijn and Pen¹⁹ for current reviews of plant-based biochemical production). Numerous papers have appeared in the literature arguing that field-grown plants provide a low-cost alternative to prokaryotic microorganism-based production of transgenic products. One argument put forth is that eukaryotic plants have a greater capacity for post translational processing of complex proteins.¹⁹ Secondly, the use of field-grown plants as “bioreactors” is a relatively cheap and almost infinitely variable system for foreign protein production. While it is true that production costs are less expensive in field-grown plants than in fermentation systems, it is equally true that subsequent isolation of foreign proteins from green plant biomass may present considerably more difficulty than extraction from bacterial cell biomass. Successful demonstration of the ability to isolate foreign proteins from green plants is critical to the ultimate success of plant bioprocessing.

At NCSU, recent research has demonstrated proof of concept for the production of a foreign gene product with commercial potential and the subsequent ability to isolate that product in high purity (Figure 13.3).⁶⁰ In this project, tobacco was transformed with a gene for expression of bovine lysozyme, a 15 KDa antibacterial protein with a broad array of uses including medicinal, agricultural, and industrial applications against animal and plant pathogenic bacteria.³⁹ The gene was coupled to a CaMV 35S promoter and a selectable marker. This construct was transferred into an *Agrobacterium* system that was then used to transform the tobacco. Following recovery of transformed plantlets, a heterogeneous array of transformed plants were grown in a greenhouse. Expression levels were calculated using SDS-PAGE densitometry for several groups of transformants. The highest level of expression was 1.8% of total F-2-p protein or approximately 1% of total plant protein. Yields of lysozyme could ultimately be higher as this system was not optimized for expression of the lysozyme gene.

Plants were harvested and immediately processed for protein using a procedure similar to that described earlier. The aqueous F-2-protein stream was processed through an ultrafiltration system equipped with a 30 KDa exclusion limit membrane filter. Subsequently, a second ultrafiltration system consisting of a 1-kDa exclusion limit membrane was used to further purify the lysozyme fraction while concurrently dialyzing the sample and reducing the volume 10-fold. The final step of the purification process was ion exchange chromatography using Hyper D-S resin. The sample was introduced using a 0.01 M acetate solution at pH 6.0. Following washing to remove other proteins, the purified lysozyme fraction was eluted with 0.1 M acetate at pH 8.0.

Separation mechanisms used in this process were chosen based upon their ability to be scaled up. This is clearly a successful demonstration of proof of concept for the production and downstream isolation of a foreign protein from the tobacco f-2-protein mixture. While the level of purity obtained in these experiments is not sufficient for medical uses of bovine lysozyme, the purity obtained is more than sufficient for its use as an industrial and/or agricultural antibacterial agent.

13.4 Other Product Streams from Bioprocessed Tobacco

In order to increase the commercial attractiveness of tobacco bioprocessing, it is critical that as many profitable product streams as possible be obtained. Thus far the production of native and foreign proteins in field-grown tobacco has been considered. Another class of biochemicals, i.e., low molecular weight tobacco bioproducts, also have been the subject of research. Economically significant biochemicals produced by tobacco include chlorophyll and carotenoids, starch, diterpenes, saccharide esters, alkaloids, polyphenols, long chain hydrocarbons and waxes, coenzyme-Q, and structural carbohydrates (biomass). Some of these products already have significant commercial potential and could fit well into an overall tobacco bioprocess. The most promising of these are discussed below.

13.4.1 Carotenoids

Most carotenoids are currently derived from synthetic chemical processes. Nevertheless, a resurgent market demand for natural vitamins and pigments has created a potential market for plant-derived carotenoids. Natural carotenoid pigments are used extensively in the poultry and fish industries.²⁸ These compounds have long been items of commerce as vitamins⁵⁹ and are increasingly recognized as an important factor in resistance to disease.¹⁸ A recent trend in carotene-based vitamins has been the use of naturally occurring vitamin mixtures, rather than single pure components. This trend could result in less need for high-cost downstream processing of carotenoid isolates.

The tobacco carotenoids consist primarily of beta-carotene with lesser amounts of xanthophyll, violaxanthin, and neoxanthin.⁵⁸ The pigment/starch "sludge" obtained during the initial centrifugation step in F-1-protein processing would be an excellent material for recovery of carotenoids. Successful isolation would require that the bioprocess minimize oxidative conditions by excluding light and oxygen. Solvent extraction of the pigment/starch mixture, followed by basic hydrolysis to remove chlorophyll would be one approach which might be taken to recover carotenoids. Tobacco starch, consisting of a mixture of amylose and amylopectin in a 1:4 ratio would be a by-product of this process.

13.4.2 Terpenoids

Tobacco-derived terpenoid secondary products have long been important to both the tobacco and flavor and fragrance industries. Tobacco essence, which is in part derived from the oxidative breakdown of the diterpenoid divatrienediols found in glanded trichomes on the leaves of *N. tabacum* are highly valued as an ingredient in fine perfumes.⁴² Similarly, certain *N. tabacum* biotypes produce the bicyclic diterpene, *cis*-abienol, while *N. glutinosa* produces sclareol, a related bicyclic diterpene. Both *cis*-abienol and sclareol can serve as precursors to sclareolide, a valuable “fixative” in the fragrance industry.

These diterpenes also have other notable properties. The Japanese have patented the use of *Nicotiana* diterpenes as both antifungal²⁴ and antineoplastic agents.⁴⁰ Research at NCSU and elsewhere^{11-15,31,38} has resulted in further identification of these leaf surface diterpenes as antimicrobials and/or plant growth regulators.

13.4.3 Sugar Esters

The so-called “sugar esters” of *Nicotiana* (typically a mixture of sucrose and fructose esters to which a mixture of short-chain linear and methyl-branched fatty acids are esterified) are the subject of serious study by scientists at the USDA. These researchers have found the sugar esters to be excellent natural insecticides.^{2,3,48} As in the case of the diterpenes, these compounds also have been shown to have significant antimicrobial and plant growth regulatory activity.^{9,12,13} A recent paper²³ determined that up to 11 lb/acre could be produced from *N. glauca* at traditional tobacco production planting densities (18 K plants/ha). Yields should be even higher in close-grown plots.

13.4.4 Hydrocarbons and Waxes

High molecular weight waxes and hydrocarbons, such as those found on the leaf surface of tobacco, have been touted⁸ as an alternative to fossil-fuel petroleum lubricants. Although the quantities of these compounds are relatively small in tobacco, they would be worth examination as a by-product stream in the production of diterpenoids and/or sugar esters. Removal of hydrocarbons and waxes using either solvent partitioning or chromatographic techniques is a key step in the purification of leaf surface diterpenes and sugar esters. Thus, these compounds might be obtained in relatively high purity with little subsequent processing beyond solvent removal.

13.4.5 Coenzyme-Q

Coenzyme-Q is a widely utilized drug for the treatment of heart disease in Japan and Western Europe.³³ This natural vitamin/cofactor has superior antioxidative properties in blood, thereby decreasing the formation of low density lipoprotein.⁵³ The Japanese currently produce coenzyme-Q in a tobacco cell fermentation system. The potential for selection of lines which produce high levels of Coenzyme-Q in the field should be examined.

13.4.6 Structural Carbohydrate

Finally, the residual cellulosic fraction (biomass) that arises from the overall processing of tobacco for alternative uses may have value for the production of alcohol fuels, paper, and

other chemicals.^{6,41} Scientists in the pulp and paper department at NCSU have produced paper-like products from the cellulosic biomass which is the final byproduct of the processing of tobacco for protein (R. C. Long, personal communication). Perhaps a more attractive use of residual biomass is in the production of alcohol or biogas fuels. The stalks from traditional tobacco production systems have been examined as a feedstock for the production of methane in a synfuels demonstration facility.

13.5 Tobacco Bioprocessing — The Future

Despite a long record of research on the uses of bioprocessed tobacco as a renewable resource for proteins and low molecular weight fine chemicals, much work remains before tobacco bioprocessing becomes a commercially viable enterprise. Critical issues must be addressed including a reduction in the costs of field production and enhancement of biomass yield. Key areas for research here will be alternatives to the economically and environmentally costly use of fumigants for bed preparation and control strategies for bacterial rot in secondary harvests.

In addition, effort must be directed to optimization of the tobacco plant for use in bioprocessing. Plant lines need to be developed which are better suited biochemically for bioprocessing. Examples include the need to develop plants with decreased polyphenol and alkaloid content. Polyphenols bind to and interfere with recovery of proteins while the presence of alkaloids raises issues of consumer and regulatory acceptance. Tobacco lines that produce enhanced levels of high-value natural products also are desirable. An example here might be the development of tobacco varieties that produce high levels of commercially valuable (and marketable) sclareol in place of the duvatrienediols.

Improvement in the initial F-1-p and F-2-p recovery process is another important goal. Improvements in protein recovery require a careful examination of homogenization conditions, the addition of protein solubilizing agents, reductants, agents for the removal of polyphenols, flocculation techniques, the use of cellulases for cell wall degradation (produced *in planta*?), and clarification procedures. Perhaps the greatest opportunity for decreasing costs is the development of relatively low-cost downstream processes for high value proteins and natural products. Production and yields of these products are the ones most likely to “make or break” tobacco bioprocessing. As has been demonstrated,⁶⁰ it is possible to obtain good yields of engineered proteins in relatively high purity using cost-effective technologies such as selective precipitation, dynamic membrane filtration and diafiltration, and ion exchange chromatography.

The bioprocessing of more exotic proteins for use in medicine is more problematic. On the one hand these products have tremendous value and could greatly increase the per acre profit. Conversely such products require a high standard of process sanitation and must be isolated in a purity which will require the use of more expensive downstream bioprocessing techniques such as aqueous two-phase partitioning and the use of highly selective membrane filtration. Despite these problems, the potential payoff for such products is so great as to warrant further study in this area. Processes that might be examined include expanded bed adsorption, size exclusion, and affinity chromatography.

The potential to enhance the product stream of bioprocessed tobacco by isolation of valuable low molecular weight products has received much less attention. A clear-cut demonstration of proof of concept is needed for these materials. Logical products to pursue include carotenoids, leaf surface diterpenes, and sugar esters. An obvious process stream

for carotenoid recovery, the green “sludge” fraction already exists. Techniques such as base hydrolysis of chlorophyll and subsequent solvent partitioning are well defined carotenoid recovery methods. The increased marketability of mixed carotenoids enhances the attractiveness of this product stream. In the case of the diterpenes and sugar esters, methods must be developed which will permit isolation of a crude mixture prior to protein isolation without effecting subsequent protein recovery. In the case of sclareol, a clear market exists. The sugar esters could be niche marketed as natural insecticides.

While tobacco bioprocessing research is important, it is equally important to identify and cultivate potential markets for the products. The likelihood of success improves tremendously when the marketplace, rather than the technology, drives development. At present, tobacco bioprocessing is largely technology driven. In order to ensure the highest potential for success of this technology, a critical, but unmet, need is market analysis and the development of wider contacts with manufacturers. Significant efforts have been made in this area,⁵⁵ but further work is required before the entire array of products that will be required for a successful economic tobacco bioprocessing “package” can be obtained. Several scientists have discussed the development of industrial crops.^{7,16,22,32,35} Invariably, these authors stress the importance of prior analysis of markets and prices before beginning work on any industrial crop development. They also emphasize the need for close coupling of research with market development and penetration strategies.

There are a number of advantages to plant-based production of biochemicals. Plants have significant advantages over most synthetic chemical production systems as they are capable of producing enantiomerically pure compounds. As eukaryotes, plants can produce more complex foreign proteins, a significant advantage over the prokaryotic bacteria used in industrial bioreactors.

While this chapter has focused upon the potential benefits to farmers of this technology, it is important to note that plant bioprocessing requires close coupling of agricultural and rural industrial development. Thus, crop bioprocessing would provide excellent returns to farmers while offering good industrial jobs to rural residents. If planned properly, plant bioprocessing also promises to be a relatively benign industry in terms of the environment. The use of renewable resources would lessen our dependence on a petrochemical-based economy, resulting in greater security for those countries who have invested in plant-based industries.

Of course, there also are disadvantages that must be addressed and significant technological hurdles to overcome. These include the risks involved in such a radical departure from current methods for producing biochemicals. Even a technology that has the many advantages outlined above is not guaranteed success in the marketplace. As development of plant-based technologies proceeds, it is imperative that a close coupling of production scale-up and marketing be maintained.

In summary, it is our belief that the potential for plant-based production of biochemicals as items of commerce is strong. Field-grown plants can indeed function as “bioreactors”. What is required is a focused research effort that includes early and careful consideration of the market potential for the proposed products. The research program must develop a complete research team which is sufficiently funded and capable of undertaking a coordinated project including basic and applied research in agronomics, plant molecular biology, chemistry and biochemistry, and process engineering. New product development must be done with careful consideration of markets and marketing. As new products are developed they must be incorporated efficiently initially into pilot-scale processes and ultimately into full-scale industrial processes. Concurrently, corporate support and market development expertise must be available to ensure the success of the product(s). Unfortunately, the short-term mindset of both industry and, yes, even academia, make such an undertaking

all the more difficult. Nevertheless, it is our strongly held belief that such an undertaking is possible, and, more importantly, has great potential to create a new paradigm for industrial biochemical production with concomitant benefits to farmers, industry, consumers, and the environment.

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Natural Products Containing Phenylalanine as Potential Bioherbicides

Mikhail M. Bobylev, Ludmila I. Bobyleva, and Gary A. Strobel

CONTENTS

- 14.1 Introduction
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ABSTRACT Our previous study of maculosin (**I**, cyclo-TyrPro), a host specific toxin produced by *Alternaria alternata* on spotted knapweed (*Centaurea maculosa*), showed that a number of phenylalanine analogs of **I** possess similar activity, the unsubstituted analog (cyclo-PhePro, **II**) being the most active. Interestingly, **II** appeared to be active against a wide variety of plants. We suggested that protected phenylalanine and not a diketopiperazine is an active moiety of **II** and other analogs. To prove this idea we synthesized and tested two compounds: (1) where the proline carbonyl was cut off of the pyrrolidine ring, and (2) where in addition the pyrrolidine ring itself was cut to form diethylamine. Both compounds produced the same symptoms on spotted knapweed plants as **II**.

KEYWORDS: *natural products; phenylalanine; herbicides; maculosin; spotted knapweed*

14.1 Introduction

Natural products comprise a voluminous source of new and strikingly diverse bioactive compounds for pharmaceutical and agrochemical development. In medicine they have already been heavily used for many years and form a very substantial part of prescribed drugs — antibiotics being one very good example. However, this is not the case in plant protection. Very few natural products or their derivatives are currently used as agrochemicals; pyrethroids are probably the only example of true success. There are three major reasons for this drastic dearth of natural products in the plant protection area: complex structure, insufficient stability, and, sometimes, high toxicity.

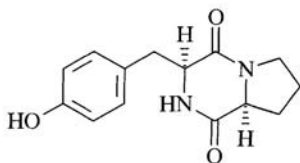
Indeed, many of the natural bioactive compounds have very complex structure and, therefore, are very difficult to synthesize in the lab and very expensive to produce industrially. This obstacle is very easy to overcome with the virtually unlimited resources of the pharmaceutical industry; people are ready to pay large sums of money for a cure. In contrast, in crop protection there is a very strict, simple, and low limit on spending. It should always be lower than the cost of the saved crops. This sole consideration makes most of the natural products prohibitively expensive.

Insufficient stability also is a very serious problem. Many active natural products cannot withstand harsh conditions of field application because sunlight and oxygen break them down before they produce the desired biological effect. Again, pyrethroides are a very good example. Their natural prototypes were extremely unstable and had to be heavily modified for agricultural application. As a result, some of the pyrethroides bear very little resemblance to the initial molecule and could hardly be considered as natural products or their derivatives.

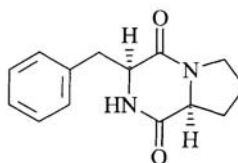
The third reason is toxicity. Contrary to widespread expectations, natural products are often very toxic and might have a devastating effect if spread over large areas of field or pasture land. Probably the most familiar example is nicotine. It is an excellent insecticide, but its application is limited because of its high mammalian toxicity. Consequently, the right candidate for a natural biopesticide must not only possess relevant biological activity, have a simple structure, be stable, but have a low mammalian toxicity as well. Maculosin (I) is one of the very few compounds completely satisfying these requirements.

14.2 Maculosin-I and -II

Maculosin {(I), (3S-cis)-hexahydro-3-[(4-hydroxyphenyl)methyl]pyrrolo[1,2-a]pyrazine-1,4-dione} is a host specific fungal toxin produced by *Alternaria alternata* on spotted knapweed (*Centaurea maculosa*).¹ It was discovered in the course of a systematic search for bioactive natural products for weed control among weed pathogens, a novel approach developed by professor Gary Strobel at Montana State University.² Initially the authors suggested the name maculosins for the entire series of related dipeptides isolated from *Alternaria alternata*. Only two compounds in the series were phytotoxic and they were assigned individual names of maculosin-1 (I) and maculosin-2 (II). However, the less active maculosin-2 was not mentioned after that and maculosin-1 became known simply as maculosin. Since the present work reveals some interesting properties of II, we return to the authors' original terminology and address the compound as maculosin-2.



I



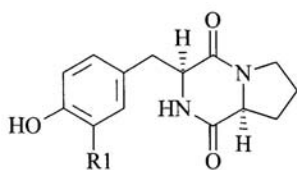
II

Maculosin-1 possesses a truly remarkable combination of useful properties. First, and most important, it is highly toxic to the target species. In primary tests maculosin-1 produced necrotic lesions on detached and punctured spotted knapweed leaves at the concentration as low as 10^{-5} mole/l. Second, its structure is very simple. It is just a combination of two

common amino acids — proline and tyrosine. Third, in maculosin-1 these amino acids form a cycle and the whole structure becomes very stable. This stability is a general quality of cyclic dipeptides³ which might be considered as a terminal product of metabolism. They form very easily as a result of metabolism or degradation of proteins, but once formed they usually resist further metabolism or degradation. This process of formation and accumulation of cyclic peptides takes place during cooking or even storage of any protein-containing food, and we consume these compounds daily throughout our lives without any adverse effect. This fact reveals the fourth important quality of maculosin-1 — its potential (although not proven) safety.

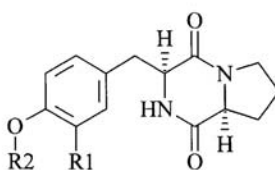
14.3 Maculosin-I and -II Analogs: Biological Activity

For this reason, 3 years ago we started a systematic investigation of maculosin-1 and its analogs with the initial goal to explore their potential as knapweed control agents and to determine primary structure–activity relationships. We synthesized a series of 17 maculosin-1 analogs (**III–VIII**) carrying different substituents on the aromatic ring and tested them on whole knapweed plants in the greenhouse.⁴ We found that neither maculosin-1 (**I**) nor any other analog with the free hydroxyl group (**IIIa,b,c**) were active against whole and intact knapweed plants. We also found that the elimination of the free hydroxyl group by any means — protection (**IVa–f**), substitution (**Va–e**), or complete removal (**II, VI–VIII**) — restores the activity. The activity greatly depended on the size of the substituent or the protecting group; the smaller the substituent, the higher the activity. The most active compound appeared to be the one without substituents, maculosin-2 (**II**). At the concentration of 6×10^{-2} mole/l, which is approximately equivalent to 1.5%, it induced sweeping necrosis on spotted knapweed leaves and destroyed up to two thirds of the foliage



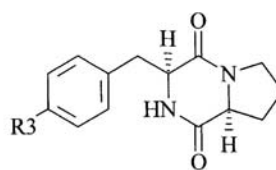
III

a: R1 = OH;
b: R1 = Cl;
c: R1 = NO₂;



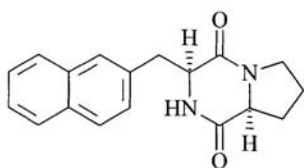
IV

a: R1 = H, R2 = Me;
b: R1 = H, R2 = Et;
c: R1 = H, R2 = Pr;
d: R1 = H, R2 = Bu;
e: R1 = H, R2 = Bn;
f: R1 = Cl, R2 = Et

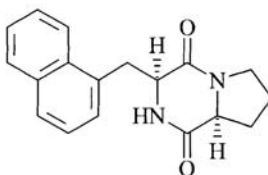


V

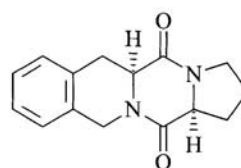
a: R3 = F;
b: R3 = Cl;
c: R3 = Br;
d: R3 = I;
e: R3 = NO₂



VI



VII



VIII

within 1 week after application. This result seemed to be of special importance because the compound had already been described as a phytotoxin and shown to be toxic to another plant.⁵ Therefore, we might expect that maculosin-2, in contrast to uniquely selective maculosin-1, possesses a broad spectrum herbicide activity.

We tested maculosin-2 first on more closely related to spotted knapweed plants, like yellow star thistle and Canada thistle, Russian knapweed, rush skeleton weed, dandelion, and sunflower. Later we tested it on a wide variety of totally unrelated weeds, like hound's tongue, lambsquarters, plantain, sulphur cinquefoil, white top, field bindweed, wild buckwheat, common mallow, leafy spurge, and hollyhock. The results of these tests are given in the Table 14.1. A more detailed report of the study is being prepared for publication in *Plant*

TABLE 14.1

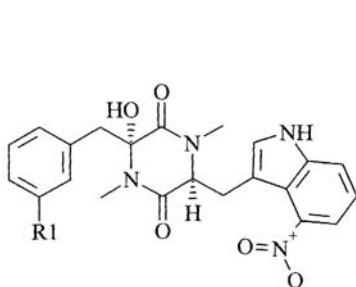
Broad Spectrum Phytotoxicity of Maculosin-2

The Highest Observed Toxicity	
<i>Sweeping necrosis</i> , more than half of the leaf surface is damaged; symptoms appear within several hours after application and fully develop within a day or two	
<i>Weeds</i>	
Canada thistle (<i>Cirsium arvense</i>) Hound's tongue (<i>Cynoglossum officinale</i>)	
High Toxicity	
<i>Sweeping necrosis</i> , more than half of the leaf surface is damaged; symptoms appear in 2 to 3 three days after application and fully develop within a week	
<i>Weeds</i>	
Yellow starthistle (<i>Centaurea solstitialis</i>) Russian knapweed (<i>Centaurea repens</i>)	
Moderate Toxicity	
2 to 3 mm necrotic spots all over leaf surface; symptoms appear in 5 to 7 days after application and fully develop within 10 days	
<i>Weeds</i>	<i>Crops</i>
Rush skeletonweed (<i>Chondrilla juncea</i>)	Potato
Dandelion (<i>Taraxacum officinale</i>)	Tomato
Broad-leaved plantain (<i>Plantago major</i>)	Sunflower
Lambsquarters (<i>Chenopodium album</i>)	
Redroot pigweed (<i>Amaranthus retroflexus</i>)	Ornamentals
Whitetop or Hoary cress (<i>Cardaria draba</i>)	Hollyhock (<i>Alcea rosea</i>)
Common mallow (<i>Malva neglecta</i>)	
Low Toxicity	
Slightly "burned" tips of the leaves	
<i>Weeds</i>	<i>Crops</i>
Sulfur (erect) cinquefoil (<i>Potentilla recta</i>)	Wheat
Leafy spurge (<i>Euphorbia esula</i>)	Barley
No Toxicity	
<i>Weeds</i>	<i>Crops</i>
Field bindweed (<i>Convolvulus arvensis</i>)	Beans (<i>Phaseolus vulgaris</i>)
Wild buckwheat (<i>Polygonum convolvulus</i>)	

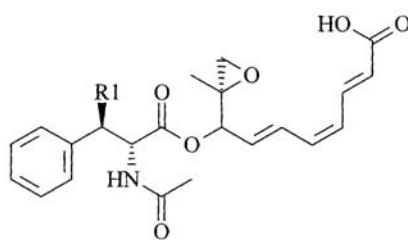
Science.⁶ As we expected, maculosin-2 appeared to be toxic to all of the tested weeds except field bindweed and wild buckwheat. This toxicity, apparently, did not depend on the plant family or genus, but on a quite unexpected quality — hairiness of the leaves. Plants with hairy leaves developed much stronger symptoms within shorter periods of time than those without or with less hair. We suppose that the presence of plant hair may somehow improve absorption of maculosin-2 by the leaves and thus facilitate its phytotoxic action. Among other plants with hairy leaves, Canada thistle and hound's tongue appeared to be the most sensitive, even more sensitive than spotted knapweed. First, necrotic spots develop on these two plants within 2 to 3 h after application, and in 24 h most of the leaves, except for one or two youngest, are completely desiccated. In this case, the level of phytotoxic action was almost sufficient for practical application and was approaching that of the commercial biopesticide "Scythe".

14.4 Role of Phenylalanine

These results, as well as proving the idea of a broad spectrum herbicide activity of maculosin-2, lead us to the suggestion that there is a much broader phenomenon than the phytotoxicity of a certain compound, or even a group of compounds, to a certain plant or group of plants. Indeed, maculosins are not the only cyclic dipeptides with phytotoxic properties. Pyriculamide (**IIIa**), a 3-nitro derivative of maculosin, was described by Russian scientists as being somewhat phytotoxic to rice.⁷ Two other nitrated dipeptides — thaxtomins A and B (**IXa,b**) — were found to be responsible for producing the symptoms of potato scab.⁸ Recently, the same two products were shown to be phytotoxic to a wide variety of seedlings.⁹ All these compounds have one component in common, namely, phenylalanine (tyrosine should naturally be considered as a substituted phenylalanine), and it is quite logical to assume that phenylalanine is responsible for their phytotoxic action. This assumption is supported by the fact that there are two other *Alternaria alternata* phytotoxins (**Xa,b**) — AK toxin I and AK toxin II — that comprise esters of phenylalanine.^{10,11}



IXa: $R^1 = \text{OH}$;
b: $R^1 = \text{H}$

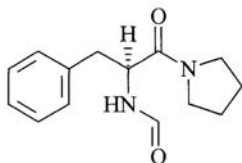


Xa: $R^1 = \text{Me}$;
b: $R^1 = \text{H}$

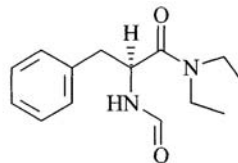
We suppose that phenylalanine may be toxic to higher plants and that this toxicity reveals itself when a properly protected molecule of phenylalanine reaches the target. In that sense there is no difference between maculosins and other phytotoxic cyclic peptides on one hand and esters like AK I and II on the other; all are just protected phenylalanine. To check this idea, we used something similar to a disconnection approach. We synthesized a compound where proline carbonyl was cut off of the pyrrolidine (**XI**), and another one

(XII) where in addition the pyrrolidine ring itself was cut to form diethylamine. Essentially, the two compounds are still very similar to maculosin-2 both in shape and size, but are no longer cyclic dipeptides. Instead, they both are just phenylalanine, protected with amido and formyl groups. As we expected, both compounds produced the same symptoms on spotted knapweed plants as did maculosin-2.

Although far from being conclusive evidence, this experiment proved to us that we are on the right track and that a special study should be done to investigate the phytotoxicity of protected phenylalanine.



XI



XII

ACKNOWLEDGMENTS: The authors thank the Montana Noxious Weed Trust Fund, the Montana Agricultural Experimental Station, and Beim Foundation for their financial support.

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Spectrum of Activity of Antifungal Natural Products and Their Analogs

Stephen R. Parker, Robert A. Hill, and Horace G. Cutler

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- 15.1 Introduction
- 15.2 The Synthesis of 6-pentyl-2*H*-pyran-2-one
- 15.3 Structure–Activity Relationships of Natural Analogs
- 15.4 Synthetic Analogs
- 15.5 Closing Remarks
- Acknowledgments
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ABSTRACT Synthetic and naturally occurring analogs of the *Trichoderma* metabolite 6-pentyl-2*H*-pyran-2-one have been tested for their activity against a range of filamentous fungi. Candidates for development as “natural” or “soft” fungicides have been identified.

15.1 Introduction

The *Trichoderma* metabolite 6-pentyl-2*H*-pyran-2-one (**I**) is a deceptively simple molecule (Figure 15.1). Interestingly, its chemical synthesis was achieved before it was identified as a natural product. In 1969 Nobuhara reported its synthesis in one of a series of papers examining the organoleptic properties of γ - and δ -lactones.^{1–4} The synthetic compound is available today as a “nature identical” product supplied by certain flavor and fragrance manufacturers. It is used as a food additive for modifying flavor/aroma. The Flavor and Extract Manufacturers’ Association (FEMA) monograph for **I** (FEMA 3696) cites its use in a wide range of food stuffs, including baked goods, cheese, and confectionery. The compound has an aroma described variously as similar to coconut or mushroom.

In 1971 Denis and Webster⁵ demonstrated the production of volatile antibiotics by *Trichoderma* isolates. The authors reported that the active isolates “were all characterized by a definite ‘coconut’ smell.” However, they also noted that not all the isolates that produced this aroma had antagonistic activity by “vapor action,” and the antibiotic activity was tentatively assigned to the production of acetaldehyde. A year later, **I** was identified as a major aroma constituent of *Trichoderma viride*.⁶ A direct assessment of the antifungal activity of the compound was not performed. However, the temporal proximity of these two publications

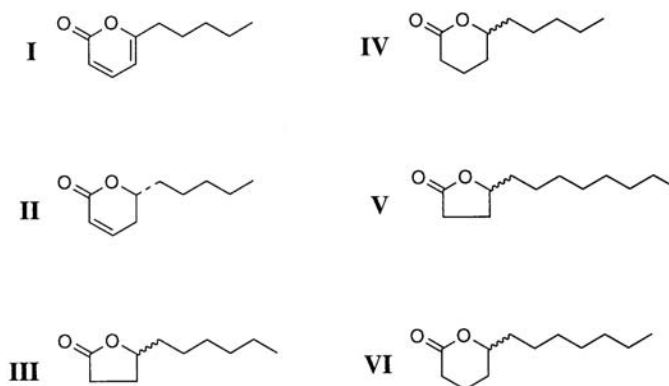


FIGURE 15.1

Structure of 6-pentyl-2H-pyran-2-one (**I**) and its analogs: massoialactone (**II**), γ -decalactone (**III**), δ -decalactone (**IV**), γ -dodecalactone (**V**), and δ -dodecalactone (**VI**). (From Parker, S.R., *J. Agric. Food Chem.*, 45, 2774-2776, 1997. With permission.)

has led to confusion as to whether or not **I** is a volatile antifungal agent. Direct investigations of the vapor action of **I** have been performed, and vapor mediated phytotoxicity has been observed *in vitro*.^{7,8} It is worth noting that the vapor pressure of **I** is around 0.006 mmHg at 20°C. Claydon et al. (1987) questioned whether the phytotoxicity observed *in vitro* would be of significance in the soil environment.

A direct demonstration of the antifungal activity of **I** was first referred to in 1983.⁹ The compound was tested in a standard agar diffusion assay following its purification from cultures of a *Trichoderma harzianum* isolate observed to be growing profusely over the surface of Slash Pine (*Pinus elliotii* Engelm.) logs.¹⁰ Interestingly the compound was initially isolated by bioassay directed fractionation on the basis of its plant growth regulatory activity in the etiolated wheat coleoptile assay.¹¹ The purified metabolite was subsequently assayed for antifungal activity. As an aside, in 1984 a European patent application was filed for the use of *Trichoderma harzianum*, and/or the products of its culture, as biocontrol agents for the control of plant pathogens.¹² Cited in its claims was the use of **I** as a phytosanitary product. This application subsequently lapsed.

The natural occurrence of **I** is now widely recognized and it has been identified as a component of fruit volatiles such as nectarines,^{13,14} peaches,¹⁵ and plums.¹⁶ Its production also has been noted for other genera of fungi including *Aspergillus*.¹⁷ It is unlikely that its antifungal activity is of any significance at the concentrations of **I** observed in fruit. However, the natural occurrence of **I**, its established use as a food additive, and its relatively simple chemical structure make it an attractive candidate for development as a "natural fungicide". However, as alluded to above, appearances can be deceptive.

15.2 The Synthesis of 6-pentyl-2H-pyran-2-one

The original synthetic route of Nobuhara is laborious.³ A number of alternative synthetic routes have since been published.¹⁸⁻²⁰ The route proposed by Pittet and Klaiber (1975) is a two-step procedure for the preparation of **I** (Figure 15.2).¹⁸ Consideration of the synthetic pathway illustrates why the synthesis of **I** is problematic. Although the route is simple, the preparation of one of the key reagents, methyl 3-butenolate, is difficult due to conjugation

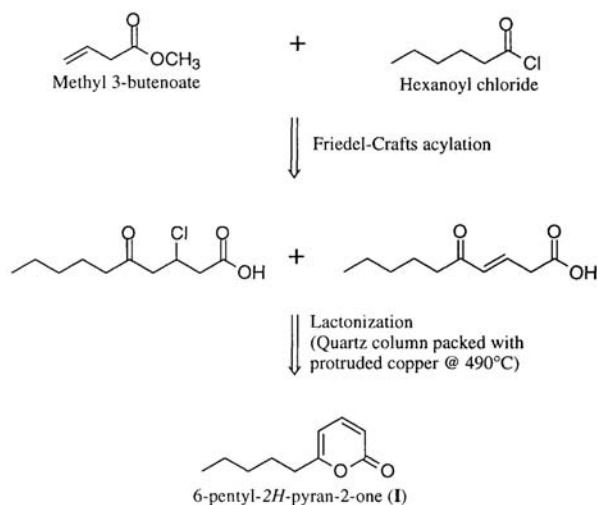


FIGURE 15.2

Synthetic scheme for the preparation of 6-pentyl-2H-pyran-2-one (I). (Adapted from Pittet, A.O. and Klaiber, E.M., *J. Agric. Food Chem.*, 23, 1189, 1975.)

of the carbonyl and olefinic bonds being favored. Although vinyl acetic acid is readily available, its esterification under standard conditions of alcohol and acid will permit migration of the terminal olefinic bond. Therefore, other methods for the preparation of methyl 3-butenote need to be employed.²¹⁻²³

The employment of a “nonstandard” method for lactonization of the mixed keto-acids obtained from the Friedel-Crafts acylation may be related to the structural requirements of an acyclic intermediate for the synthesis of I. The olefinic bonds of such an acyclic precursor are required to be in a *cis-trans* configuration. Whereas, if a *trans-trans* configuration is adopted, the lactonization will not occur. (It is interesting to consider how such a step is achieved biosynthetically by *Trichoderma* where it would be reasonable to assume that I is derived from a single acyclic precursor molecule.) By comparison the lactonization of 5-hydroxydecanoic acid to form the corresponding δ -decalactone is spontaneous in the presence of acid.

Recognizing that these difficulties in the preparation of I might represent obstacles to its commercial development as a natural fungicide, we were prompted to consider what other structurally related candidates could be examined for this application. We sought compounds that shared the favorable attributes of I, but were readily available and less costly.⁸

15.3 Structure–Activity Relationships of Natural Analogs

Earlier structure–activity relationships determining the antifungal activity (by agar diffusion) of a range of synthetic analogs of I demonstrated that the structural requirements for activity appeared to be stringent.²⁴ Shortening of the 6-alkyl substituent resulted in a marked loss of activity, as did saturation of the Δ^2 -bond of the pyrone ring (Figure 15.3). The 6-pent-1-enyl substituted analog of I,²⁵ which is frequently observed as a co-metabolite of I in *Trichoderma* cultures, had activity comparable with that of I.

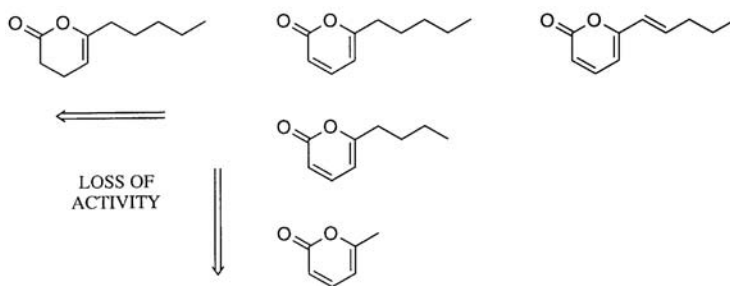


FIGURE 15.3

Summary of structure activity relationships for selected compounds in an agar diffusion-based antifungal assay. (Adapted from Dickinson, J.M., Ph.D. thesis, University of Sussex, U.K., 1988.)

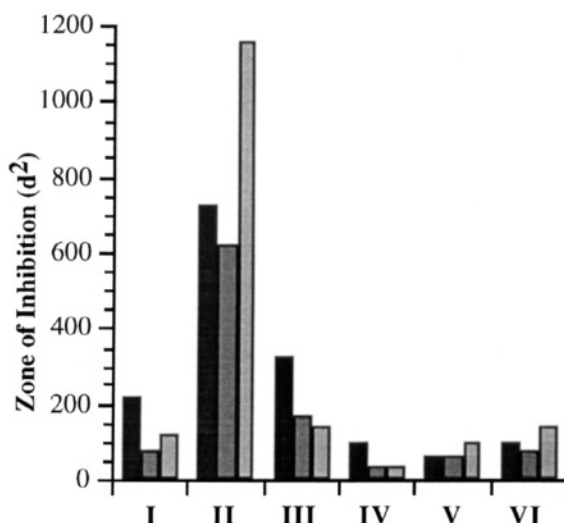


FIGURE 15.4

Antifungal activity of 6-pentyl-2H-pyran-2-one (I) and its analogs (II–VI) in an agar diffusion assay. Suspensions of *Penicillium* spores (Solid — *P. digitatum*, coarse shading — *P. expansum*, fine shading — *P. italicum*) were prepared by washing PDA slopes with two 5 ml volumes of aqueous sterile 0.1% (v/v) Tween 80. The spore density of the combined volumes was determined for a 20-fold dilution using an improved Neubauer hemocytometer. The calculated volumes of spore suspension required to yield final spore densities of 10^5 , 10^6 , and 10^7 spores ml^{-1} were added to 20 ml volumes of molten PDA. For each spore concentration 3 ml aliquots were transferred to each of the wells of a six-well microtitre plate (Nunc) and allowed to solidify. Solutions of test compounds were prepared in acetone at a concentration of 25 mg ml^{-1} . Twenty microliters of each test compound solution, containing 500 nl (c. 500 μg) of each test compound, was applied to a 5 mm diameter sterile filter paper (Whatman No.1) disc. After allowing the solvent to evaporate, the impregnated filter paper disc for each of the test compounds was placed at the center of each of a well. Plates were incubated for 48 h at 20°C . The diameters (d) of the resulting zones of total inhibition were measured and recorded. (From Parker, S.R., *J. Agric. Food Chem.*, 45:7, 2775, 1997. With permission.)

Extending these studies to a group of compounds that were all available commercially and used as food flavoring compounds, we were surprised to observe the greater antifungal activity of massoialactone (II) relative to I (Figure 15.4). Unlike the synthetic and racemic, saturated γ - and δ -lactones (III–VI) tested, II is a purified botanical extract obtained from the bark of the tree *Cryptocaria massoia*. Like I, it has a potent flavor and its use is cited in a similar range of processed foods (FEMA 3744). The compound is the main component (as

Control	I	II	III	IV	V	VI
91	76	84	96	97	94	92

Lettuce seed germination assay. The assay was performed as described by Claydon et al. (1987). A glass coverslip was placed at the center of a petri dish lined with moist filter paper and 25 lettuce seeds (cv. Webbs Wonderful) were randomly scattered over the filter paper surface. A volume of 5 μ l of test compound was applied to the glass coverslip and the petri dish lid replaced. Petri dishes, four per test compound, were incubated in the dark at 20°C for 3 days. The number of seeds germinating was recorded. See Parker et al. (1997) for additional details.

Molar concentration of test compound	Wheat coleoptile growth (% control)					
	I	II	III	IV	V	VI
10 ⁻³	0*	0*	0*	59*	0*	0*
10 ⁻⁴	100	63*	100	100	98	100

Etiolated wheat coleoptile assay. The assay was performed as previously described. Briefly, wheat seeds (*Triticum aestivum* L. cv., Wakeland) were sown on moist vermiculite and incubated in the dark for 4 days at 22°C. From individual coleoptiles 4 mm sections were excised, 2 mm below the tip, and placed in test tubes with 2 ml of a phosphate-citrate buffer (pH 5.6) containing 2% (w/v) sucrose. Test compounds were added in 10 μ l acetone to yield final concentrations of 10⁻³, 10⁻⁴, and 10⁻⁵ M. Length of the sections was measured after incubation for 18 hours. Data were statistically analyzed and the 0.01 level of confidence accepted. Assays were performed in duplicate. Inhibition of the growth of wheat coleoptile sections is reported for only two concentration, for each of the test compounds. *Denotes significant inhibition (P<0.01).

FIGURE 15.5

Assessments of phytotoxicity of 6-pentyl-2H-pyran-2-one (I), massoialactone (II), γ -decalactone (III), δ -decalactone (IV), γ -dodecalactone (V), and δ -dodecalactone (VI). (From Parker, S.R., *J. Agric. Food Chem.*, 45:7, 2776, 1997. With permission.)

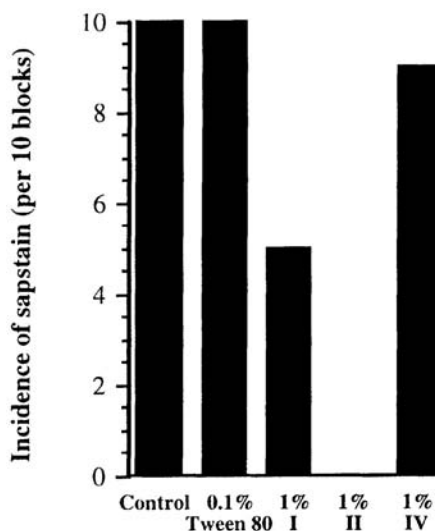
judged by gas chromatography) of massoia bark oil (FEMA 3747). More recently, the microbial production of this metabolite has been reported in yields anticipated to make the bio-synthetic production of this chiral molecule economical.^{26,27} The compound therefore has all the favorable attributes of I, with greater *in vitro* antifungal activity and the potential for economical production as a “natural”.

Early trials of II alerted us to the potential phytotoxicity of this compound. When applied to leaf surfaces as a 1.0% (v/v) aqueous emulsion, localized tissue necrosis was observed within 24 h of application. However, it was noted that the same effects were observed with each of the lactones (I, III-VI) when applied in this manner over the same concentration range. The phytotoxicity was not systemic as judged by the continued healthy growth of untreated parts of the plant. This “nonspecific” mode of phytotoxicity contrasted with the relative activity of the compounds in both the etiolated wheat coleoptile assay and the lettuce seed germination assay (Figure 15.5).

Seeking an application where the potential phytotoxicity of II would not be an issue, we evaluated the compound for its ability to control sapstain in sawn timber (*Pinus radiata*). Sapstain, as its name implies, is a staining of the sap wood of sawn timber. It is caused by a heterogeneous collection of fungi that grow through the wood and become pigmented, thus degrading its visual appearance. Marked differences were observed between the relative ability of I, II and δ -decalactone (IV) to control the development of sapstain in a laboratory based trial (Figure 15.6). These results are particularly striking when one considers that each compound in the series differs only in its degree of desaturation.

FIGURE 15.6

Control of sapstain by 6-pentyl-2*H*-pyran-2-one (**I**), mas-soialactone (**II**), and δ -decalactone (**IV**). Freshly sawn wood blocks ($50 \times 50 \times 7$ mm) were sterilized by γ -irradiation. Blocks were dipped individually in a 1% (v/v) emulsion of test compound prepared in sterile 0.1% (v/v) Tween 80. Each block was dipped for 30 seconds with gentle agitation and then placed on edge and allowed to drain. Single blocks were inoculated with 200 μ l of a spore suspension (c. 10^6 spores ml^{-1}) of sapstaining organisms FK64 and FK150 and placed in 500 ml glass jars. Each glass jar contained a filter paper disc moistened with 2 ml sterile distilled water and was sealed. Wood blocks were not in direct contact with the filter paper discs. Ten wood blocks were employed per treatment set. The wood blocks were incubated at 25°C for 7 to 10 days and scored for the presence or absence of sapstain.



15.4 Synthetic Analogs

An alternative approach to examining the structure–activity relationships of naturally occurring analogs of **I**, was to assess the synthetic obstacles to the economical production of **I** and determine what, if any, synthetic analogs could be prepared more readily. The 4-methyl substituted analog of **I**; 4-methyl-6-pentyl-2*H*-pyran-2-one (**VII**), may be prepared by a route analogous to that proposed for the synthesis of **I** by Pittet and Klaiber (1975) (Figure 15.7).²⁸ In the preparation of this methyl substituted analog difficulty in preparation of the esterified reagent is mitigated, and lactonization of the mixed keto-acids formed by the Friedel-Crafts acylation of hexanoyl chloride proceeds under standard conditions.

The ease with which **VII** could be prepared was confirmed and the vacuum distilled product tested for antifungal activity. The *in vitro* activity of **VII** was comparable with that of **I** (Figure 15.8). Recognizing that although innate biodegradability is an attractive aspect of natural products for use as agrochemicals, too short a biological half-life may render their use impractical. Structural modification of the “lead compound” (**I**) in this manner may yield compounds of practical use in the field, both in terms of their relative cost and rate of biodegradation.

15.5 Closing Remarks

The research reviewed here serves to underscore the adage that bioactive natural products serve as “lead compounds” for discovery. From the identification of **I** as a “natural fungicide” two promising candidates for further development have been identified. Along the way we are generating data that will help us understand the key structural requirements that define antifungal activity for this family of compounds. However, we should be cautious not to be too simplistic in our approach. Although a simple molecule, the behavior of

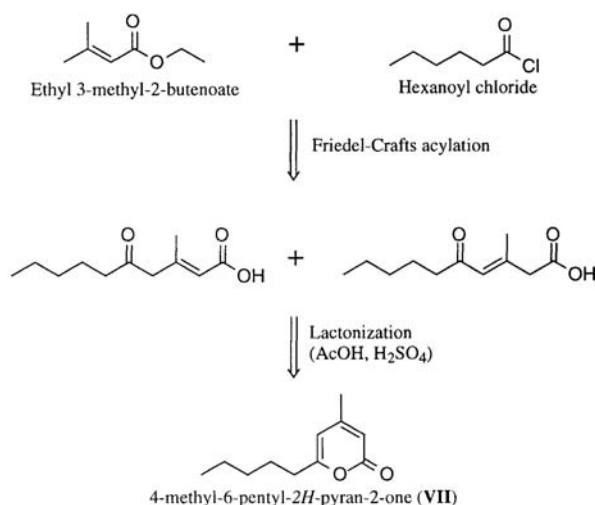


FIGURE 15.7

Synthetic scheme for the preparation of 4-methyl-6-pentyl-2H-pyran-2-one (VII). (cf. Figure 15.2.) (Adapted from Lohaus, G. et al., *Chem. Ber.*, 100, 658, 1967.)

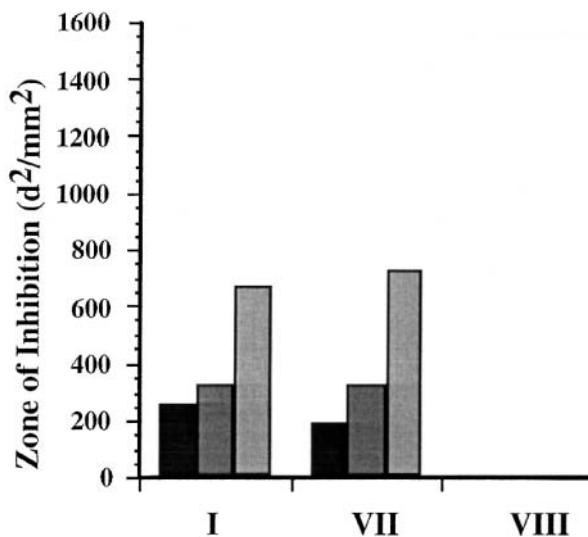


FIGURE 15.8

Spore suspensions were prepared by washing sporulating plates or slopes of the test organism with 10 ml sterile 0.1% (v/v) Tween 80. The spore density of the aspirated volume was determined using an improved Neubauer hemocytometer. The spore suspension was used to inoculate molten potato dextrose agar (PDA) maintained at 45°C. (Solid — *Penicillium digitatum* at 10⁶ spores ml⁻¹, coarse shading — *Botrytis cinerea* at 10⁵ spores ml⁻¹, fine shading — *Monilinia fructicola* at 10⁴ spores ml⁻¹.) Ten milliliters of the inoculated PDA was poured over the surface of a petri dish (90 mm dia.) containing a uniform base layer of 10 ml 1% (w/v) water agar and allowed to solidify. Solutions of test compounds (6-pentyl-2H-pyran-2-one (I), 4-methyl-6-pentyl-2H-pyran-2-one (VII), or 4,6-dimethyl-2H-pyran-2-one (VIII)) were prepared in acetone and applied to sterile 6 mm diameter filter paper discs (Whatman No. 3). After allowing the solvent to evaporate, the impregnated filter paper discs were placed on the surface of the solidified agar. Three discs were used per plate placed equidistant from each other and the center of the plate. Plates were incubated at 25°C for 24 h and the diameters (d) of the resulting zones of inhibition measured.

6-pentyl-2H-pyran-2-one is complex. Understanding its mode of action may prove more challenging than one might anticipate, if indeed 6-pentyl-2H-pyran-2-one is the physiologically relevant species and not simply an artifact of our extraction methods.

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Aflastatins: New Streptomyces Metabolites that Inhibit Aflatoxin Biosynthesis

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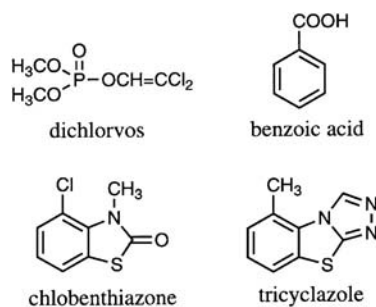
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16.1 Introduction

Aflatoxins are a group of mycotoxins produced by some strains of the fungi, *Aspergillus parasiticus*, *Aspergillus flavus*, and *Aspergillus nomius*. These aflatoxin-producing fungi are present ubiquitously in the world, but they don't always produce the toxin. Under some environmental conditions of high temperature and humidity, especially at tropical or sub-tropical zones, they infect agricultural products such as peanuts or corn, and produce aflatoxins not only on the outside, but also on the inside. Aflatoxins were first found in 1960 as toxic metabolites produced by *A. flavus* which killed numerous turkeys in England. They also were shown to have an extremely potent carcinogenicity toward mammals and found as contaminants in a wide variety of food commodities. Aflatoxin is now generally recognized not only as an extremely toxic contaminant in foods and feeds, but also as one of the certain risk factors for liver cancer in humans.¹ Thus, control and management of aflatoxins have become issues of concern.²

FIGURE 16.1

Structures of some inhibitors of aflatoxin biosynthesis.



To protect foods and feeds from aflatoxin contamination, some antibiotics which inhibit the growth of the producing fungi are useful. However, effective fungicides are usually toxic to mammals to some extent and their use can easily produce drug resistant strains. On the other hand, it is known that the production of aflatoxin is not essential for the growth of its producing strain, which is similar to cases observed in many other secondary metabolites produced by microorganisms. Therefore, a specific inhibitor of aflatoxin biosynthesis that does not inhibit the growth of its producer may be a good candidate for an effective drug to depress aflatoxin contamination without rapid emergence of a resistant strain.

Until now, many substances including pesticides or extracts of plants have been bioassayed to find inhibitory activity on aflatoxin production (Figure 16.1).³⁻⁶ Among them, organophosphorous insecticides with cholinesterase inhibitory activity, such as diclorvos, were found to show unique inhibitory effects on aflatoxin production. They inhibit some esterases involved in the biosynthetic pathway of aflatoxin resulting in the inhibition of aflatoxin biosynthesis.⁷ Some inhibitors of pentaketide-derived melanin biosynthesis in fungi, such as tricyclazole and chlobenthiazone, were recently shown to have an inhibitory activity toward aflatoxin production in *A. flavus*.^{8,9} They may inhibit a reductase in the biosynthetic pathway of aflatoxin.¹⁰ However, none of these chemicals has been used in protecting agricultural products from aflatoxin contamination.

Secondary metabolite production of fungi usually starts at a stationary growth phase which is regulated by an unknown mechanism. Several environmental and nutritional factors, such as temperature, humidity, carbon sources, and metal ions, are thought to play a role in the regulation. But, since such factors are constantly important throughout a life cycle of fungi, they are not specific regulatory factors for secondary metabolite production. Aflatoxin is one of the typical secondary metabolites produced by fungi. Therefore, a specific inhibitor of aflatoxin production that acts on the regulatory system of aflatoxin biosynthesis may be very useful, not only as a lead compound for developing a drug to protect foods from aflatoxin contamination, but also as a probe to investigate the regulatory mechanism of secondary metabolite production in fungi.

From the viewpoints mentioned above, we have been screening for specific inhibitors of aflatoxin production among metabolites of microorganisms. During the course of the screening, mycelial methanol extracts of *Streptomyces* sp. MRI142 were found to strongly inhibit aflatoxin production in *A. parasiticus*. The active principle, named aflastatin A, was then isolated and its structure was characterized as **1**,^{11,12} which is a novel tetramic acid derivative with a highly oxygenated long alkyl chain. In this article, we describe the isolation, characterization, biosynthesis, and biological activities of this new inhibitor of aflatoxin production aflastatin A, and also describe structures and biological activities of related compounds that were recently characterized after discovery of aflastatin A.

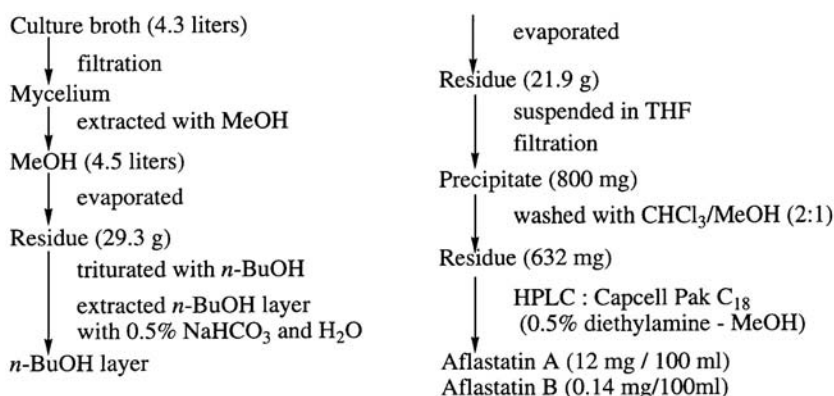


FIGURE 16.2
Isolation procedure for aflastatins.

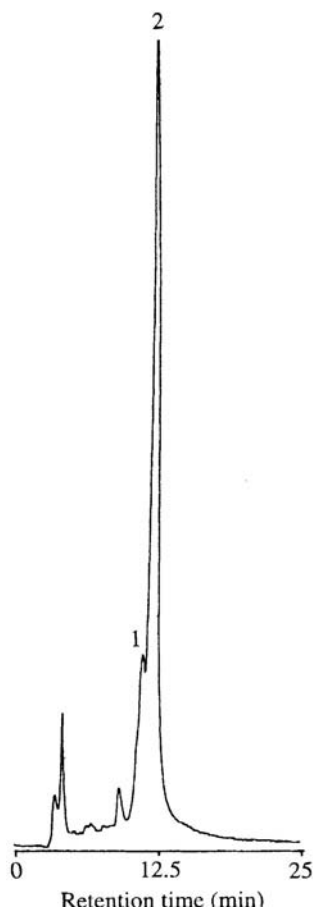
16.2 Discovery and Isolation of Aflastatins

To start a screening search for inhibitors of aflatoxin production, we first constructed a simple bioassay system for the screening. In the system, *A. parasiticus* NRRL 2999 and *Bacillus megaterium* ATCC 25848, an aflatoxin sensitive bacteria, were used as the aflatoxin producer and the indicator of aflatoxin production, respectively. A test sample was aseptically added to a flask containing a medium suitable for aflatoxin production, and spores of *A. parasiticus* were inoculated into the flask. After incubation for 7 days, the growth of the fungus was visually observed and the aflatoxin content of the culture broth was measured by a paper disk method based on the antibiotic activity of aflatoxin against *B. megaterium*. Using this method, we searched for inhibitors of aflatoxin production of *A. parasiticus* among metabolites of streptomycetes. Strains, whose culture broths did not inhibit the fungal growth but inhibited its aflatoxin production, were selected. During the course of this screening, a methanol extract of strain MRI142 mycelia showed biological activity.¹¹

Strain MRI142 was identified as *Streptomyces* sp. by taxonomic studies. It seemed to be similar to *Streptomyces griseochromogenes*, but as yet this has not been confirmed. This strain was fermented in a jar fermenter for 7 days at 27°C under conventional conditions for *Streptomyces* cultivation. Since the inhibitory activity was detected only in the mycelium, the culture broth was filtered to collect the mycelial cake which was extracted with hot methanol. The isolation procedure of the active principle from this extract is summarized in Figure 16.2. In brief, after removing the solvent from the extracts, the residue was triturated with *n*-butanol, and the *n*-butanol layer was concentrated. The residual oil was suspended in tetrahydrofuran and an active amorphous precipitate was obtained. After this precipitate was washed with a solution of chloroform-methanol (2:1), the residue was finally purified by reverse-phase HPLC under basic conditions to afford the main active principle, aflastatin A. As indicated in the HPLC profile of the last step (Figure 16.3), a minor component, named aflastatin B, also was isolated. Aflastatin A was obtained as a white powder after lyophilization. The yield of aflastatin A was relatively high, about 12 mg from 100 ml culture broth of *Streptomyces* sp. MRI142. However, repeated runs of the HPLC were necessary to obtain a sufficient amount because of poor solubility of aflastatins in the mobile phase.

FIGURE 16.3

HPLC purification of aflastatins. Peaks: 1, aflastatin B; 2, aflastatin A. HPLC conditions: column; Capcell-Pak C₁₈ (4.6 × 250 mm), mobile phase; 0.5% diethylamine/methanol (37:63), flow rate; 0.5 ml/min, detector; UV 300 nm.



16.3 Structure of Aflastatin A

Structural elucidation of aflastatin A which was obtained as a main component was attempted first. Since aflastatin A had a relatively high molecular weight (1257), complex polyol functions, and a tautomeric nature, it was difficult to rapidly determine its structure. Structural determination of aflastatin A was performed mainly by NMR and chemical degradation procedures as described in this section.

Aflastatin A **1** is soluble in a limited number of solvents. It is slightly soluble in methanol and aqueous ethanol, and insoluble in water, but dimethyl sulfoxide is a good solvent. Positive and negative FABMS spectra and HR-FABMS spectra, as well as elemental analysis, strongly suggested that the molecular formula of **1** was C₆₂H₁₁₅NO₂₄. This was finally confirmed after completing analysis of NMR spectra of **1** mentioned later. The ultraviolet spectrum of **1** was very characteristic. It showed a bathochromic shift from the absorption maxima at 299 and 247 nm in neutral and basic solution to those at 314 and 237 nm in acidic solution. The IR and ¹H NMR spectra of **1** in DMSO-*d*₆ solution suggested that **1** contained a lot of hydroxyl groups. The NMR signals of the carbons involved in the chromophore moiety of **1** were not clearly observed because of signal broadening when ¹³C NMR was

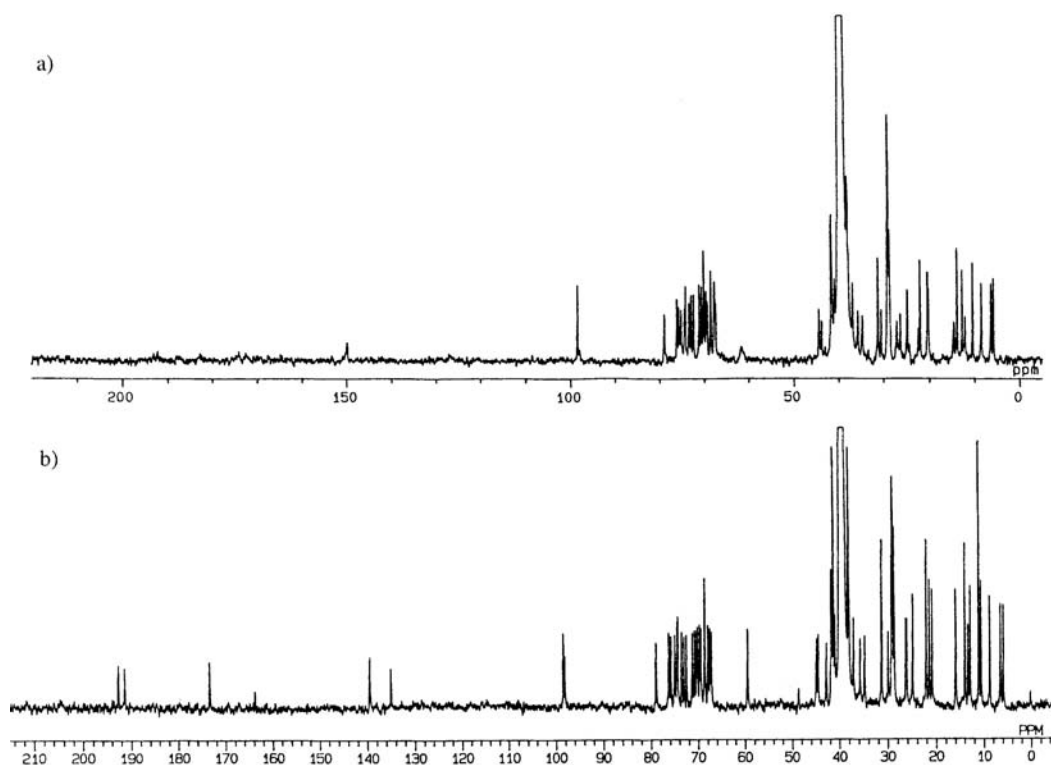


FIGURE 16.4

(a) ^{13}C NMR spectrum of free acid of **1** (in $\text{DMSO}-d_6$), and (b) ^{13}C NMR spectrum of diethylamine salt of **1** (in $\text{DMSO}-d_6$).

measured with a solution of free acid of **1**, but they could be detected when it was measured using a sample of diethylamine salt of **1** (Figure 16.4). The diethylamine salt of **1** was produced by addition of diethylamine to the mobile phase of the HPLC under basic conditions at the final purification step of **1**. This salt was used for measurement of all NMR spectra of **1** thereafter. A solvent suitable for the NMR measurement was practically restricted to only $\text{DMSO}-d_6$ because of the limited solubility of **1** mentioned above.

By analyzing a variety of NMR spectra of **1**, such as DQF-COSY, DQF-relayed COSY, HMQC, and HMBC, the presence of the partial structure (A) was clarified (Figure 16.5). Structure A was highly oxygenated and by analysis of a coupling of each hydroxy proton

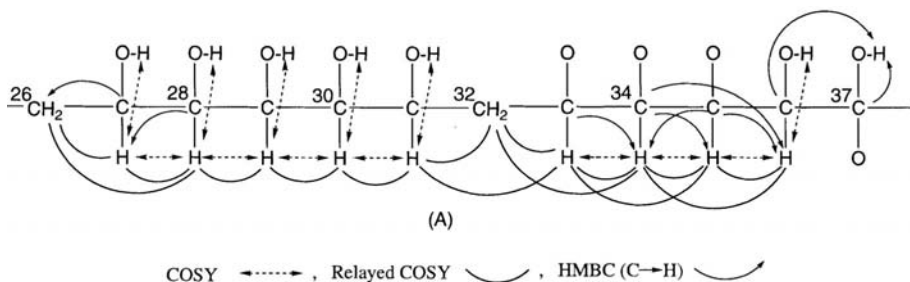


FIGURE 16.5

COSY, relayed COSY, and HMBC correlations observed in partial structure A.

observed in the COSY and HMBC spectra of **1** in DMSO- d_6 solution, the presence of several hydroxyl groups was verified (Figure 16.5). Other small partial structures also were identified from the spectra mentioned above, but it was difficult to determine the total structure of **1** by further NMR analysis with the original molecule because of hard signal overlapping. Therefore, the preparation of a useful derivative of **1** for the structure determination was attempted. Methylation of **1** with HCl-MeOH easily afforded a single product possessing a methoxyl group. This derivative suggested that the tertiary hydroxyl group in structure A might be present as a part of a hemiketal function, but it was not useful for further structural analysis of **1**. Acetylation with pyridine and acetic anhydride also was performed, but significant main products could not be obtained by the reaction. These results prompted us to do degradation experiments to obtain fragment molecules for determination of the remainder of the structure of **1**.

Since the partial structure (A), which has several diol moieties, was clarified, oxidation of **1** with NaIO₄ was carried out to obtain fragment molecules. First, **1** was oxidized with NaIO₄, followed by NaBH₄ reduction, and acetylation. The crude products obtained were purified by Sephadex LH-20 column chromatography and reverse-phase HPLC to afford two main products, **2** and **3**. A fragment was easily identified as a α,β -unsaturated carboxylic acid having the structure of **2** by analysis of its MS and NMR spectra. Another fragment had no characteristic absorption maximum in its UV spectrum, and the molecular formula was determined as C₄₄H₇₀O₂₀ by its HR-FABMS spectrum. By analysis of COSY and HMQC spectra, 10 partial structures, AcOCH₂CH(CH₃)–, –CH(CH₃)CH(OAc)– \times 4, –CH₂CH(OAc)– \times 4, and –CH₂CH₂OAc, which contained all atoms involved in the fragment were identified. Because of the close structural similarity in these partial structures, it was difficult to determine their relationship. However, detailed analysis of the HMBC spectrum could help the process. Thus, the structure of the fragment was assigned as a polyacetylated polyol **3**. Since a fragment molecule containing the chromophoric moiety of **1** was not obtained as a product from the reaction mixture, **1** was next oxidized with NaIO₄, followed only by NaBH₄ reduction. In this case, after purification of the crude products by reverse-phase HPLC under basic conditions, the fragment, whose UV spectrum showed the same absorption maxima as **1**, was obtained as a major product. The HR-FABMS spectrum of fragment **4** indicated that the molecular formula was C₁₇H₂₇NO₄. The UV spectrum of **4**, with a characteristic bathochromic shift in acidic solution and the chemical shifts of four carbon signals (δ_c 195.5, 175.8, 101.0, and 196.0 in MeOH- d_4 solution for C-1, -2', -3', and -4', respectively) contained in **4**, strongly suggested the presence of a tetramic acid skeleton. This was confirmed by HMBC experiments, and further analysis of COSY, HMQC, HMBC, and NOESY spectra lead to determine its structure as **4**. Though it is known that a tetramic acid moiety of the chromophore also is labile to excess NaIO₄, fragment **4** was obtained in the yield of about 50% by the above reaction. The degradation experiments of **1**, to afford the three fragment molecules, **2**, **3**, and **4**, are summarized in Figure 16.6.

The total carbon skeleton of **1** could be constructed with the four structures mentioned above (structure A, fragment **2**, **3**, and **4**) since fortunately all 62 carbon atoms of **1** were involved. Based on the fact that **1** had no α,β -unsaturated carboxylic acid, acetoxymethyl, or hydroxymethyl groups in its molecule, the carbon skeleton of **1** was easily reconstructed with the four structures as follows. The α,β -unsaturated carboxylic acid skeleton of **2** could be produced by cleavage of the hemiketal (ketone)-hydroxyl moiety in partial structure A, followed by reduction, acetylation, and β -elimination of the acetoxyl group. Thus, the connection between structure A and **2** was clarified. The acetoxymethyl groups at both ends of **3** indicated that two sets of vicinal diols were originally oriented in **1** adjacent to the group producing **3**. Fragment **4** should also originate by cleavage of a vicinal diol in **1**. Therefore, the connections between **3** and structure A, and **3** and **4** were used to construct the carbon skeleton of **1**. The presence of a methylene group at C-26 of **1**, confirmed by the correlation

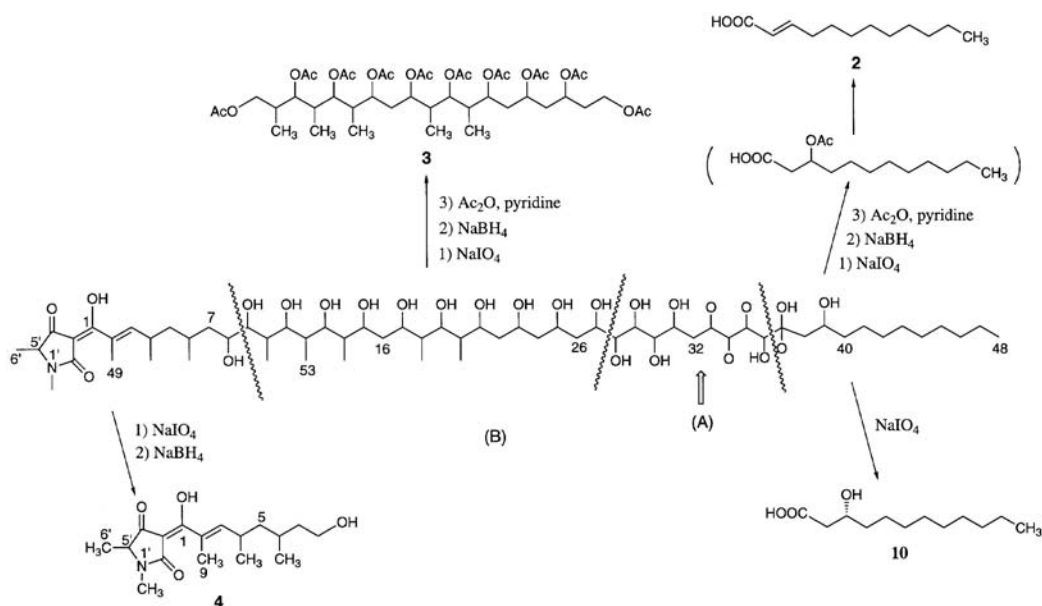
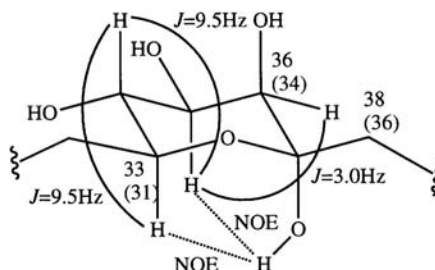


FIGURE 16.6
Degradation experiments of compound **1**.

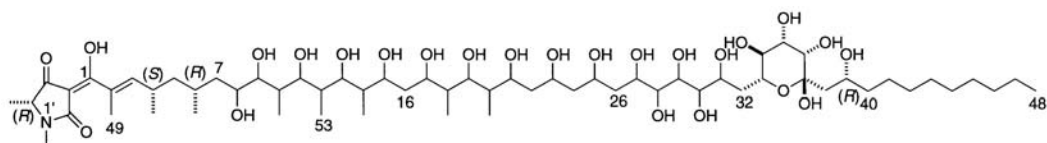
FIGURE 16.7
Relative stereochemistry of the tetrahydropyran ring of compounds **1** and **12**. Carbon numbers in the case of **12** are shown in parentheses.



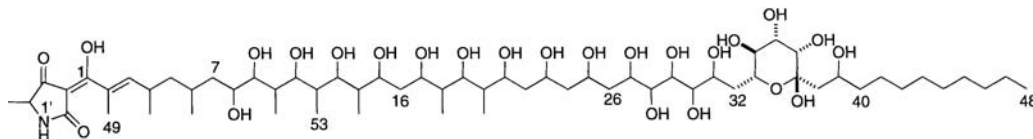
between the methylene proton at C-26 and the methine carbon at C-27 observed using HMBC, could determine the direction of linkage between **3** and structure A.

From the above results, a large partial structure (B) containing all carbon atoms of **1** was clarified (Figure 16.6). This structure and molecular formula of **1** indicated that the only remaining problem was the position of an ether linkage. The formation of a tetrahydropyran ring by the ether linkage between C-33 and C-37 was revealed by the J values and NOEs around the ring protons as shown in Figure 16.7. Thus, the total planar structure of aflastatin A was determined as **1**. It is a tetramic acid derivative with a highly oxygenated long alkyl chain. A saturated hydrocarbon skeleton forms the end part of the alkyl chain. Until now, a large number of natural products containing a tetramic acid skeleton have been isolated,¹³ but the structure of aflastatin A is unique among them.

Aflastatin A has numerous chiral centers. Determination of its absolute configuration is very important for further studies, such as chemical synthetic work or investigation of its mode of action. We have attempted to prepare crystal or crystalline derivatives of **1**, but no crystal suitable for x-ray analysis has been obtained. Therefore, we also have started work to determine the absolute configuration of **1** by using other chemical methods. At present, configurations of the four chiral centers at C-5', C-4, C-6, and C-39 of **1** have been clarified.

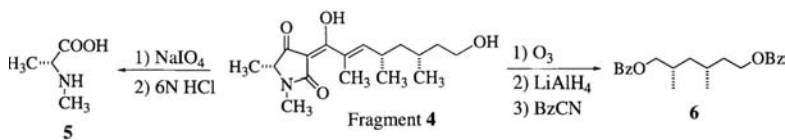


aflastatin A **1**

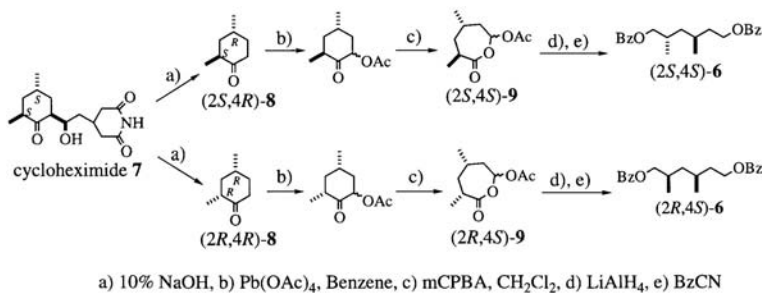


aflastatin B **11**

First, to determine the absolute stereochemistry of fragment **4**, **4** was degraded to afford *N*-methylalanine **5** and 2,4-dimethyl-1,6-hexanediol dibenzoate **6** according to the methods in Scheme 1. *N*-Methylalanine **5** was obtained by oxidation of **4** using excess NaIO_4 , followed by hydrolysis with 6N HCl. The absolute configuration of **5** was determined as D by a method with Marfey's reagent. On the other hand, O_3 oxidation of **4**, followed by LiAlH_4 reduction and benzoylation, afforded **6**. Since data to determine the absolute configuration of chiral **6** were not available in the literature, optically active (2*S*,4*S*)- and (2*R*,4*S*)-**6** were prepared as authentic samples from cycloheximide **7** (Scheme 2). Alkaline degradation of **7** afforded (2*S*,4*R*)- and (2*R*,4*R*)-2,4-dimethylcyclohexanone **8**, which were separated by normal-phase HPLC. Compound (2*S*,4*R*)-**8** was then acetoxylated and converted to a lactone (2*S*,4*S*)-**9** using the Baeyer-Villiger reaction. LiAlH_4 reduction of this lactone, followed by benzoylation afforded (2*S*,4*S*)-**6**. Similarly, (2*R*,4*S*)-**6** was obtained from (2*R*,4*R*)-**8**. Since the retention times of natural **6** and (2*R*,4*S*)-**6** on normal-phase HPLC were identical, it was indicated that natural **6** had *syn* stereochemistry. Finally, comparison of the CD spectrum of natural **6** with that of (2*R*,4*S*)-**6** showed that they were enantiomers. Thus, configuration of natural **6** was assigned as (2*S*,4*R*). To determine the absolute configuration of C-39, **1** was



SCHEME 1



SCHEME 2

oxidized with NaIO₄ and the reaction mixture was extracted with CH₂Cl₂. From the extracts, a β-hydroxycarboxylic acid **10** was obtained (Figure 16.6). The optical rotation value of **10** revealed that it has 3-(*R*) configuration. Work to elucidate the absolute configurations of the remaining chiral centers of **1** is now in progress.

16.4 Structure of Aflastatin B

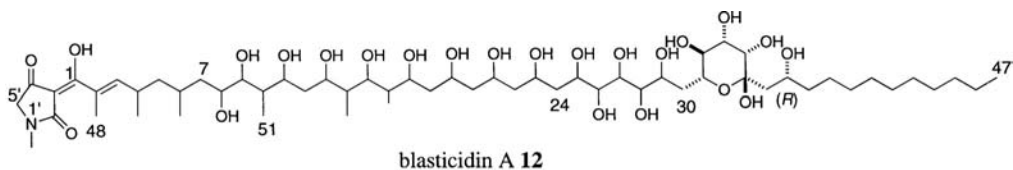
Aflastatin B **11** was isolated as a minor component of aflastatins produced by *Streptomyces* sp. MRI142 as mentioned above (Figure 16.3). The molecular formula of **11** was determined as C₆₁H₁₁₃NO₂₄ by its high-resolution FAB-MS spectrum that was smaller than that of **1** by one CH₂ unit. The UV spectrum of **11** was the same as that of **1**, indicating that **11** and **1** have a very similar chromophore. The ¹H and ¹³C NMR spectra of **11** also were almost the same as those of **1**. Only one difference was observed on the signals of *N*-methyl group, i.e., the signals of *N*-methyl group of **1** were not observed in the NMR spectra of **11**. These data clearly demonstrated that aflastatin B was the *N*-demethyl derivative of **1** having structure **11**.

When crude aflastatin was analyzed by HPLC equipped with a diode array detector, several minor peaks with the same UV absorption as aflastatin A were observed. However, since each of such extremely minor components of aflastatins was produced in a very low amount by *Streptomyces* sp. MRI142, they have not been characterized or used as derivatives to study structure–activity relationship of aflastatins.

16.5 Structure of Blastictimin A

During the course of our study on the chemical structure of aflastatin A, we discovered blastictimin A in the literature. Blastictimin A was an antibiotic discovered in 1955 in the culture broth of *Streptomyces griseochromogenes*.¹⁴ Then, Kono et al. reported its isolation and detailed physicochemical properties in 1968,¹⁵ but its structure has not been elucidated. Because close homology was observed between the physicochemical properties of blastictimin A and aflastatin A, the biological activity of blastictimin A was reexamined. It became clear that blastictimin A inhibits aflatoxin production by *A. parasiticus* like aflastatin A. These facts prompted us to elucidate the structure of blastictimin A.¹⁶ In this section, structural elucidation of blastictimin A is described.

Blastictimin A **12** was isolated as a white powder from a methanolic extract of *S. griseochromogenes* mycelia. Its isolation procedure was nearly identical to that of aflastatin A **1**. Analysis of the HR-FABMS spectrum and NMR spectra showed the molecular formula of **12** was C₅₈H₁₀₇NO₂₃, which is smaller than that of **1** by C₄H₈O. The UV spectrum of **12** was the same as that of **1**, indicating that **12** and **1** have a similar chromophore. The presence of a tetramic acid moiety in **12** was suggested by the UV and NMR spectra. ¹H and ¹³C NMR



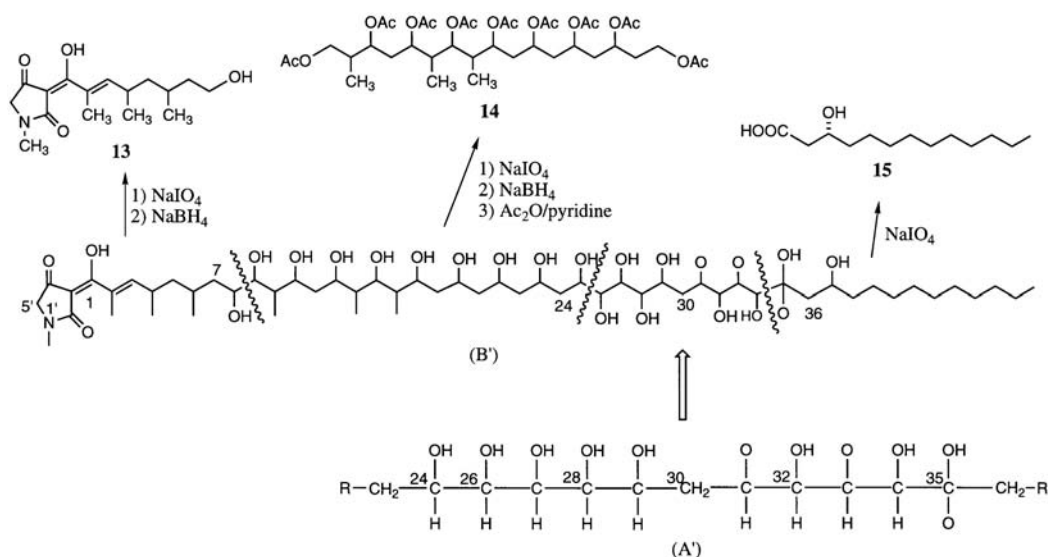


FIGURE 16.8

Partial structure (A') and degradation experiments of compound **12**.

spectra of **12** closely resembled those of **1**, and by analyzing a variety of NMR spectra of **12**, the presence of a partial structure (A') (Figure 16.8), which corresponds to the partial structure (A) in **1**, was clarified. From the NMR spectra, it was noticed that the remainder of structure **12** also resembled the counterpart of **1**. However, it was difficult to determine the total structure of **12** by further NMR analysis with the intact molecule as in **1**. Therefore, oxidation of **12** with NaIO_4 was carried out to obtain fragment molecules according to the method with slight modifications used for the preparation of fragments of **1** mentioned above.

The degradation experiments to afford fragment **13**, **14**, and **15** are summarized in Figure 16.8. First, the chromophoric fragment **13** was obtained by oxidation with NaIO_4 , followed by NaBH_4 reduction. Based on the result obtained from the similar oxidation experiment with **1**, this reaction was stopped when the amount of product **13** reached a maximum. Next, the polyol fragment **14** was obtained by NaIO_4 oxidation followed by NaBH_4 reduction and acetylation. Finally, the β -hydroxycarboxylic acid **15** was obtained only by oxidation with NaIO_4 . Structures of these three fragments were determined as **13**, **14**, and **15**, respectively, by analysis of MS and NMR spectra. The optical rotation value of **15** revealed that it has an 3-(*R*) configuration, which is the same as that of **10** from **1** mentioned above.

Since all carbon atoms of **12** were involved in structure A' or fragments **13**, **14**, or **15** as in the case of **1**, its total carbon skeleton could easily be reconstructed yielding a large partial structure (B') (Figure 16.8). From structure B' and the molecular formula of **12**, determination of the position of an ether linkage was the only remaining problem. The formation of a tetrahydropyran ring by the ether linkage between C-31 and C-35 was revealed by the *J* values and NOEs around the ring protons. Thus, the total structure of blasticidin A was determined as **12**. The relative stereochemistry of the tetrahydropyran ring of **12** was the same as that of **1** (Figure 16.7).

The structure of blasticidin A is similar to those of aflastatins. They may make a new small group of antibiotics from *Streptomyces*. There are a few differences between structure **1** and **12** as follows. A methyl group in the tetramic acid moiety of **1** is not present in the

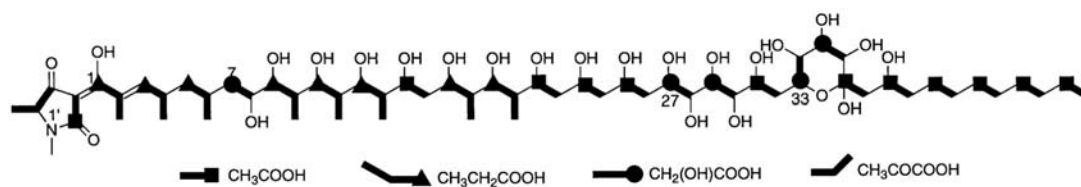


FIGURE 16.9
Biosynthetic origin of aflastatin A.

corresponding part of **12**. The length of the carbon chain and the number or position of methyl or hydroxyl groups in the part corresponding to fragment **14** are different from those in the counterpart of **1**. The length of the hydrocarbon end of **1** is shorter than that of **12** by one carbon unit. Comparison of biological activities between them is described in a later section.

16.6 Biosynthesis of Aflastatin A

Aflastatin A contains many hydroxyl groups in its alkyl side chain. If we assume that the alkyl chain is biosynthesized according to a usual polyketide pathway, several hydroxyl groups in **1** seem to attach to unexpected positions. Especially, the polyol skeleton present in partial structure A is not usually observed in polyketide metabolites. This prompted us to investigate the biosynthesis of **1**. In this section, we describe our feeding experiments with ^{13}C -labeled precursors to clarify the biosynthetic origin of carbon atoms involved in **1**.

First, feeding experiments using $[1-^{13}\text{C}]$ - and $[2-^{13}\text{C}]$ -acetate, and $[1-^{13}\text{C}]$ propionate were carried out. A labeled precursor was added, in one portion, to *Streptomyces* sp. MRI142 culture after the 48 h of cultivation. This is the approximate time when production of **1** started. The ^{13}C NMR spectrum of labeled **1** was used to determine the carbon enrichment. The ^{13}C -labeled pattern deduced indicated that most of the C_2 and C_3 units involved in the portion of C-1 to C-48 and C-2', 3', originated from acetic and propionic acid according to an expected polyketide pathway. However, no enrichment was observed at the C_2 unit of C-7,8 and two C_4 units of C-27,28,29,30, and C-33,34,35,36 (Figure 16.9). Concerning the origin of the C_2 unit of C-7,8, a glycolic acid or a related molecule might be the most probable candidate as with geldanamycin and leucomycin.^{17,18} On the other hand, two molecules of glycolic acid or a tetraose derivative might be incorporated into the two C_4 units. To clarify the origin of these unusual groups, a feeding experiment with $[U-^{13}\text{C}_6]$ glucose was carried out. In this incorporation experiment, soluble starch was used as the carbon source in place of glucose to avoid dilution of isotopically labeled glucoase. It was difficult to assign each ^{13}C - ^{13}C coupling observed due to signal overlappings in the ^{13}C NMR spectrum of labeled **1**. Thus, ^{13}C - ^{13}C COSY and J -resolved 2D NMR spectra were measured to analyze the couplings. In these results, ^{13}C - ^{13}C coupling was observed between a carbon pair in the C_2 unit of C-7,8 as well as between each carbon pair in the all C_2 units that were derived from acetic acid. Coupling occurred between each carbon pair in the C_2 units C-27,28, C-29,30, C-33,34, and C-35,36, but no coupling between C-28 and C-29 or between C-34 and 35 was observed. This indicated that a tetraose derivative such as erythrose, whose whole carbon skeleton directly originated from the glucose carbon skeleton, was not incorporated intact into the two C_4 units. Two other ^{13}C - ^{13}C couplings also were observed between C-4' and C-5', and

TABLE 16.1

Effect of Aflastatin A on Aflatoxin Production of *A. parasiticus* in Liquid Culture

Aflastatin A ($\mu\text{g/ml}$)	Aflatoxin ($\mu\text{g/ml}$)	Mycelial Weight (mg/10 ml)
0	4.1	58
0.03	2.3	63
0.13	2.0	61
0.50	Not detected	63

C-5' and C-6', indicating that the C₃ unit of C-4',5',6' came from the glucose molecule. Thus, it was presumed that the origin of the C₃ unit should be alanine biosynthesized via pyruvate.

The results obtained suggested that a glycolic acid or its derivative might be incorporated into the C₂ unit of C-7,8 and the C₄ units of C-27-30 and C-33-36. Therefore, a feeding experiment using [1-¹³C]glycolate was performed. In this case, a high level of ¹³C incorporation was not observed, but enrichment of C-7,27,29,33, and 35 was clearly evident.

From the results obtained above, we have summarized the biosynthetic origin of aflastatin A (Figure 16.9). The origin of all carbon atoms of **1**, except for C1 unit at C-7', have been clarified. Since aflastatin B (*N*-demethyl derivative of **1**) was obtained as a minor component, *N*-methylation of aflastatin B might be the final step in the biosynthetic pathway of aflastatin A. Biosynthetic origin of the two tetraol moieties of **1** is unique. To our knowledge, this is the first case of a tetraol skeleton that is biosynthesized from two molecules of glycolic acid.

16.7 Inhibitory Activities of Aflastatins and Blasticidin A on Aflatoxin Production

Aflastatin A inhibited aflatoxin production by *A. parasiticus* with high specificity at low concentrations. First, the effect of **1** on aflatoxin production was examined *in vitro*. When *A. parasiticus* NRRL2999 was cultured with a liquid medium containing **1** at a concentration of 0.03 $\mu\text{g/ml}$ for 7 days at 27°C, the amount of aflatoxin produced was reduced to about half of that of the control which was cultured without **1** (Table 16.1). When the concentration of **1** added to the medium was elevated to 0.5 $\mu\text{g/ml}$, **1** completely inhibited aflatoxin production in *A. parasiticus*. At that time, however, mycelial weight of the fungus was not reduced by the same concentration compared to that of control (Table 16.1). This indicated that aflastatin A at this concentration could inhibit aflatoxin production in the liquid culture without inhibiting the growth of its producer. Aflastatin A completely inhibited aflatoxin production of *A. parasiticus* also on agar plates at 0.5 $\mu\text{g/ml}$. In this case, the hyphal extension rate was reduced on the plate together with some morphological changes. But even at 100 $\mu\text{g/ml}$ on the plate, fungal growth was not completely inhibited though the hyphal extension rate was reduced by 70% of control.

Inhibitory activity of aflastatin B and blasticidin A also was tested using the same system as for **1** above. Both aflastatin B and blasticidin A showed about the same activity as aflastatin A, i.e., complete inhibition of aflatoxin production by *A. parasiticus* at 0.5 $\mu\text{g/ml}$ with no fungal growth inhibition. We now have some information about structure–activity relationships of aflastatin. Except for aflastatin B and blasticidin A, inhibitory activity of only one compound (fragment **4**) was examined. It showed no inhibitory activity toward aflatoxin production even at 20 $\mu\text{g/ml}$.

TABLE 16.2Effect of Aflastatin A on Aflatoxin Production of *A. parasiticus* in Peanut Plants

Aflastatin A (mg/plant)	Plant Weight (g)	Peanut Weight (g/7 pieces)	Growth of <i>A. parasiticus</i>	Aflatoxin Contents ($\mu\text{g/g}$)
0	485	11.0	++	4.15
2	414	11.6	++	2.08
10	432	11.5	++	0.08

A. parasiticus cultivated on raw peanuts produces aflatoxin. We examined the effect of aflastatin A using this model infection system of *A. parasiticus* on peanuts. Aflastatin A was topically applied to peanuts before inoculation of *A. parasiticus*. After cultivation of the fungus with or without aflastatin A, the peanuts were analyzed for aflatoxin content. Growth of the cultivated fungus was not affected by aflastatin A, but aflatoxin production in the peanuts was reduced. When applied to peanuts at 20 $\mu\text{g}/10\text{g}$ (peanut weight), aflatoxin was produced in only trace amounts. This suggested that aflastatin A may be effective in preventing peanuts from aflatoxin contamination during storage.

The effect of aflastatin A on aflatoxin production was next tested *in vivo* with the whole plant. Since it was difficult to perform a field experiment due to the pathogenicity of *A. parasiticus*, we used the following model *in vivo* assay system to examine the effect of 1. Peanut plants were cultivated from seed for 6 months at which time peanuts in the soil grew to maturity. Then, aflastatin was sprayed on plant leaves. Three days after application, peanuts were harvested and inoculated with *A. parasiticus* spores in the laboratory. After cultivation of the peanuts for 7 days at 27°C, aflatoxin content in the peanuts was determined. When 2 mg or 10 mg of aflastatin A was sprayed on peanut leaves, plant weight and peanut weight as well as growth of *A. parasiticus* on the peanuts, were not affected as compared to control. However, aflatoxin content was reduced depending on the dose of 1 sprayed (Table 16.2). This indicated that aflastatin A could be translocated from leaves to the developing peanuts 3 days after foliar application, and that it or its metabolite is involved in inhibiting of aflatoxin production by *A. parasiticus* inoculum. This may suggest that application of aflastatin A to leaves during cultivation of peanut plants can prevent aflatoxin contamination of peanuts by *A. parasiticus*.

16.8 Effects of Aflastatin A on Production of Other Polyketide Metabolites by Fungi

To investigate the mode of action of aflastatin A on aflatoxin production, we examined its effects on production of other secondary metabolites which are biosynthesized according to a polyketide pathway in various fungi. We first selected two strains (*Aspergillus parasiticus* ATCC24690 and *Aspergillus nidulans* IFO30872) that produced norsolorinic acid **16** and sterigmatocystin **17**, respectively. Both compounds were biosynthetic intermediates of aflatoxin¹⁹ (Figure 16.10). Strain ATCC24690 was cultured on agar plates to produce **16** (with or without aflastatin A) and content of **16** in the ethyl acetate extracts of the plates was analyzed by HPLC. Aflastatin A completely inhibited production of **16** at 0.25 $\mu\text{g}/\text{ml}$ without inhibiting fungal growth. Using a similar method, aflastatin A at 0.5 $\mu\text{g}/\text{ml}$ completely inhibited production of **17** by strain IFO30872. The fact that aflastatin A inhibited norsolorinic acid production is very important since norsolorinic acid is an intermediate in the earliest step of the aflatoxin biosynthetic pathway (Figure 16.10).

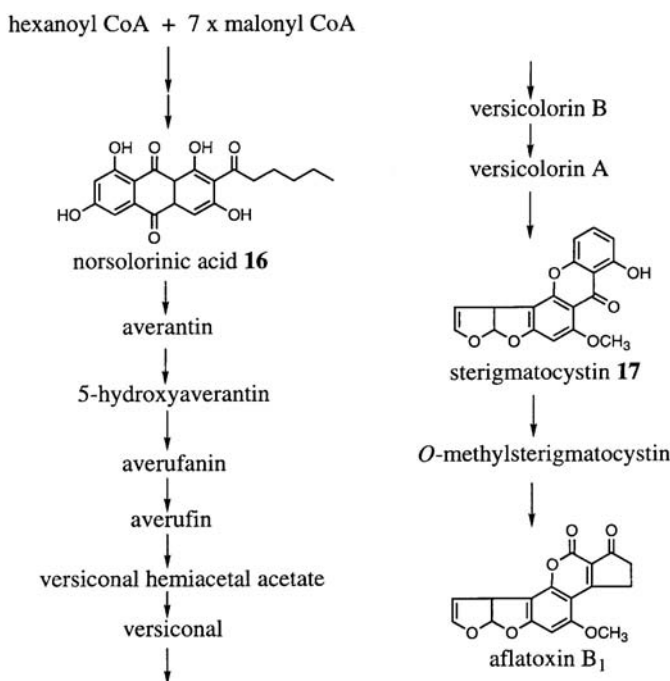


FIGURE 16.10

Biosynthetic pathway of aflatoxin B₁.

Next, effects of aflastatin A on production of citrinin, patulin, and 6-methylsalicylic acid by *Penicillium citrinum* ATCC8506, *Penicillium urticae* NRRL2159A, and *Penicillium griseofluorum* ATCC9260, respectively, were examined. Solid culture on agar plates was used to produce each metabolite, and the quantity of each compound was determined by HPLC. In these cases, inhibitory activity of aflastatin A on production of these three compounds was much weaker than that on aflatoxin production mentioned above. However, aflastatin A (50 µg/ml) caused a clear reduction of the amount of each compound. We are now examining the effects of aflastatin A on the production of other secondary fungal metabolites (such as terpenoids or alkaloids) produced by a variety of biosynthetic pathways.

16.9 Mode of Action of Aflastatin A

We now have some information about the mode of action of aflastatin A. Results from a norsolorinic acid-producing strain suggested that aflastatin A should act at a very early step of aflatoxin production. Furthermore, inhibitory activities toward production of polyketides other than aflatoxin suggested that aflastatin A may widely inhibit production of secondary metabolites biosynthesized by fungi via polyketide pathways.

Many genes regulating aflatoxin production have been identified. They include not only those encoding enzymes involved in aflatoxin biosynthesis, but also a regulatory gene whose product regulates expression of some enzyme genes for aflatoxin biosynthesis.^{19,20} The study with these genes provide a very important approach to investigating the mode of action of aflastatin A. We are performing some experiments to clarify whether or not the expression of these genes is affected by addition of aflastatin A. In a preliminary study,

mRNA of a gene that codes for one of the biosynthetic enzymes located in a later step of the pathway was found not to be expressed in *A. parasiticus* under conditions where aflatoxin production was completely inhibited by aflastatin A. Further studies to identify a target step or a target molecule of aflastatin A are now in progress.

16.10 Concluding Remarks

Since aflastatin has a novel structure and specific biological activity, it is expected to be a lead compound for new aflatoxin production inhibitors useful in protecting foods and feeds from aflatoxin contamination by fungi. It also may be a good probe to investigate mechanisms of polyketide biosynthesis in fungi since it inhibits production of some polyketide metabolites other than aflatoxin. Work to elucidate the mode of action of aflastatin A is thought to be an important next step.

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Practical Natural Solutions for Plant Disease Control

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ABSTRACT The viability of New Zealand's plant-based industries depends on the efficient production of high-quality produce for export. Development of natural systems for disease control will enhance the reputation of New Zealand produce on world markets and protect the market access. Biological control provides an alternative to the use of synthetic pesticides with the advantages of greater public acceptance and reduced environmental impact. The use of microorganisms as biological control agents (BCAs) seeks to restore the beneficial balance of natural ecosystems which is often lost in the crop situation.

Trichoderma has proved to be a useful BCA, the best strains producing high quantities of 6-pentyl-alpha-pyrone (6PAP). This compound inhibits the growth of sapstain fungi, including *Ceratocystis picea*, both *in vitro* and on wood. In field trials at mill sites various extracts from fungi and higher plants have given longer control of sapstain than standard commercial products. *Trichoderma* BCA strains also can be used to control *Botrytis cinerea* on kiwifruit and *Armillaria* spp. on trees, kiwifruit vines, and other woody plants. The *Trichoderma* metabolite 6PAP also has been used successfully to control these pathogens. Composts containing *Trichoderma* suppressed *Armillaria* in kiwifruit vines and encouraged more vigorous growth. The ideal delivery system for bioactive natural products often proves to be the BCA itself. In *Pinus radiata*, dipping the roots in a suspension of *Trichoderma* gave

good protection against *Armillaria*. Growing mixes containing *Trichoderma* controlled *Phytophthora fragariae* in strawberries in pot trials. *Botrytis cinerea* infects stem wounds of greenhouse tomatoes and can cause serious economic losses. *Cladosporium cladosporioides* has reduced infection from 80 to 100% to 0 to 10%. Stem-end rot of kiwifruit caused by *Botrytis cinerea* has been responsible for substantial post-harvest storage losses. The source of this inoculum is *B. cinerea* growth on necrotic tissue in the orchard. Biological suppression of *Botrytis* on necrotic kiwifruit leaf disks has been demonstrated. Selected fungal antagonists (*Epicoccum* spp. and *Ulocladium* spp.) reduced *Botrytis* spore production by up to 100% and some isolates were more effective than the fungicide, iprodione. In grapes *Botrytis* also is a major problem. Research in progress, partially funded by industry, is focused on suppression of *Botrytis* by selected microorganisms and the potential for control with antimicrobial natural products and elicitors.

17.1 Introduction

Disease suppression using composts and mulches has long been used successfully by “organic” gardeners and growers. While the natural suppression of plant diseases has been recognized but imperfectly understood for at least a century,¹ the deliberate use of biological control agents for disease control is a recent relative to that of biological control of insects and weeds.² Many of the soils that naturally suppress plant diseases are high in organic matter that support the growth of beneficial microorganisms.^{3,4} These include *Trichoderma* and *Pseudomonas* which are known to suppress the activity of soilborne plant pathogens. Two types of disease control have been identified: a short-term suppression of pathogens brought about by increased microbial activity, and long-term suppression which is influenced by a number of factors. Mechanisms of biological control include: induced resistance of the plant to the pathogen; parasitism, where beneficial organisms invade and consume pathogenic species; antibiosis/toxin production by beneficial organisms which may kill or inhibit disease agents; and exclusion or suppression of the pathogen by nonpathogenic strains and other organisms.

17.2 The Potential of *Trichoderma* and its Metabolites in Biological Control

Trichoderma species have been used successfully in field trials to control many crop pathogens. Examples include the control of *Nectria galligena* in apples;⁵ *Sclerotium rolfsii* in tobacco, bean and iris;⁶⁻⁹ *Rhizoctonia solani* in radish, strawberry, cucumber, potato, and tomato;¹⁰⁻¹³ *Sclerotium cepivorum* in onion;¹⁴ *Macrophomina phaseolina* in maize, melon, and bean;¹⁵ *Fusarium oxysporum* in tomato and *Chrysanthemum*;¹⁶⁻¹⁸ *Verticillium albo-atrum* in tomato;¹⁹ *Chondrostereum purpureum* in stone-fruit and other crops;²⁰⁻²² and *Botrytis cinerea* in apple.²³ Papavizas (1985) comprehensively reviewed the potential of *Trichoderma* as a biocontrol agent.

The most effective biological control agents for *Armillaria* in New Zealand include isolates of *Trichoderma hamatum* (Bon.) Bain, *T. harzianum* Rifai, *T. viride* Pers. ex S.F. Gray, and other *Trichoderma* spp., particularly those collected from *Armillaria*-infected orchards and forest sites. Some *Trichoderma* strains were isolated from situations in which they were

growing and consuming *Armillaria* mycelium and rhizomorphs. On transfer to the laboratory, *in vitro* tests confirmed the activity of the *Trichoderma* isolates against *Armillaria* and, following many further tests, superior strains were selected for field use.

Trichoderma species produce a number of antibiotics. One of these is 6-pentyl- α -pyrone (6PAP) which has antifungal activity (Chapter 15, Parker et al.). It is a common metabolite of our *Trichoderma* isolate. A 1:40 dilution of the purified metabolite applied at the rate of 15 μ l/4 mm disk inhibited the growth of *Aspergillus flavus* which produces aflatoxins.^{24,25}

17.3 *Armillaria* Control in *Pinus Radiata* and Kiwifruit with *Trichoderma* and 6PAP

Before 1980, the incidence of *Armillaria* in New Zealand kiwifruit was only occasional and considered to be a minor problem. Between 1980 and 1990 a dramatic increase occurred in the number of infected orchards and by 1995 over 2000 orchards were infected at an estimated annual cost of \$20 million.

Soil fumigation in California orchards gave some control of *Armillaria*. However, the mechanism for this control was found to be through increased activity of *Trichoderma viride* in fumigated soils.^{26,27} Dubos et al. (1978) found that *Trichoderma* reduced the initiation and growth of *Armillaria* rhizomorphs and five species of *Trichoderma* were isolated from forest soils in Ontario with a small incidence of *Armillaria* root decay.²⁸ All species of *Trichoderma* tested were found to be capable of inhibiting growth of mycelia and rhizomorphs of *Armillaria*. Some killed rhizomorphs and trials showed that *Trichoderma* effectively reduced the growth rate and infectivity of *Armillaria*.

Crude extracts of *Trichoderma* containing 6PAP were evaluated with *in vitro* assays against *Armillaria novae-zelandiae*. Potent antimicrobial activity was seen with 4 μ l per disk of 6PAP purified from the active crude extract. Other microorganisms also were strongly inhibited, including *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Chondrostereum purpureum*, *Phytophthora* spp., *Pythium* spp., and *Corticium rolsfii*, all of which are important phytopathogens. These results led to field trials in their respective crops.

In *Pinus radiata* tissue cultured plantlets, high 6PAP-producing *Trichoderma* isolates were tested in laboratory bioassays. No pathogenicity or toxicity was seen except in very aged cultures where nutrients were exhausted. Following this, forest trials were initiated in January 1991. Following treatment with *Trichoderma*, trees showed less mortality and were more vigorous compared to controls. Far fewer treated trees were infected and died from *Armillaria* (5.9%) compared with controls (22%). Treated trees were taller and had thicker trunks and wider canopies than untreated trees.

In kiwifruit orchards the stumps of shelter trees that had been cut down and were possible sources of *Armillaria* infection were treated with *Trichoderma* formulations. Soil amendments with *Trichoderma* inhibited or prevented the spread of the organism within kiwifruit orchards and, in addition, soil treatments in barrier trenches between infectious *Armillaria* sites and kiwifruit plantings have been very successful. Soil drenches also were effective. Injections with formulations of *Trichoderma* directly into the trunks of kiwifruit vines have shown that infected plants may recover. Pastes made up of *Trichoderma* applied directly to infected areas, where as much as four fifths of the vascular cambium has been destroyed, caused the vines to regain their vigor and become productive. Root treatments with *Trichoderma* reduced mortality in kiwifruit vine replants at diseased sites from over 50% of untreated plants to less than 5% of treated ones.

TABLE 17.1

Sporulation of *B. cinerea* and *Trichoderma* spp. on Postharvest Kiwifruit After Inoculation with *Botrytis cinerea* and Treatment with Six Isolates of *Trichoderma* spp

Isolate	<i>Trichoderma</i> spp.	<i>Botrytis cinerea</i>		<i>Trichoderma</i>	
		Spores/fruit ($\times 10^4$ /ml)		Spores/fruit ($\times 10^5$ /ml)	
TV	<i>T. viride</i>	0	b ^a	30.2	a ^a
M1037	<i>T. hamatum</i>	0	b	7.9	b
TBHPP7	<i>T. hamatum</i>	0	b	3.8	c
MTM	<i>T. hamatum</i>	0.5	b	3.6	c
D	<i>T. harzianum</i>	1.8	b	0.1	c
KEK	<i>T. hamatum</i>	13.9	a	0	c
Untreated		12.8	a	0	c

^a Means in a column followed by the same letter are not significantly different at $P = 0.05$.

Vine injection also has been employed as a method of application. *Armillaria*-infected kiwifruit vines in the Bay of Plenty were injected in February 1992 with treatments ranging from 10 to 100 μ l per vine and 300 μ l of a crude extract known to contain 6PAP from a high yielding isolate of *T. hamatum*. Other infected vines were injected with mixed-strain *Trichoderma* formulations with proven efficacy against *Armillaria*. Untreated *Armillaria*-infected vines died within 6 months. The 6PAP treatment significantly increased the survival rate (to ~50%) in infected vines. However, *Trichoderma* formulations were even more effective, and over 80% of the infected vines survived. The crude extract was approximately as active as 6PAP.

17.4 Control of *Botrytis* Stem-End Rot of Kiwifruit with *Trichoderma*

Stem-end rot of kiwifruit in storage caused by *Botrytis cinerea* Pers. is a major problem for the New Zealand kiwifruit industry²⁹ and has caused serious losses (\$10 to 15 million) in some years. Six isolates of three species of *Trichoderma* were evaluated for control of *B. cinerea* and their ability to inhibit *B. cinerea* spore production on fruit. *Botrytis cinerea* cultures were established on potato dextrose agar (PDA), each from a single spore isolated from a diseased fruit. Spore suspensions were made by washing spores from a 10- to 14-day-old PDA culture with water to inoculate the fruit. No *Botrytis* rot developed on any of the *Trichoderma*-treated fruit, but *B. cinerea* sporulation occurred at the wound site of some treated fruit. Three isolates of *Trichoderma* — TV (*T. viride*), M1037 (*T. hamatum*), and TBHPP7 (*T. hamatum*) — gave complete inhibition of *B. cinerea* spore production (Table 17.1).³⁰

17.5 Control of *Botrytis* in Greenhouse Tomatoes with *Cladosporium*

Botrytis cinerea infects stem wounds of greenhouse tomatoes and can cause serious economic losses. A bioassay using stem sections was developed to study wound infection and to screen potential fungal antagonists for activity against *Botrytis*. Isolates of *Cladosporium*

TABLE 17.2

Effect of Gel-Applied Antagonists on *Botrytis* Infection of Terminal Wounds of Whole Tomato Plants (Six Replicates)

BCA Isolate Number	Percent <i>Botrytis</i> Infection	(95% Confidence Interval)
Control	100	54.1–100.0
95-1 (<i>Trichoderma</i>)	33	4.3–77.7
806 (<i>Trichoderma</i>)	50	11.8–88.2
677 (<i>Cladosporium</i>)	0	0–45.9
712b (<i>Cladosporium</i>)	0	0–45.9
724 (<i>Cladosporium</i>)	0	0–45.9

cladosporioides reduced infection from 80 to 100% to 0 to 10%. *Trichoderma harzianum* isolates gave a smaller reduction. Similar results were obtained on whole plants. *Penicillium* isolates varied widely in activity. The concentration of *Cladosporium* and *Trichoderma* that gave the highest level of protection was c.10⁸ cfu/mL (Table 17.2). When only half the wound was treated, simulating a poor spray coverage, *Cladosporium* isolates still prevented infection. By contrast, the *Trichoderma* isolates and four fungicides failed to give the same level of protection. The ability of certain fungal isolates to colonize the wound surface was thought to be partly responsible for this activity. Antagonists were applied successfully to whole plants using both aqueous suspensions and gel secateurs.³¹ The efficacy of these treatments has been confirmed in field trials.

17.6 Biological Suppression of *Botrytis* in Kiwifruit

Botrytis cinerea has been responsible for substantial post-harvest storage losses. Biological control of *Botrytis* on kiwifruit tissues was described by Menzies et al. (1989),³² and this research was followed up in New Zealand in the early 1990s.³³ A biological suppression program was initiated which is aimed at reducing the ability of *Botrytis* to produce spores on necrotic tissues and subsequent contamination of fruit surfaces.³⁴ In 1995 an international collaborative research program between HortResearch and IPO-DLO (Wageningen, The Netherlands) was established with the primary aim of selecting fungi from New Zealand kiwifruit orchards that were capable of surviving field conditions; rapidly colonizing necrotic kiwifruit tissues, and suppressing *Botrytis* sporulation in the kiwifruit canopy.

Selected antagonists applied 24 h after *Botrytis* inoculation and exposed to field conditions for 7 days reduced *Botrytis* spore production on necrotic leaf disks by 90 to 100%. The majority of antagonists suppressed *Botrytis* spore production more effectively than iprodione (Rovral) (Figure 17.1). These field experiments were repeated 11 times during the 1996/97 growing season at two geographic locations and the findings were consistent and repeatable.³⁵

17.7 Silver-Leaf Disease Control with *Trichoderma* and 6PAP

Effective disease control using high 6PAP-producing strains of *Trichoderma*, especially *T. hamatum*, has been achieved in New Zealand against silver-leaf disease (*Chondrostereum*),

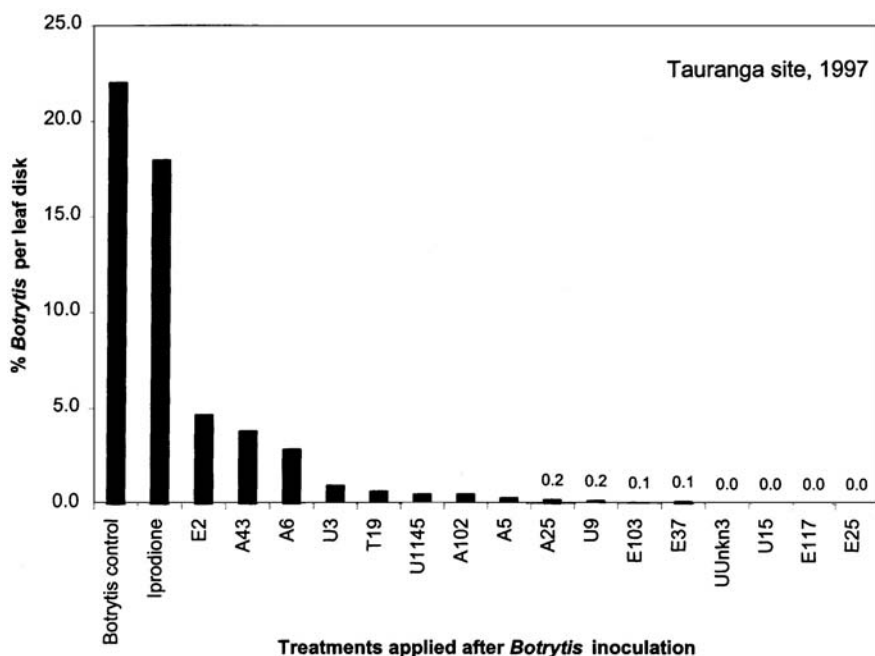


FIGURE 17.1

Effect of antagonists on *Botrytis* sporulation on necrotic kiwifruit leaf disks exposed to field conditions. (% *Botrytis* measured after 7 days exposure to field conditions then incubation in *Botrytis*-conductive conditions. *Botrytis* applied 24 h prior to the antagonists. A = *Alternaria* spp; E = *E. purpurea*; U = *Ulocladium* spp; T = *Trichoderma* spp; Iprodione = Rovral.)

an organism that was controlled *in vitro* by *Trichoderma* isolates in laboratory assays. Injections with liquid formulations of *Trichoderma* gave rapid control of silver-leaf in *Pyrus serotina* (nashi, Asian pear) with even severely affected trees recovering completely. Most treated trees remained disease free 2 years following the treatment. In addition, a pruning paste containing *Trichoderma* greatly reduced the spread of silver-leaf in infected nashi orchards. Similar results were obtained with 6PAP.

17.8 Sapstain Control with Natural Products and Biological Control Agents

Sapstain is caused by pigmented fungi growing in the sapwood. After tree felling, logs and sawn timber may develop sapstain in less than 1 week in mid-summer. *Pinus radiata* is particularly susceptible to sapstain which results in multimillion dollar losses annually through downgrading or rejection of wood. Research in progress aims to achieve cost-effective sapstain control in *Pinus radiata* logs and sawn timber for 6 months with environmentally friendly natural products and/or biological control agents (BCAs). All biological control and natural product treatments decreased the incidence of sapstain and the best natural product formulations gave good control of sapstain for over 6 months.

17.9 Disease Suppressive Pine Bark Composts

In addition to the direct application of beneficial microorganisms as BCAs, composts may be used to encourage the growth of these organisms. Research in progress aims to gather the basic biological knowledge on bark-based composts to develop economic and environmentally acceptable ways of dealing with ever-increasing volumes of waste bark and establish how the composting process can be manipulated to maximize disease suppressiveness and wettability in soilless potting mixes. Such composts reduce production costs by substituting for peat, a nonrenewable resource, and add value by using biological methods of disease suppression. Bark composts prepared *in situ* in orchards may provide a cost-effective method of controlling soil-borne fungal and bacterial diseases and suppress weeds, offering a realistic alternative to the use of methyl bromide, an ozone-reducing bio-cidal soil fumigant. The potential of pine-bark-based compost mixes to be disease-suppressive has been proven and a range of new techniques have been successfully developed to quantify the degree of disease suppressiveness obtained. New techniques also have been successfully developed to introduce known disease-suppressive organisms such as *Trichoderma*. These all represent significant contributions to the field of compost and disease suppressive research and open up new fields for the use of a renewable New Zealand resource. These research findings have been extended into the field to evaluate their practical application. This has required the development of a range of new techniques and methodologies. The resultant large-scale field trials now established offer the promise of improved orchard management, without recourse to chemicals, for several otherwise intractable and commercially important disease problems.

17.10 Inducing Plant Resistance to Powdery Mildew

As a final consideration, plants themselves may resist infection by using a combination of physical and chemical defenses. A failure or a delay in the deployment of these defenses can result in disease development. Another of our approaches for disease control, “induced resistance”, involves triggering the plants’ defenses by the application of compounds called elicitors. Elicitor-treated plants are said to be “sensitized” and thereafter respond more rapidly and intensely to subsequent attack by plant pathogens.

It has been shown that elicitors obtained from yeast cell walls showed broad specificity and could induce resistance against major fungal pathogens including powdery mildew on barley, grey mould and stem rot on lettuce, and chocolate spot on beans.³⁶ More recently we successfully induced resistance to shoot die-back in *Pinus radiata* and *Sclerotinia* in kiwi-fruit leaves.³⁷ All plants have inducible disease resistance mechanisms and it is likely that nonspecific elicitors could offer broad-spectrum disease control across several plant species. In these studies we tested an elicitor shown to be effective in *Pinus radiata* and kiwi-fruit for its potential to induce resistance to powdery mildew in grapes.

Elicitors reduced powdery mildew severity on green leaves on potted grape vines (cv. Chardonnay; Figure 17.2). The reduction of mildew was equivalent to that of sulphur applications. Key to the treatments: Untreated, water control (“pulse”), elicitor 1, elicitor 2, algan (seaweed-based extract sourced from Europlant B.V., The Netherlands), and a sulphur

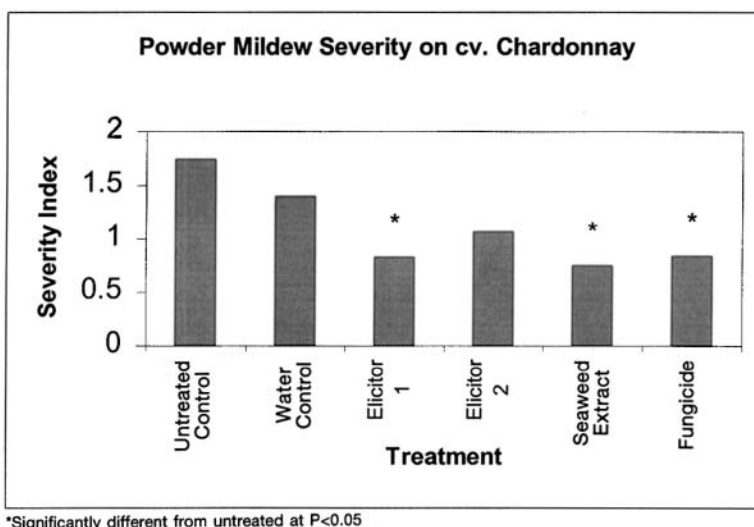


FIGURE 17.2

The effect of elicitors and a seaweed-based product on the severity of powdery mildew on grape leaves (cv. Chardonnay). The powdery mildew disease severity index was 0 = healthy, 1 = 1–5% leafy area, 2 = 5–25% leaf area infected. *Significantly different from untreated at $P < 0.05$.

standard fungicide (“Super six”). The treatments were applied at 10 day intervals. The benefits of induced resistance include:

- Reduction in pesticide use and increased environmental sustainability
- Broad specificity — elicitors enhance overall plant resistance and thus have the potential to induce resistance against a range of pathogens
- Durability — Induced resistance relies on a range of plant defenses and so development of pathogen resistance is unlikely
- Compatibility — Induced resistance can be integrated with other disease control methods

17.11 Conclusions

The integration of natural control measures that promote plant health and reduce plant disease, including biological control agents, natural products, and elicitors, with more conventional control methods can offer an economic and environmentally safe crop protection strategy for New Zealand crops.

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Cotton Pest Resistance: The Role of Pigment Gland Constituents

R. D. Stipanovic, A. A. Bell, and C. R. Benedict

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- 18.2 Terpenoid Aldehydes and Insect Resistance
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- 18.4 Conclusion
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ABSTRACT *Gossypium* species, of which *G. arboreum*, *G. barbadense*, *G. herbaceum*, and *G. hirsutum* (Upland cotton) are cultivated for production of cotton fiber, belong to the tribe Gossypieae of the family Malvaceae. The genera in the tribe are distinguished from other genera in the family by the production of lysigenous glands that contain gossypol, a polyphenolic terpenoid (Figure 18.1), in seed.¹ The glands are usually referred to as pigment glands because they appear as dark dots in leaves and stems and as yellow to orange dots on seeds and roots. Cottonseed is toxic to monogastric animals, such as humans, swine, poultry, fish, and rodents; the cause of this toxicity was associated with the lysigenous glands early in the 20th century. However, it was not until the 1940s that the major toxin was identified as gossypol and its structure determined.² Gossypol in seed also may be accompanied by small amounts of its 6-methyl and 6,6'-dimethyl ethers, as well as their precursors, hemigossypol and hemigossypol-6-methyl ether as shown in Figure 18.1.³ This same mixture also is found in roots. Studies on the toxicity of gossypol to animals have been reviewed.⁴

18.1 Biosynthesis of the Glandular Sesquiterpenoids

Terpenoids are derived via the isoprenoid pathway from mevalonic acid. In the case of the cotton sesquiterpenoids, (+)- δ -cadinene has been shown to be the first cyclized product in the pathway⁵ (Figure 18.1). Gossypol and thus δ -cadinene is derived from E,E-farnesyl diphosphate⁶⁻⁸ via nerolidyl diphosphate.⁹ Intermediates between δ -cadinene and desoxy-hemigossypol (dHG) have not been identified.

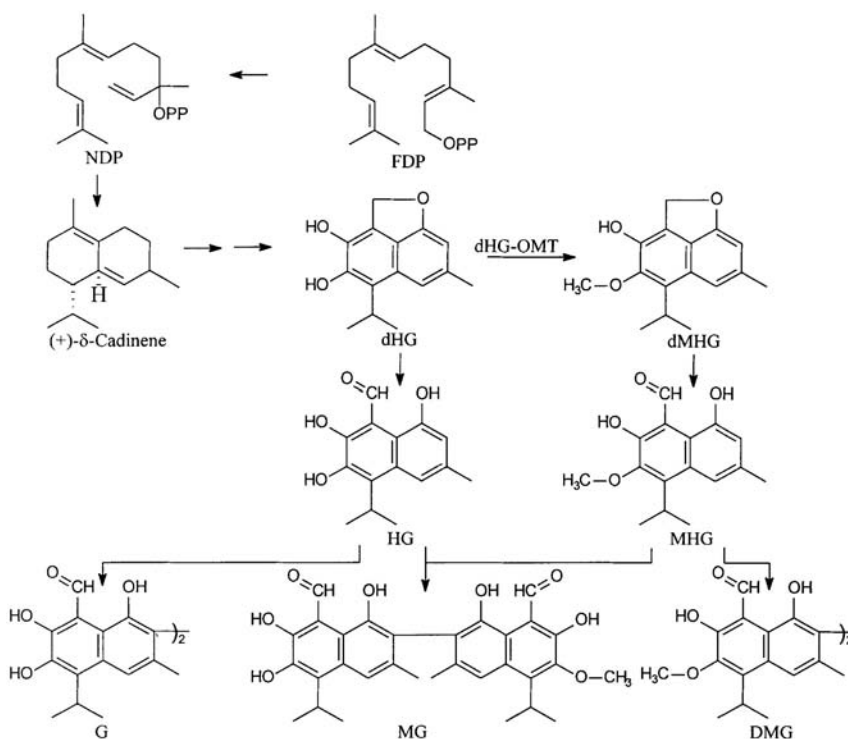


FIGURE 18.1

Structures and proposed biosynthetic pathway of cotton phytoalexins and cottonseed terpenoids (FDP = *E,E*-farnesyl diphosphate; NDP = nerolidyl diphosphate; dHG = desoxyhemigossypol; dMHG = desoxyhemigossypol-6-methyl ether; HG = hemigossypol; MHG = hemigossypol-6-methyl ether; G = gossypol; MG = gossypol-6-methyl ether; DMG = gossypol-6,6'-dimethyl ether; dHG-OMT = desoxyhemigossypol-O-methyltransferase).

dHG is converted to hemigossypol (HG) which is efficiently converted in the seed to gossypol presumably via a peroxidase enzyme¹⁰ (Figure 18.1). The peroxidase dimerization of HG yields a mixture of (+)- and (–)-gossypol. In *G. hirsutum*, the ratio of (+) to (–) is usually about 3:2, although cultivars with higher levels of the (+)-isomer have been identified. The (–)-isomer appears to be the more biologically active¹¹ and this isomer may be the primary cause of toxicity in nonruminant animals. Gossypol is also the predominate terpenoid aldehyde in root glands. In foliar plant parts, dHG is converted to hemigossypolone (HGQ)¹² (Figure 18.2). HGQ presumably undergoes a Diels-Alder reaction with either myrcene or β-ocimene to form heliocides H₂ and H₃^{13,14} from the former, and heliocides H₁ and H₄¹⁵ from the latter. The compounds HGQ, heliocides H₁, H₂, H₃, and H₄ together with gossypol constitute the major terpenoid aldehyde components in foliar glands of *G. hirsutum* plants.¹⁶

In *G. barbadense*, these compounds together with a group of O-methylated derivatives are produced¹⁷ (Figure 18.2). In *G. barbadense* the phenolic C-6 position of dHG is methylated via S-adenosyl-L-methionine (SAM) by an O-methyltransferase.¹⁸ The methylated derivative, desoxyhemigossypol-6-methyl ether (dMHG), is subsequently converted to hemigossypol-6-methyl ether (MHG). dMHG can undergo the same set of transformations as dHG giving rise to gossypol-6-methyl ether and gossypol-6,6'-dimethyl ether³ (Figure 18.1), and to hemigossypolone-6-methyl ether (MHGQ), and heliocides B₁, B₂, B₃, and B₄¹⁷ (Figure 18.2).

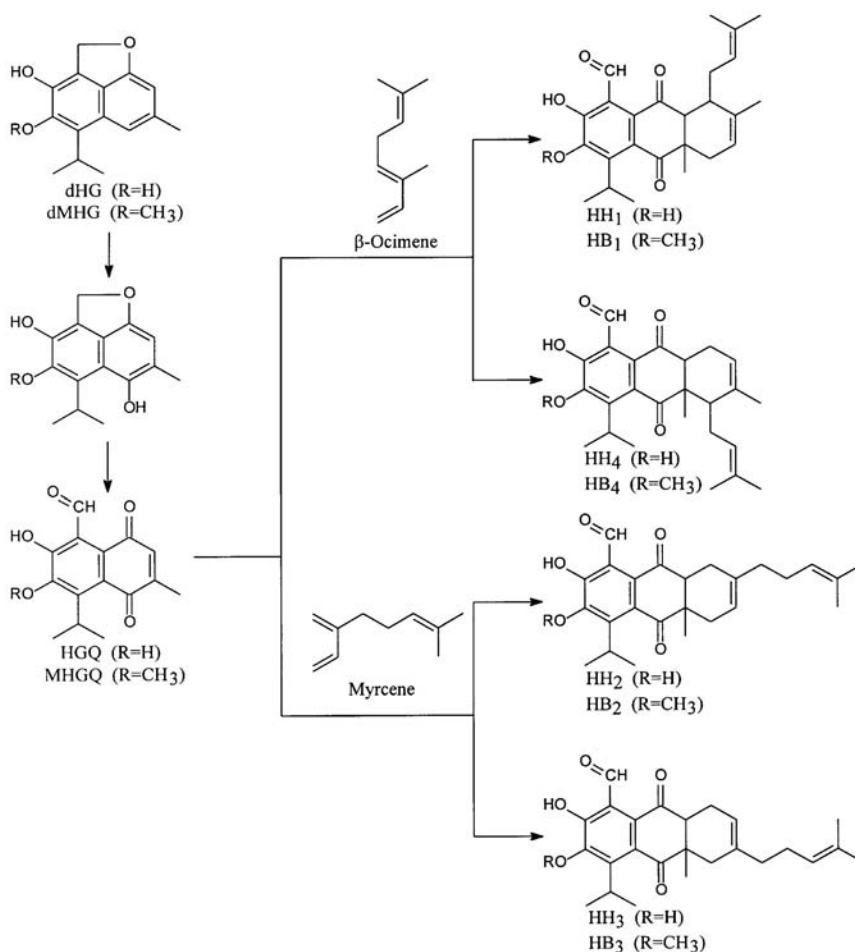


FIGURE 18.2

Structures and proposed biosynthetic pathway of the terpenoid in cotton leaves and flower buds (dHG = desoxyhemigossypol; dMHG = desoxyhemigossypol-6-methyl ether; HGQ = hemigossypolone; MHGQ = hemigossypolone-6-methyl ether; HH₁, HH₂, HH₃, HH₄ = Heliocides H₁, H₂, H₃, H₄; HB₁, HB₂, HB₃, HB₄ = Heliocides B₁, B₂, B₃, B₄).

Stem tissue in both *G. hirsutum* and *G. barbadense* is normally devoid of the terpenoids. However, invasion by a pathogen induces the synthesis of dHG and HG and their methyl ether derivatives. The concentrations of the methyl ether derivatives are higher in *G. barbadense* than in *G. hirsutum*. The levels of methylation vary greatly not only among *Gossypium* species, but also among tissues in a given species. Methylation is introduced into the terpenoid pathway at only one point, i.e., the transfer of a methyl group to desoxyhemigossypol to form desoxyhemigossypol-6-methyl ether (dMHG) as shown in Figure 18.1. All of the methylated terpenoid aldehydes, in turn, are derived from dMHG. The methyl group is transferred from SAM by desoxyhemigossypol-O-methyl transferase (dHG-OMT).¹⁸ HG does not act as substrate for this enzyme. dHG-OMT has been partially purified.¹⁸ All *Gossypium* species contain the structural gene for the synthesis of dHG-OMT. However, in *G. hirsutum* this structural gene is under the control of a dominant regulator gene, designated *TM1*, which apparently restricts the synthesis of the enzyme in leaf, stem,

and young boll tissues.^{19,20} Regulator genes also have evolved in many of the other *Gossypium* species to restrict methylation of the terpene aldehydes to some degree, especially in leaves.^{17,20,21} The cultivated species *G. barbadense* does not contain a regulator and consequently has relatively high percentages of methylated terpene aldehydes in all tissues.

The *TM1* regulator gene in *G. hirsutum* usually does not function well in juvenile tissues that serve as ports of entry for most pathogens. Thus, there is little restriction of methylation in root tips where fungal wilt pathogens, root rot pathogens, and nematodes usually penetrate to gain access to less protected tissues.²² Hunter²³ reported that there is little restriction of methylation in the young hypocotyl and root that is attacked by various seedling pathogens. There is some degree of regulation in xylem tissue but even here as much as 20% of the terpene is methylated in some cultivars of *G. hirsutum*.²⁴

18.2 Terpenoid Aldehydes and Insect Resistance

The discovery that gossypol in seed was localized strictly in glands and was responsible for cottonseed toxicity led to the search for glandless mutant cottons. It was hoped that cottonseed from glandless cottons could be used in human foods and in greater amounts in animal feeds. Such cottons were developed by crossing lines that contained very few glands. The completely glandless character was shown to be due to two recessive genes designated *gl2* and *gl3*. These genes were soon incorporated into many different commercial breeding lines. When the glandless plants were planted in the field, they were damaged more severely by insects and rodents known to feed on cotton. In addition, the glandless plants were attacked by various herbivores, such as beetles, rodents, and birds, that previously were not known to attack cotton.²⁵⁻²⁷ These studies show that the lysigenous glands provide protection against a wide range of herbivores. In Jenkin's²⁸ 1995 study of 56 accessions of *G. hirsutum* resistant to *Heliothis* spp., 33 were high in terpenoid aldehydes.

The experiences with glandless cottons led to the development of "high gossypol" or highly glanded cotton lines for increased resistance to insects. While highly glanded breeding lines were indeed more resistant to insects, the level of resistance could not always be explained by the gossypol content of leaves and flower buds. Efforts to explain this discrepancy led to the discovery that the major terpenoid aldehydes in glands of leaves and young bolls of Upland cotton were the terpenoid aldehyde quinone, hemigossypolone, and its derivatives, heliocides H_1 - H_4 , which are formed by a Diels-Alder reaction of the quinone with the volatile monoterpenes β -ocimene or myrcene as shown in Figure 18.2. Bell¹⁷ showed that several *Gossypium* species also contain the 6-methyl ethers of hemigossypolone and its heliocides as major terpenes in leaves and bolls. The methyl ethers of heliocides H_1 - H_4 are referred to as heliocides B_1 - B_4 because of their original discovery in *G. barbadense*. Elzen et al.²⁹ and Bell et al.³⁰ subsequently showed that all of the volatile monoterpenes and sesquiterpenes, such as β -ocimene, myrcene, α - and β -pinenes, γ -terpinene, β -caryophyllene, humulene, and β -bisabolene, also are stored in the lysigenous glands located in green tissues. Thus, the glands in aerial parts of the plant that contain chlorophyll contain unique terpenoid aldehydes, in addition to gossypol, dissolved in essential oils. Resistance of leaves or bolls to insects correlates best with the concentrations of hemigossypolone and heliocides H_1 and H_2 .³¹ Also, in artificial diets hemigossypolone-6-methyl ether and the methylated heliocides are less than one half as toxic as their unmethylated counterparts.³²

18.3 Terpenoid Aldehydes and Disease Resistance

In the U.S. two species of cotton are grown commercially, *G. hirsutum* or Upland cottons and *G. barbadense* or pima cotton. The overwhelming majority of acreage is devoted to Upland cotton production.

Traditionally, the *G. barbadense* cultivar Seabrook Sea Island (SBSI) has been considered to be the premiere cultivar in resistance to *Verticillium dahliae*. However, even this cultivar can be rendered susceptible by growing the plants at slightly cooler temperatures. It is during the growing season when night time temperatures are cooler that this pathogen is most devastating. Thus, no cotton is immune to this pathogen and strategies are required to provide breeders with the tools to increase resistance to the wilt pathogens that are applicable to the diverse *G. hirsutum* germplasm which is grown across the cotton belt.

Early experiments by Bell³³ showed that xylem tissues and boll endocarp tissue, which normally are devoid of terpenoids, rapidly synthesize gossypol and related terpenoid aldehydes in response to infection by fungal pathogens. This reaction occurred both in glanded and glandless cottons, showing that the gland alleles affect the storage of gossypol but not its biosynthesis. These studies also showed that terpenoid aldehydes are synthesized as part of the active defense against microbial infections and thus should be considered as phytoalexins. Bell³⁴ then showed that when xylem vessels were infected with *V. dahliae* more than 50% of the terpenoid aldehydes were exuded into the xylem vessels.

Many of the details of the role of terpenoid aldehydes as phytoalexins (active defense agents) in response to infection by wilt fungi have been reviewed.³⁵⁻³⁸ The terpenoids are synthesized by the perivascular cells³⁹⁻⁴¹ appressed to the xylem vessels and are exuded first into the vessels and then into the surrounding intercellular spaces. The most abundant compound formed in *G. hirsutum* is hemigossypol. Its biosynthetic precursor, desoxyhemigossypol (Figure 18.1) occurs at about one third the concentration of hemigossypol 48 to 72 h after inoculation. In *G. barbadense*, the 6-methyl ethers of these compounds (Figure 18.1) are usually the predominant compounds and the desoxyhemigossypol-6-methyl ether concentration is 2 to 3 times greater than that of hemigossypol-6-methyl ether.

Accumulation of terpenoid aldehydes in xylem vessels occurs more rapidly in resistant than susceptible cultivars in response to the wilt pathogen *Fusarium oxysporum* f. sp. *vasinfectum* (F.o.v.) as well as to *V. dahliae*.^{42,43} The onset of rapid phytoalexin accumulation in resistant cultivars coincides almost perfectly with the time that fungal spread is curtailed in the xylem vessels.⁴⁴ The above observations along with the demonstration that hemigossypol is deposited on fungal hyphae in vessels *in situ*³⁹ strongly indicate a determinative role for terpenoid aldehydes and their naphthofuran precursors in resistance to wilt fungi. Two recent experiments further support this conclusion. Bell et al.⁴⁵ introduced recessive and dominant genes from *G. barbadense* and *G. sturtianum*, respectively, into *G. hirsutum* to increase the percentage of the 6-methyl ethers from less than 5% to more than 50% in the leaves of one set of cotton near-isolines while leaving the normal levels in the sister set of near-isolines. The increase in methylation decreased the toxicity of the total terpenoids because the less toxic ethers replaced their more toxic unmethylated counterparts. In all four pairs, the lines with enhanced methylation had decreased resistance to *Verticillium* wilt. Furthermore, Eldon and Hillocks⁴⁶ were able to break resistance to *Verticillium* wilt by using an enzyme inhibitor that disrupts an early step (i.e., HMGR-CoA reductase) in the terpenoid biosynthetic pathway.

The relationship between phytoalexins and disease resistance appears to be controlled by the speed of response and quality of the phytoalexins. Thus, plants that respond to an infection

by the rapid synthesis of phytoalexins are expected to be more resistant. Furthermore, plants that produce the highest concentration of the most toxic phytoalexin likewise are expected to be more resistant. Progress in understanding and developing these complementary processes are discussed below.

18.3.1 Speed of Response

Bell³⁴ showed that the time at which phytoalexins began to accumulate was about 24 h sooner in vessels of a resistant *G. barbadense* compared to a more susceptible *G. hirsutum*. He also showed that the fungus grew from the vessels into surrounding tissue of *G. hirsutum* only in the young terminal stem tissue which had very limited ability to make terpenoid aldehydes. He proposed that the speed of terpenoid aldehyde synthesis relative to the speed of secondary colonization by the fungus was a critical determinant of resistance.³⁵ In a kinetic analysis of cotton stele tissue from resistant *G. barbadense* (SBSI) infected with *V. dahliae*, Alchanati et al.⁹ showed that δ -cadinene synthase mRNA, δ -cadinene synthase activity, and formation of sesquiterpenoid phytoalexins were induced 12 h after inoculation with the fungus. mRNA was already at a peak level at 12 h, while δ -cadinene synthase activity was at 54%, with peak level occurring at 48 h. Phytoalexins were not detected until 24 h.

Cui et al.^{43,47} compared the mRNA levels of β -1,3-glucanase, chitinase, phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), caffeic acid O-methyltransferase (C-OMT), 3-hydroxy-3-methyl glutaryl CoA reductase (HMGR), and δ -cadinene synthase (δ -CS) in each of the four cultivars at 12, 24, 36, 48, 72, and 96 h after inoculation with a fungal suspension or sterile water. Low levels of β -1,3-glucanase mRNA were observed in both fungal and water-treated plants. Chitinase mRNA was detected only in inoculated plants, but in relatively low levels. Thus, these enzymes may be important, but do not appear to be critical for an incompatible response in cotton. PAL mRNA was constitutively expressed in all plants from all treatments. PAL removes an amino group from phenylalanine to form cinnamic acid. Cinnamic acid is utilized by the plant to produce either flavonoids or lignin. In cotton, flavonoids are converted to condensed tannins as part of the active defense response.⁴⁸ CHS is a key enzyme needed to synthesize condensed tannins while caffeic acid o-methyltransferase (C-OMT) converts cinnamic acids to lignin precursors. A high PAL mRNA level constitutively expressed in all four cultivars suggests there is always enough enzyme activity for flavonoid and/or lignin synthesis. CHS mRNA levels were higher in the resistant plants but did not reach their highest levels until 60 h post inoculation (PI). mRNA levels of C-OMT, a key enzyme leading to lignin precursors, were higher in early samples from resistant cultivars as compared to susceptible cultivars with maximal activity measured at 12 h PI. This may in part account for xylem vessel plugging which physically restricts the fungus and prevents its spread.^{41,49-53} HMGR is a key enzyme in the formation of mevalonate, the sole precursor for terpenoid biosynthesis.^{54,55} δ -CS is the enzyme that catalyzes the formation of δ -cadinene, the first compound unique to the cotton terpenoid phytoalexin synthesis.⁵ Maximal levels of both HMGR mRNA and δ -CS mRNA were higher in the resistant cultivars with maximal activity measured at 12 h PI.

These results show that currently available resistant cotton cultivars are very quick to recognize the presence of the pathogen. At 12 h, PI, C-OMT mRNA involved in the biosynthesis of lignins and HMGR and δ -CS mRNA's involved in phytoalexin biosynthesis are already at maximal levels. At 12 h, the conidia used to inoculate the plant have just begun to germinate. Thus, in new resistant *G. hirsutum* cotton cultivars, early recognition is not a problem. In order to further augment resistance, increasing the potency of the phytoalexins offers an attractive option.⁵⁶

18.3.2 Quality of the Phytoalexins

Since the quickness in recognizing the pathogen and mobilizing defense biosynthetic pathways in new *Verticillium* wilt resistant *G. hirsutum* cultivars appear to be reaching its maximum potential, it is appropriate to target the quality or toxicity of the phytoalexins. Two approaches can be envisioned — the introduction of foreign genes from a *V. dahliae* immune plant or an alteration in the biosynthetic pathway to increase the toxicity of the existing phytoalexins in cotton. We view the latter approach as more easily attainable at this time.

The toxicity of the phytoalexins has been determined against a number of fungi. The ED₅₀ of desoxyhemigossypol, the most potent antibiotic, ranges from 5 to 30 ppm against different fungi. The naphthofurans are usually about twice as toxic as their aldehyde derivatives.^{42,45,57-59} However, it takes twice as much of the 6-methyl ethers to be as toxic as their unmethylated counterparts. Similarly, the methylated terpenoids in leaves, hemigossypolone-6-methyl ether and the O-methylated heliocides, are less than one half as toxic as their unmethylated counterparts.³¹ Thus, methylation has an undesirable effect on toxicity to both pathogenic fungi and insects. This observation suggests that preventing or lowering the rate of methylation would enhance the natural defenses of the plant to many pests. The evolution of regulatory genes to “shut down” methylation in leaves in some *Gossypium* species such as *G. hirsutum*, which is discussed in the section on biosynthesis, support this conclusion.^{17,21}

Our immediate goal is to identify the dHG-O-methyltransferase (dHG-OMT) gene and use antisense technology to lower expression of the dHG-OMT gene. Partial purification of the dHG-OMT enzyme has been accomplished.¹⁸ Suppressed expression of the dHG-OMT gene is expected to enhance resistance of cotton to multiple pests.

18.4 Conclusion

Because of the toxicity of gossypol, its inclusion in the glands of cottonseed is viewed as an undesirable attribute by the cottonseed industry. An active research effort is currently underway to overcome this problem using molecular biology to block synthesis of gossypol or at least of its (–)-isomer which is thought to be the toxic component. However, the glandular terpenoid aldehydes in the foliar plant parts constitute an important component in the plant's defense to insects. Similarly, current evidence suggests that the phytoalexins produced in the xylem tissue are essential for protecting the plant from pathogenic fungi. This knowledge has led us to believe that a significant increase in resistance to pathogens can be accomplished by blocking methylation of desoxyhemigossypol using antisense constructs.

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Phytochemical Modification of Taste: An Insect Model

J. Alan A. Renwick

CONTENTS

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- 19.2 Repellents, Antifeedants, and Toxins
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19.1 Introduction

Plant life in its various forms is widely recognized as the most readily available, abundant source of new chemicals or leads for synthetic chemicals to meet the growing needs of the agricultural and pharmaceutical industries. The array of chemicals produced by plants is enormous, but the number of known compounds represents only a fraction of the total. Furthermore, the diversity of biological activities exhibited by different groups of compounds continues to offer countless opportunities for practical applications. While we have become more aware of this chemical treasure and we increasingly exploit the unique properties of individual phytochemicals, especially as pharmaceuticals, the adaptive significance of these natural chemicals in the evolution of plants tends to be either ignored or forgotten.

Although some controversy exists over the relative importance of pathogens and herbivores in the evolution of secondary metabolite production by plants, it is clear that the selective pressure exerted by invading organisms has played a major role.¹ Most chemicals that we now value for their biological activity are, therefore, likely to be products of the evolution of chemical defenses against such attacks. The properties that cause a specific phytochemical to deter oviposition by an insect pest, to function as an antibiotic, or to exhibit anticancer activity, may be considered as side benefits of this evolutionary process of self-protection. Compounds that defend a plant against invertebrates or vertebrates may now be used to protect crop plants in an agricultural setting. Other compounds that are highly toxic to vertebrates have proved to have valuable pharmacological properties, and phytochemicals that combat pathogen attack are often active against human pathogens (Figure 19.1). In many cases, common biochemical or physiological pathways may be involved in the activity of a chemical in different organisms. Such similarities point to the possible value of using one organism as a model for studying the reaction of another organism, especially humans, to phytochemicals.

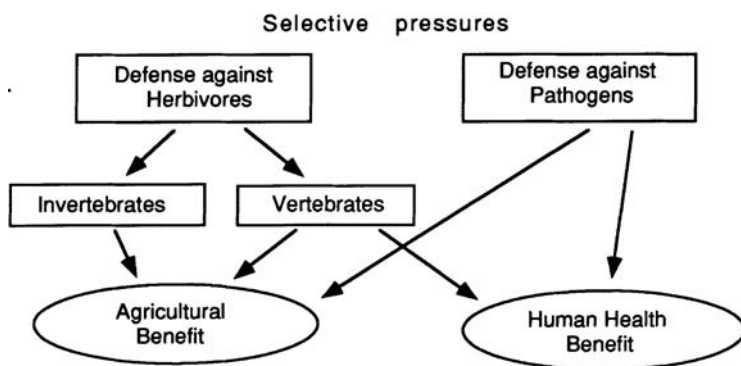


FIGURE 19.1
Evolution and human benefits of secondary metabolite production in plants.

19.2 Repellents, Antifeedants, and Toxins

Many studies designed to develop crop plants that are resistant to insects or to find new control agents have utilized basic information about the mechanisms used by plants to protect themselves against herbivores.² Chemical defenses of a plant against insects can be roughly categorized as repellents, antifeedants, or toxins. Repellents generally prevent landing by adult insects or movement onto plant surfaces by larvae. Antifeedants inhibit or deter feeding by those insects that venture onto the plant, whereas toxins either kill or immobilize those insects that do feed. However, the production of these chemicals by a plant may be dramatically influenced by many environmental factors, both abiotic and biotic.³ Nutrition, particularly, nitrogen, sulfur, and phosphorus levels, can affect the biosynthesis of compounds that are rich in any of these elements.^{4,5} Allelochemical production may be induced by herbivory, pathogens, or mechanical damage,⁶ and exposure to UV-B radiation or air pollutants may have profound effects on some biosynthetic pathways (Figure 19.2).

When we examine the way that plant chemistry may influence the selection of hosts by phytophagous insects, we are dealing with basic principles of sensory evaluation and palatability of potential foods. As humans, our discriminatory eating habits depend on perception of both olfactory and gustatory chemical signals associated with the food, and personal preferences or individual reactions depend on the way that this chemical information is processed by the brain. Similarly, insects are often guided by the volatiles from a potential food plant for their initial approach (or avoidance), and then by nonvolatile gustatory stimuli for acceptance or rejection of the plant. The sense of taste, therefore, plays a major role in determining host ranges, or dietary discrimination, of insects as well as higher animals.

19.3 Cabbage Butterfly, *Pieris rapae*

The cabbage butterfly, *Pieris rapae*, has been used as a model for studying the interplay of gustatory cues that affect the host selection behavior of a specialist insect. The female butterfly has contact chemoreceptors on its tarsi that are responsible for perceiving both positive and negative gustatory stimuli at the plant surface.^{7,8} Before accepting a plant for

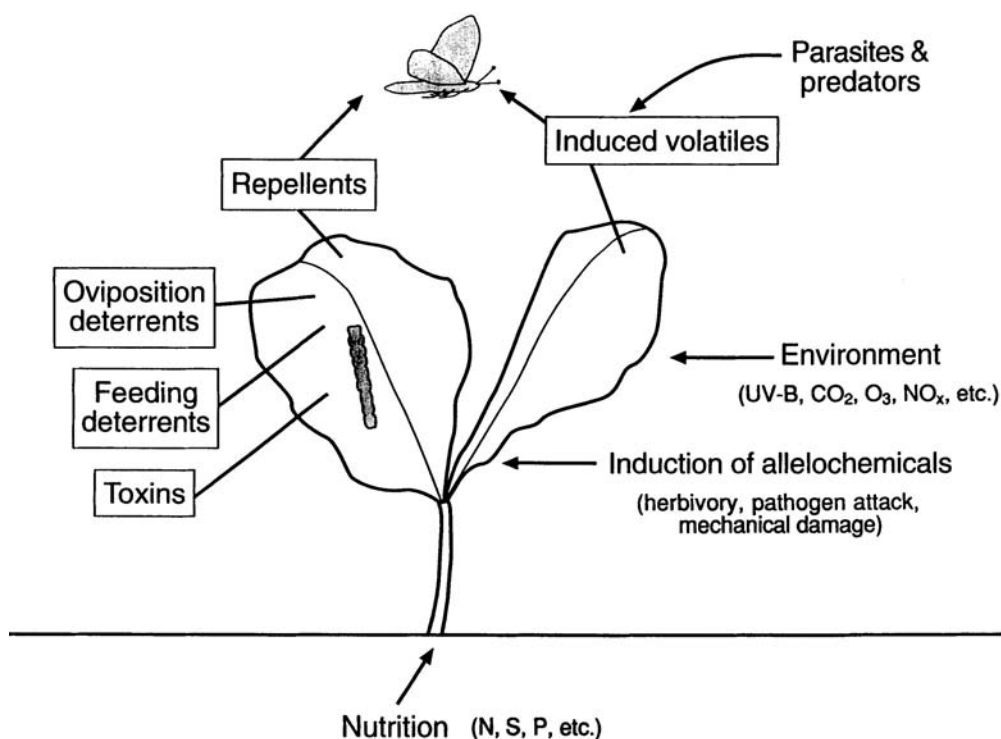


FIGURE 19.2

Dynamics of allelochemical production in the defense of plants against insects. (Adapted from Renwick, J.A.A., in *Phytochemical Diversity and Redundancy in Ecological Interactions*, Plenum Press, New York, 1996.)

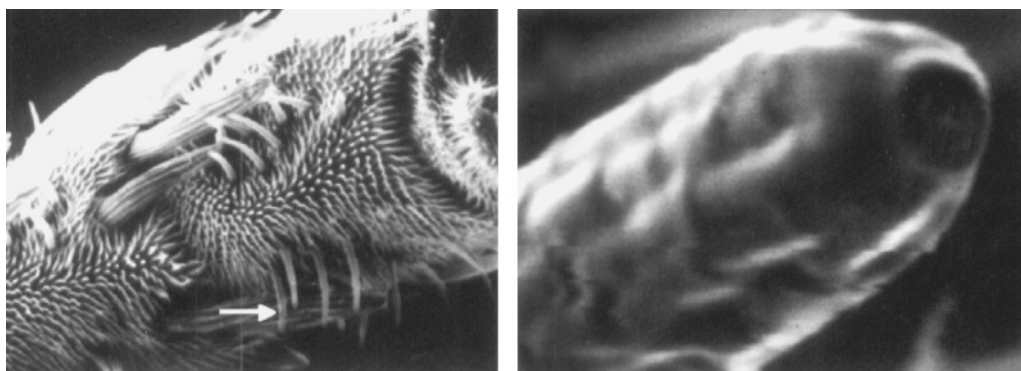


FIGURE 19.3

(Left) SEM of ventral side of the distal segment of a female *Pieris rapae* foretarsus showing a cluster of contact chemosensory sensilla (arrow). (Right) Enlarged view of a sensillum showing the single pore at the tip, typical of contact chemoreceptors.

oviposition, the female often exhibits rapid movement or tapping with the forelegs. This behavior has been called “drumming”, and presumably allows more receptor hairs to come in contact with the chemicals that stimulate oviposition.^{9,10} These receptors, or sensilla, are peg-like structures which are arranged in rows between spines on the foretarsi (Figure 19.3). A single pore at the tip of the sensillum is typical of contact chemoreceptors, and electrophysiological recordings have been used to confirm that receptor cells associated with these sensory hairs respond to active plant constituents.^{7,8}

Tasting by larvae of the cabbage butterfly also depends on the use of contact chemoreceptors that perceive the active chemicals at the surface of a plant. These receptors are located on the galea within the mouth of the caterpillar. Two pairs of sensilla styloconica, medial and lateral, are generally involved in the process of food recognition and discrimination, resulting in either stimulation or inhibition of feeding.¹¹ As in the case of tarsal receptors, electrophysiological recordings can be performed to show good correlations between the responses of sensory receptor cells and behavioral responses to specific compounds.¹²

Several compounds responsible for acceptance of suitable host plants and for rejection of unsuitable plants by adults and larvae of *P. rapae* have now been identified. For example, wormseed mustard, *Erysimum cheiranthoides*, is protected from attack by several specific cardenolides.¹³ However, the most effective oviposition deterrents are not the same as the most effective feeding deterrents. The best oviposition deterrents are strophanthidin glycosides, whereas the strongest feeding deterrents are glycosides of digitoxigenin.^{14,15} When extracts of *E. cheiranthoides* are subjected to solvent partitioning to remove the deterrents, aqueous extracts actually become stimulatory to ovipositing females of *P. rapae*.¹⁶ This has served to demonstrate the fact that plants may contain both stimulants and deterrents and that the balance of these positive and negative chemical messages determines whether a plant is accepted or rejected.¹⁷ This balance is likely to be influenced by the physiological state of an individual insect, perception of the compounds, and processing of the information that reaches the central nervous system (Figure 19.4).

The possibility of manipulating the balance of positive and negative sensory cues to cause an insect to reject plants that they would normally accept has been suggested as a means to reduce crop losses to insect pests. Oviposition or feeding deterrents could conceivably be applied to protect the plants or they might be introduced through plant breeding or genetic engineering to produce resistant plants. Alternatively, we might attempt to interfere with the ability of an insect to taste chemical constituents of plants that they encounter. If an insect loses its sensitivity to stimulants in a plant, it would no longer be capable of recognizing a good host on the basis of secondary plant chemistry. On the other hand, a loss of sensitivity to deterrents might result in feeding on unsuitable food plants that would have a deleterious effect.

19.4 Diet-Dependent Sensitivity

Recent studies on larvae of *P. rapae* have resulted in the discovery of diet-dependent sensitivity to an antifeedant in a normally acceptable host plant. The butterflies readily lay eggs on garden nasturtium, *Tropaeolum majus*, and the hatching larvae feed and develop normally. However, if larvae that have fed and developed on cabbage plants are transferred to nasturtium, they refuse to feed. The effect is so extreme that the larvae will starve to death rather than feed on the nasturtium.¹⁸ Similar results were obtained when larvae were transferred from a range of other host plants, including both crucifers and noncrucifers, to nasturtium. This rejection behavior was explained by the presence of chlorogenic acid and some additional, unidentified constituents of *T. majus*.¹⁹ Chlorogenic acid was strongly deterrent to cabbage-reared larvae, but only slightly deterrent to nasturtium-reared individuals. Furthermore, larvae that were reared on a wheat germ artificial diet were almost completely insensitive to chlorogenic acid. It appears, therefore, that *P. rapae* larvae are insensitive to deterrents at the time of hatching and will feed on a wide range of plants or artificial diet. However, as they feed on cabbage or other crucifers, they develop sensitivity that results in their refusal of food that contains deterrents. We have concluded that continuous exposure

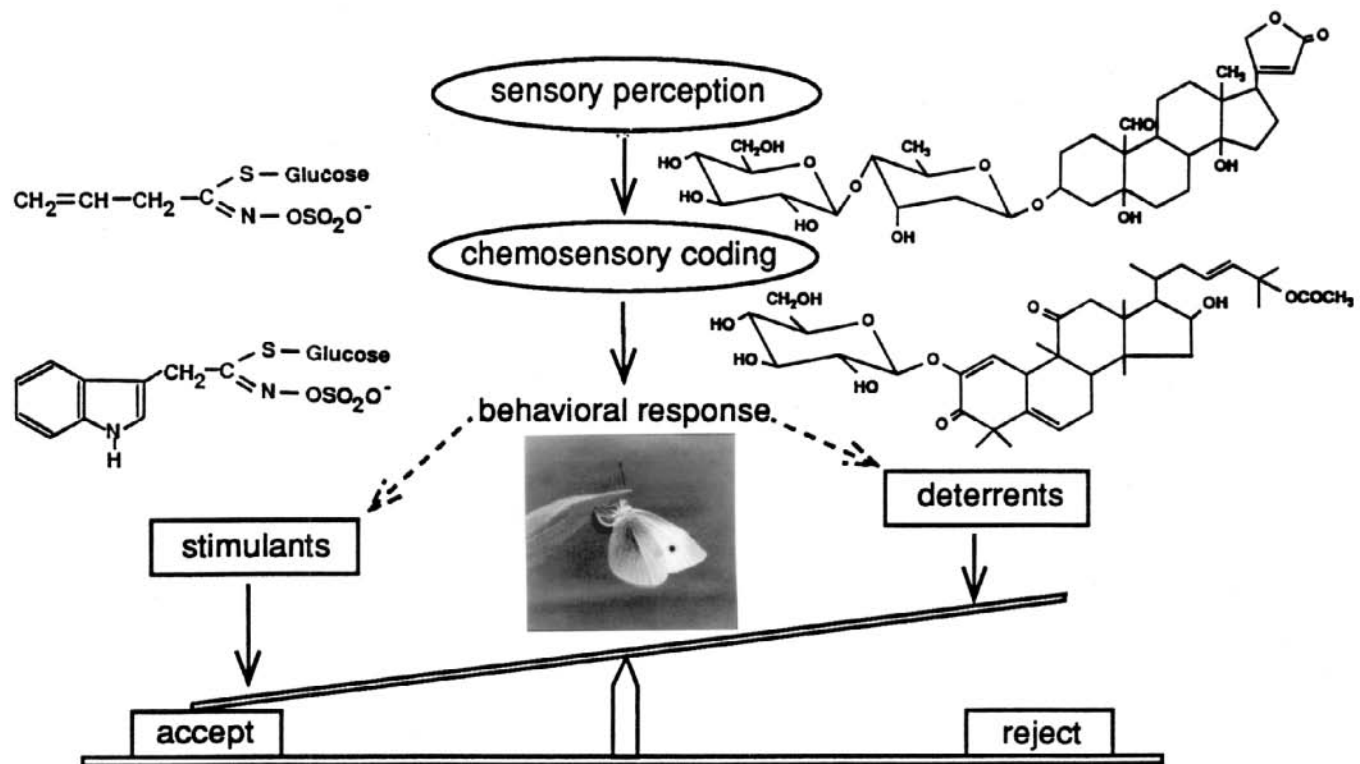


FIGURE 19.4

Factors affecting the balance of positive and negative chemical cues that influence acceptance or rejection of a potential host plant by an insect. (Adapted from Renwick, J.A.A. and Huang, X., in *Functional Dynamics of Phytophagous Insects*, Oxford & IBH Publishing, New Delhi, 1994.)

to and/or consumption of food containing deterrents results in a type of habituation, or more precisely, suppression of sensitivity development.¹⁹

Since habituation is usually defined as a “waning of response to a repeatedly presented stimulus over time,” the term is not precisely applicable to the lack of sensitivity development seen in this insect. However, experiments conducted to test the effects of dietary experience with one or more compounds on responses to unrelated compounds seem to indicate that a type of “cross-habituation” is possible. When larvae were reared on nasturtium, they were less sensitive than cabbage-reared larvae to a range of feeding deterrents.²⁰ Furthermore, larvae reared on wheat germ diet were almost completely insensitive to the same compounds. When larvae fed and developed on cabbage leaves that had been treated with selected deterrents, including cardenolides and cucurbitacin glycosides, they remained less sensitive to the nasturtium deterrents.²⁰

The development of sensitivity to feeding deterrents can be followed at any larval stage. When larvae were transferred from nasturtium to cabbage, they became progressively more sensitive to the nasturtium deterrents. Although all instars show the same general effect, second instars of *P. rapae* appear to be most plastic in their development of sensitivity as they feed on cabbage.²¹ The possible involvement of an “inducer” in cabbage has been discounted, since larvae that were reared on cabbage foliage treated with nasturtium extract remained insensitive to the deterrents from that plant. It is likely, therefore, that sensitivity develops naturally as larvae feed on host plants that lack deterrents or other compounds that suppress this development. This phenomenon of increased sensitivity within an instar after removal from plants containing suppressors has prompted the proposal to use *P. rapae* as a model organism for future analysis of the physiological and biochemical processes involved in chemosensory development.²²

The idea of feeding experience changing the characteristics of taste receptors is not completely new. Already in 1969, Schoonhoven²³ found that the sensitivity of a so-called deterrent receptor in *Manduca sexta* larvae to salicin was considerably lower for larvae reared on artificial diet that contained this compound than for larvae reared on control diet. Similar electrophysiological results have since been obtained for larvae of *Pieris brassicae* in response to chlorogenic acid, proline, and cyanin chloride,²⁴ and for larvae of *Spodoptera* species in response to azadirachtin, nicotine, or sinigrin.²⁵

The effect of wheat germ diet on the ability of insects to taste and respond to deterrents is particularly interesting. Artificial diets containing wheat germ have long been used by entomologists for rearing a wide range of insects for various purposes. However, many insects that are normally reared on wheat germ diets will refuse this artificial food if they have previously fed on a host plant. Extracts of an artificial diet containing wheat germ were found to be deterrent to *Manduca sexta* larvae, and wheat germ itself was thought to be the source of deterrent components, as omission of the wheat germ increased the acceptability of the diet.²⁶ Similarly, larvae of the cabbage butterfly refuse to feed on wheat germ diet when transferred from a cabbage plant, and the presence of deterrents has been demonstrated to explain this behavior. Both hexane extracts and butanol-soluble material from aqueous extracts of the whole wheat germ diet were highly deterrent to cabbage-reared larvae of *P. rapae*.²⁷

Wheat germ diet for insect rearing generally consists of seven components in addition to wheat germ itself and water. These include agar, aureomycin, casein, methylparaben, salt mix, sorbic acid, and a vitamin mix. When tested in a feeding deterrent assay using fourth instars of *P. rapae*, only the sorbic acid was slightly deterrent. However, extracts of the wheat germ itself were highly deterrent. Since strong deterrent activity was found in hexane as well as butanol extracts, a separation scheme was developed to examine both active fractions (Figure 19.5). Seven compounds were isolated by HPLC separation of the butanol

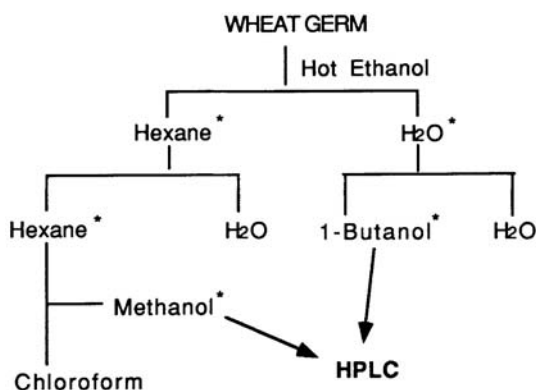


FIGURE 19.5

Extraction and isolation scheme for feeding deterrents (sensitivity suppressors) in wheat germ. *Denotes deterrent activity.

fraction of the aqueous material. The UV spectra suggested that most of these are apigenin-based flavones. Fractionation of the hexane-soluble material by sequential solvent partitioning revealed that most of the active compounds were methanol-soluble, and HPLC of the methanol fraction showed that some compounds were common to both the butanol and hexane fractions, but others were present only in one of the fractions. When neonate larvae were allowed to feed on cabbage leaves that were treated with individual fractions collected from the HPLC, they remained insensitive to deterrents. Thus, individual constituents of wheat germ could account for the “cross-habituation” that is necessary for larval acceptance of nasturtium.²⁷

The sensitivity suppressing activity of specific compounds in wheat germ would suggest that these phytochemicals are acting as taste modifiers. This could have considerable practical significance, since modification of taste in humans is an effect that is often desired and is poorly understood. The value of using insects as models for the investigation of higher animal taste mechanisms has already been suggested, and the validity of comparing the two biological systems is supported by the fact that bitter taste to humans has been used as a guide in the search for insect feeding deterrents in plants.²⁸ Insects are stimulated to feed by sugars that taste sweet to humans, and the interaction of sweet and bitter tastes in humans may be compared with the interactions between stimulants and deterrents in insects (Figure 19.6). Selected compounds that are bitter tasting to humans have been found to deter both oviposition and feeding by the tobacco budworm, *Heliothis virescens*, and preliminary electrophysiology has suggested that responses of a sucrose-sensitive neuron in the gustatory sensilla of the ovipositor are inhibited by these compounds.²⁹ In addition, both insects and vertebrates exhibit a rather general phagostimulatory response to low molecular weight amino acids such as glycine, β -alanine, α -aminobutyric acid, γ -aminobutyric acid (GABA), L-arginine, and L-proline.³⁰

19.5 Conclusions

Modification of taste is of interest for several reasons. Specific inhibitors of bitter taste are continually sought for pharmaceutical or food science applications, whereas bitter taste might be considered desirable in other products, such as beer. Suppression of bitterness may

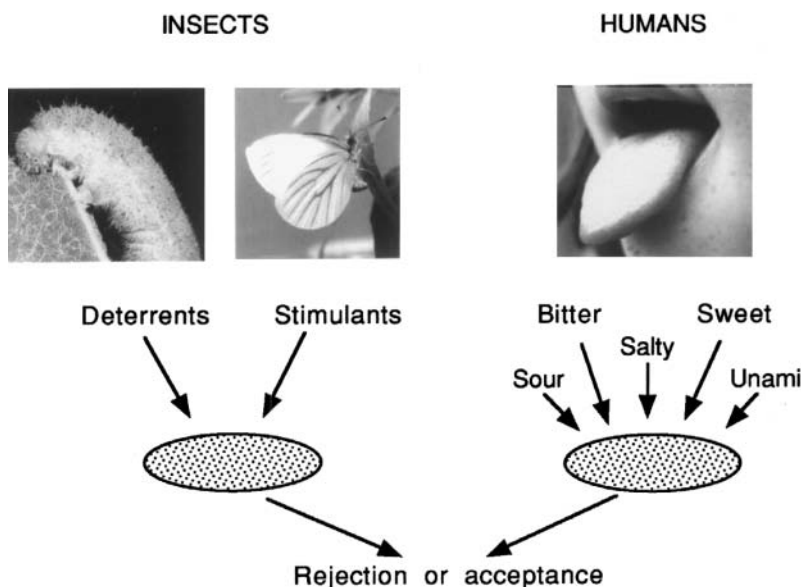


FIGURE 19.6

The role of taste in food selection and discrimination by insects and humans.

be accomplished simply by the addition of salt,³¹ or the perception of bitter compounds may be modified by the presence of citrate or malate that have a sour taste.³² However, most studies in this area have focused on modification of sweet taste. Many plant-derived chemicals have been shown to act as either sweetness inhibitors or sweetness inducers or enhancers. As a result, much of the work on taste modification has capitalized on this knowledge to examine mechanisms that govern sweet taste, and considerable progress has been made in explaining how sweetness modifiers might work using an insect model system.³³

Although information about the biochemistry of taste receptors in insects as well as mammals is still rather fragmentary, significant advances in the molecular biology of taste transduction in mammals have recently been made.³⁴ Some key component G proteins involved in taste transduction in mammals have been cloned. Electrophysiological, biochemical, and molecular biological studies have suggested that bitter as well as sweet taste is transduced by second messenger-mediated pathways involving cyclic adenosine monophosphate (AMP).³⁵ However, G proteins have yet to be isolated and identified from insect taste receptors. As soon as taste cell proteins can be cloned from insects, the restriction imposed by the palate of human tasters will be removed to allow for the design and screening of novel taste agents.³⁴

The development of taste sensitivity in mammals or invertebrates is a phenomenon that still remains a mystery. Observations with human newborns have indicated that responses to a bitter compound increase within a period of 14 to 180 days.³⁶ These results appear to parallel the gradual development of sensitivity to deterrents found in newly hatched larvae of *P. rapae*. These caterpillars, therefore, represent an ideal model for detecting taste receptor proteins as the sensory system develops. Such critical information about the processes involved in taste modification may then provide a starting point for the needed isolation and cloning of taste receptor proteins. The identities and exact role of phytochemicals in the modification of taste will be key components of this puzzle that now seems possible to solve in the foreseeable future.

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Exploring the Potential of Biologically Active Compounds from Plants and Fungi

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ABSTRACT Our research concentrates on screening extracts from plants and insect-pathogenic fungi for novel pesticidal chemistries with high target selectivity and environmental compatibility. Our search is directed to narrowly defined and relatively untapped biological organisms. We also are interested in improving techniques for extracting and quantifying known secondary metabolites to refine our understanding of the roles these compounds play in nature and agroecosystems, and their potential roles in managing crop pests and diseases. Extracts of culture broth from the fungal genus *Aschersonia* sp., exhibited insecticidal activity in a *per os* assay against *Drosophila melanogaster*. Two new cyclic depsipeptides, Destruxins A4 and A5, were isolated by bioassay-guided fractionation. These are the first biologically active metabolites reported from an *Aschersonia*. Concentrations which resulted in 50% mortality of the population of *D. melanogaster* (LC₅₀) were estimated at 41 (A4) and 52 (A5) ppm. Avenacins, triterpenoid saponins from oats, may be important determinants of disease resistance and a source of pathogen-suppressive activity in soils. Analytical protocols developed to quantify avenacins were used to show inter-varietal differences in production of these compounds, their location in the root zone, and their susceptibility to turnover.

20.1 Introduction

Growing public concerns of undesirable effects of pesticides on human health and the environment have led to the withdrawal of some commercial pesticides, increasing the vulnerability of crops to pests and diseases. In the scientific community, concern also extends to the increasing difficulty of managing pesticide resistance. These concerns have spurred efforts to develop crop-protection methods that do not depend on chemical pesticides alone. Furthermore, these efforts include a search for new pesticides that will (1) be efficacious, (2) have minimal environmental impact, and (3) support efforts to manage pesticide resistance. We seek compounds with high target selectivity and novel modes of action to serve these goals.

The rate of discovery of new agrochemicals has declined from 1 in every 5000 compounds tested to 1 in every 20,000 over the past two decades.¹ This decline highlights the need to identify new sources of pesticidal chemistries. Narrow, heretofore overlooked biological niches, such as entomopathogenic fungi or suppressive cover crops and soils, may be extremely useful in providing leads for biologically effective compounds. Mining these unique niches for useful natural products offers the added benefit of identifying new ways to use renewable natural resources.

20.2 Fungi as Sources of Novel Chemistries

Microorganisms produce a chemically diverse array of secondary metabolites, some of which have been developed as therapeutic agents, with antibiotics ranking as premier among these metabolites.^{2,3} Since the mid-1940s, over 5000 antibiotic agents have been identified, primarily from bacteria and actinomycetes.⁴ Fungi are the second largest microbial producers of antibiotics (ca. 1600 known compounds), leading bacterial producers (ca. 950), but trailing behind the actinomycetes (ca. 4600). Of the 10 fungal antibiotics commercially produced, the penicillins, cephalosporins, griseofulvin, and fusidic acid have a majority of their use in clinical settings.⁴ More recently fungi have been looked to as sources of novel secondary metabolites,⁵ although no fungal-derived products have yet been developed as biorational pesticides. Fungal biodiversity (an estimated 1.5 million fungal species worldwide), of which only 65,000 species (or 5%) of this biota have been described^{6,7} far outstrips that of the bacteria (estimated 40,000 species),² so fungi will likely prove to be the richest microbial source of useful natural products. The vast pool of undescribed fungal species represents an immense untapped resource for novel metabolites of agricultural and biomedical importance.

20.2.1 Entomopathogenic Fungi

Pathogens of insects and other invertebrates have been identified in more than 100 fungal genera with >700 species recognized that attack insect hosts.^{8,9} These pathogens produce a variety of biologically active secondary metabolites. For the most thoroughly investigated of these metabolites, the cyclic depsipeptidal destruxins from *Metarhizium anisopliae* and beauvericin from *Beauveria bassiana*, a wide range of biological effects have been demonstrated from suppression of insect immune responses¹⁰ to acute toxicity.^{11,12} The mitochondrial ATPase inhibitors, leucinostatins from *Paecilomyces*¹³ and the efrapeptins from

Tolypocladium spp.,¹⁴ are peptides composed of unusual amino acids and terminal blocking groups. Evidence that these compounds are toxic to insects¹⁵ suggests that they may be determinants of virulence for the entomopathogenic fungi that produce them.

Metarhizium flavoviride produces viridoxins, novel diterpine derivatives of polysubstituted gamma-pyrones that exhibit insecticidal activity against Colorado potato beetle.¹⁶ These compounds are closely related chemically to phytotoxins produced by plant pathogenic fungi in the genus *Colletotrichum*. One of these phytotoxins, colletochin, which differs from viridoxins by the absence of an α -hydroxy acid moiety, is much less toxic to insects than the viridoxins.¹⁶ This preliminary observation of a structure/activity relationship suggests that *M. flavoviride*, an insect pathogen, produces a toxin with more directed activity against insects. This is an encouraging example of the potential for finding insecticidal compounds with high target selectivity among the toxins of entomopathogenic fungi.

The extant literature on toxins of entomopathogenic fungi, although substantial, deals with relatively few species. Virtually nothing is known of the secondary chemistry of many important fungal pathogens of invertebrates, even those that have demonstrated toxicity for which toxins may play a role in pathogenesis. Conversely, where mechanisms of pathogenesis are well understood for some species,⁹ the role of toxins in the infection process is still largely a matter of speculation.¹⁷⁻¹⁹ It is not yet possible to draw convincing parallels to the clear-cut roles that host-selective toxins play as determinants of pathogenicity and/or virulence in some plant disease systems.²⁰

20.2.2 Screening Entomopathogenic Fungi for Novel Metabolites

The USDA-ARS Collection of Entomopathogenic Fungal Cultures (ARSEF) housed in Ithaca, NY has the world's largest and most diverse germplasm repository for fungal pathogens of insects and other invertebrate pests affecting agriculture, encompassing nearly 5500 accessions of more than 300 fungal taxa from nearly 900 hosts and 1200 locations worldwide.²¹ For our screening purposes, stock mycelial cultures are maintained on solid media. Mycelial plugs are taken to inoculate several liquid media to optimize growth and production of active extracts. Cultures are harvested for extraction usually after 10 to 30 days of growth.

When sufficient biomass has been produced, fungal mycelium and culture broth are separated and extracted to yield polar and nonpolar fractions for a total of four extracts from each fungal strain. Crude extracts that show sufficient activity are then purified using appropriate bioassay-guided separation techniques. In some assays biological activity can be rapidly assessed, e.g., fungicidal activity can be visualized directly on TLC plates sprayed with a fungal spore suspension in dilute agar. Once a biologically active compound is purified in sufficient yield (usually >1 mg), structural data can be acquired by various spectrometric methods.

20.2.3 An Example from the Entomopathogenic Fungi: New Destruxins from *Aschersonia* sp.

Members of the genus *Aschersonia* (Coelomomycetes, Deuteromycotina) have been investigated as potential biological control agents of important aleyrodid homopteran pest species such as the citrus whitefly, *Dialeurodes citri*, greenhouse whitefly, *Trialeurodes vaporarum*, and the sweet potato whitefly, *Bemisia tabaci*.²² Some species of *Aschersonia* have been linked to their perfect stages in the genus *Hypocrella*.²³ *Hypocrella bambusea* is known to produce a group of biologically active compounds, hypocrellins, which are photoactive perylenequinones under evaluation as potential anticancer and antiviral agents.²⁴

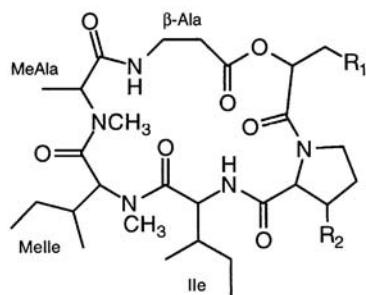


FIGURE 20.1

Structures of destruxins isolated from the entomopathogenic fungus *Aschersonia* sp. (From Krasnoff, S. B. et al., *J. Nat. Prod.*, 59, 485, 1996. With permission.)

1	$R_1 = \text{CH}=\text{CH}_2$, $R_2 = \text{H}$
2	$R_1 = \text{CH}=\text{CH}_2$, $R_2 = \text{CH}_3$
3	$R_1 = \text{CHMe}_2$, $R_2 = \text{H}$

20.2.3.1 Purification of Active Principles

A 2 mg/ml solution in 10% sucrose of a methylene chloride extract of culture broth from an undescribed *Aschersonia* sp. collected as a pathogen of *D. citri* in the Phillipines produced 80% mortality against fruit flies, *Rhagoletis pomonella* and *Drosophila melanogaster*.²⁵ The extract was flash chromatographed on silica gel using a methylene chloride-methanol gradient. Activity was detected in the fraction eluting in 2% MeOH in methylene chloride. This was then purified via semi-preparative reversed-phase HPLC using a 5 μm C18 (250 \times 10 mm) column, eluted with acetonitrile:water (50:50). Two major components accounting for all the biological activity were isolated (Figure 20.1); a third major component also was purified, but displayed no activity.

The molecular formulae $\text{C}_{30}\text{H}_{49}\text{N}_5\text{O}_7$, $\text{C}_{31}\text{H}_{51}\text{N}_5\text{O}_7$, and $\text{C}_{31}\text{H}_{53}\text{N}_5\text{O}_7$ assigned to compounds 1, 2, and 3, respectively, were deduced from high resolution mass spectra. Based on proton, C13, and HMBC, and HMQC NMR experiments, structures for two novel compounds, destruxins A4 and A5, were assigned to compounds 1 and 2, respectively.²⁵ Compound 3 was identified as homodestruxin B which was previously reported from the plant pathogenic fungus *Alternaria brassicae*.²⁶

20.2.3.2 Biological Activity of Destruxins

LC_{50} values for the new destruxins in the *Drosophila melanogaster* bioassay were estimated using the probit model (Figure 20.2). Destruxin A4 was more active than Destruxin A5, with an LC_{50} of 41 ppm (95% confidence interval: 32 to 50 ppm) vs. 52 ppm (95% confidence interval: 44 to 63 ppm). Homodestruxin B was inactive at 400 ppm. These data are consistent with previous structure/activity work on the known destruxins,²⁷ suggesting that the olefinic side chain in the α -hydroxy acid moiety of A group of destruxins confers greater biological activity than the saturated side chain seen in the B family of destruxins.

Destruxins A4 and A5 are the 28th and 29th destruxins to be characterized, and the first biologically active metabolites reported from the genus *Aschersonia*.²⁵ Various biological activities have been reported for destruxins, including phytotoxicity,²⁸ antitumor,²⁹ and antiviral³⁰ activity, as well as toxicity to insects.¹¹ Insects injected with destruxins exhibit a tetanic paralysis thought to be due to an effect on calcium channels in muscle.¹⁸ Others have reported that destruxins degranulate insect hemocytes *in vitro* and, thus, may have an immunosuppressive effect,¹⁰ although it is not clear whether destruxins mediate this process during infection *in vivo*. The potential role of destruxins as virulence factors for *Aschersonia* fungi should be explored in light of the economic importance of their insect hosts.

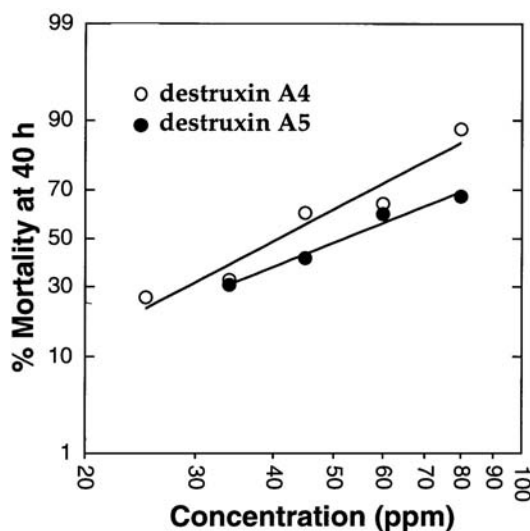


FIGURE 20.2

Insecticidal effect of destruxins A4 and A5 against *Drosophila melanogaster*. Percentage of mortality is plotted on a probability scale and concentration of formulated destruxins on a \log_{10} scale. Each point represents a sample of 40 to 65 insects. Control mortality (response to 4.75% EtOH/10% sucrose solution) was zero in this experiment. The probit regression lines shown (probit mortality = 3.51 (\log_{10} dosage -0.66); probit mortality = 2.73 (\log_{10} dosage) + 0.31) were used to estimate effective doses for destruxins A4 and A5, respectively. (From Krasnoff, S. B. et al., *J. Nat. Prod.*, 59, 485, 1996. With permission.)

Due to regulatory considerations, toxigenic strains have been avoided up to now by those who seek to develop entomopathogenic fungi as biocontrol agents. These strains, however, may hold promise as pinpoint delivery systems for insect selective toxins (cf. Bt toxin from *Bacillus thuringiensis*) and should be evaluated in this context.

20.3 Plant-Derived Biopesticides

There is a plethora of current and ethnobotanical literature listing plants with known pest control properties. Over 2000 plant species are known to have insecticidal properties,³¹ and many of these plants are still used by natives in developing countries. It is estimated, however, that only 5 to 15% of the 250,000 to 500,000 known plant species have been assessed for biological activity, and there is little information concerning biological properties in the vast majority of tropical plant species.³² Potentially useful biological compounds remain undiscovered, uninvestigated, undeveloped, or underutilized from this reservoir of plant material.³³ Likewise, knowledge of production and location of these compounds during plant development may aid in effective strategies to incorporate their use for crop protection, either for screening germplasm for resistance or deployment to generate suppressive soils in crop rotation strategies.

Roots perform many vital functions for plants and constitute 3 to 40% of the total biomass. They share the subterranean environment with fungi, bacteria, and nematode parasites that include some of the major pests of crop plants. Plant roots are known to be rich sources of allelochemicals;³⁴ these chemicals are plant secondary compounds that exhibit biological effects on other organisms and are thought to function in plant defense. However, few ecological and

physiological studies of the effects of allelochemicals on root parasites have been published to date, although the root zone may be the arena determining resistance or suppression. Since much of the chemical domain of plant roots remains unexplored, work in this area holds promise for the identification and characterization of natural compounds with high target selectivity and environmental compatibility for agricultural use.

20.3.1 An Example from Plants: Oat Roots as a Source of Active Principles

Field studies have shown that using oats as a rotation crop can suppress parasitic infections on primary crop species.³⁵⁻³⁷ Although susceptible to *Gaeumannomyces graminis* var. *avenae*, the form of takeall that affects oats, wheat, and barley,³⁸ oats are resistant to *G. graminis* var. *tritici*. Consequently oat rotations have been used successfully to reduce the incidence of var. *tritici* in wheat and barley.^{36,39}

Oats produce fungitoxins, including steroidal and triterpenoid saponins and phytoalexins which may play a role in their disease-resistance properties.⁴⁰⁻⁴³ The avenacins, triterpenoid saponins, are thought to account for the resistance of oats to *G. graminis* var. *tritici*.⁴³⁻⁴⁵ Avenacins were initially isolated as general antimicrobials by Maizel et al.,⁴⁶ and the complete structural characterization of the avenacins and their relationship to takeall disease was reported by Crombie's group.^{43,44,47} Of the four structurally related avenacins, A-1 is the most fungitoxic and predominant avenacin produced by oats. Avenacins are produced in oat roots with highest concentrations in young root tips. Levels decline gradually from 12 µg/mg (1.2%) dry weight in 3-day-old roots to 0.005% in 77-day-old root tissue.⁴⁸ No appreciable differences in avenacin content in root tissue were detected in a study of 30 oat varieties,⁴⁹ although an oat species, *Avena longiglumis* (lacking detectable levels of avenacin and highly susceptible to *G. graminis* var. *tritici*), has been reported.³⁸

Since oat varieties vary in their disease resistance to a number of plant pathogens,⁴⁹⁻⁵³ it was of interest to determine whether avenacins are the major biological metabolites conferring resistance. Our work in this system illustrates the need to carefully prepare biological materials in order to quantify differences in secondary metabolites as well as to understand the ecological and physiological relevance of active compounds at their site of action.

20.3.1.1 Extraction of Avenacins

Initial results using traditional methanol extractions of root material produced highly variable amounts of avenacins in replicated samples, although ranges were similar to those reported previously.^{44,48} A preliminary evaluation of these methanolic extracts by HPLC revealed a high level of mono-deglucosyl avenacin A-1 (mono-dG-A1), indicating that extensive hydrolysis was taking place, even under cold storage, possibly via endogenous glycosidase activity. Also, extracts were highly colored due in part to the presence of flavonoids and polyphenols.

Immediate blanching of the harvested roots in hot 20 mM potassium metabisulfite, followed by dilution to 50% methanol, and boiling for an additional 5 min, minimized endogenous glucosidase and polyphenol oxidase activities, as detected by reduction in enzymatic activity (data not shown), increased undeglucosylated avenacin concentrations, and a decrease in mono-dG-A1 (Figure 20.3). Yields of A-1 and A-2 are approximately 10 times higher with the improved extraction procedure, while yields of B-1 are approximately 2 times higher. Decreased B-2 levels in the 20 mM potassium bisulfate/methanol (PBM) extracts may indicate that B-2 exists in the plant as a hydrolysable complex that can be released via glycosidase activity in the 50% methanol extract.

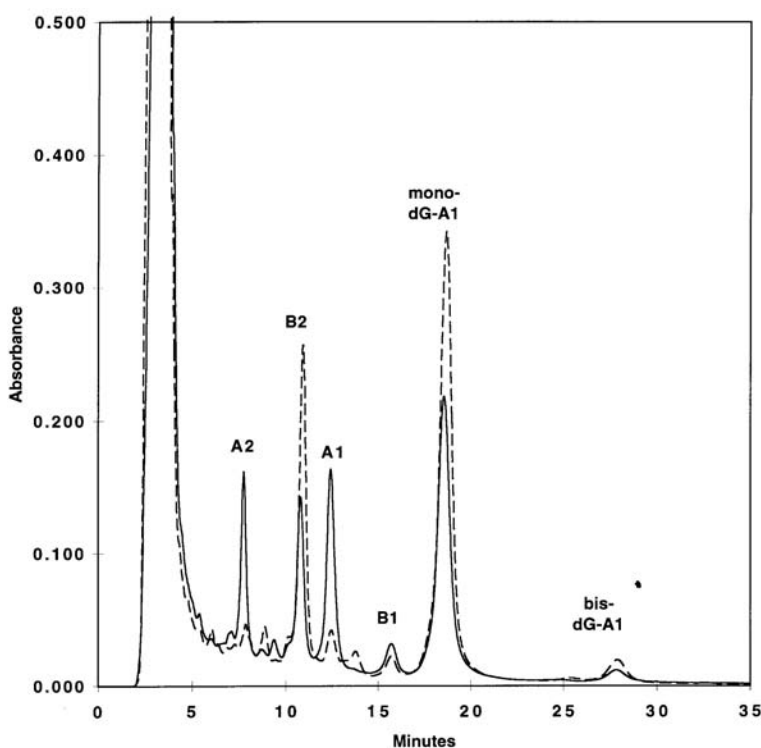


FIGURE 20.3

HPLC analysis of avenacins extracted from oat roots using either 50% methanol (---) or heated 20 mM potassium bisulfate/methanol (PBM) (—). Samples were analyzed using a C18 reversed phase column, mobile phase of (0.1% $(\text{NH}_4)_2\text{CO}_3$ in H_2O : acetonitrile (61:39), flow rate of 1 ml/min, column temperature of 21°C, and detection at both 226 and 283 nm. Avenacins are eluted in the following order: A-2, 7.89 min; B-2, 10.59 min; A-1, 12.49 min; B-1, 15.73 min; monodeglucosyl A-1, 18.71 min; and bisdeglucosyl A-1, 27.9 min.

20.3.1.2 Comparison of Avenacin Profiles among Varieties

A selected group of oat varieties was chosen to represent a range of cultivars, each with known resistance or susceptibility to soil fungi or nematodes. Oat variety Newdak is resistant to crown and stem rust⁵² while Porter displays moderate resistance.⁵³ Saia shows field resistance to *Pratylenchus* species, especially *P. penetrans* (root lesion nematode).⁴⁹ Variety Pennuda is an older variety with low to moderate resistance to crown and stem rust. Five replicate extracts were prepared from 7-day-old root samples from 4 oat varieties, using both extraction procedures, and subsequently analyzed for avenacins via HPLC (Tables 20.1 and 20.2). Total active avenacins included A-2, B-2, A-1, and B-1, while total avenacins included active avenacins and mono-dG-A-1 which has some residual fungicidal activity. The concentrations of avenacins A-1, B-1, and A-2 were greater in all cases with the PBM extraction, and in some cases, over 10 times greater levels were detected. For example, with the variety Pennuda, 50% methanol extracts contained only 16.8 μg A-1/g fresh weight, while the PBM extract contained 704.6 μg A-1/g fresh weight. A pairwise comparison test between extraction methods showed significant differences ($p = 0.05$) between concentrations of individual avenacins and total active avenacins, although total avenacins did not differ between extraction methods or variety. Active avenacin concentrations ranged from 0.6 to 1.1 mg/g fresh weight (12 to 22 mg/g dry weight) and total avenacins ranged from 0.8 to 1.2 mg/g fresh weight (16 to 24 mg/g dry weight), or approximately 5 to

TABLE 20.1

Avenacin Content of Oat Varieties ($\mu\text{g/gfw}$ Roots) Prepared Under Minimal Hydrolytic Conditions

Variety	Avenacin ($\mu\text{g/gfw}$ Root)		
	A-1	Active Avenacins	Total Avenacins
Newdak	534.2 \pm 151.2ab	815.0 \pm 212.1ab	845.5 \pm 213.0a
Pennuda	704.6 \pm 177.2ab	1074.4 \pm 254.9a	1226.9 \pm 331.7a
Porter	688.1 \pm 176.5a	965.1 \pm 259.9ab	1204.5 \pm 257.6a
Saia	349.5 \pm 146.6b	594.9 \pm 181.4b	1018.8 \pm 150.7a

Note: Concentrations were determined by HPLC.

TABLE 20.2

Avenacin Content of Oat Varieties ($\mu\text{g/gfw}$ Roots) Prepared Under Conditions Allowing Hydrolysis

Variety	Avenacin ($\mu\text{g/gfw}$ Root)		
	A-1	Active Avenacins	Total Avenacins
Newdak	80.5 \pm 13.3a	294.2 \pm 53.7a	1117.8 \pm 201.9a
Pennuda	16.8 \pm 24.6b	164.3 \pm 67.1b	1041.1 \pm 418.7a
Porter	85.0 \pm 40.3a	284.0 \pm 98.9a	1484.9 \pm 379.6a
Saia	38.3 \pm 12.9b	237.1 \pm 25.3ab	944.06 \pm 99.0a

Note: Concentrations were determined by HPLC.

8 times higher than levels reported previously (3.13 mg/g dry weight in 10-day-old roots).⁴⁸

20.3.1.3 Developmental Expression of Avenacins

Utilizing the fluorescent qualities of the avenacins, several varieties were monitored by direct autofluorescence of root tissue up to 6 weeks in age. Bright zones of autofluorescence were visible at the growing root tips in all varieties examined. The most notable overall difference among the varieties examined, however, was in the amount of lateral root development over time. Roots of the oat variety Saia were more highly branched with a larger number of autofluorescent root tips, compared with varieties Pennuda or Porter, indicative of greater levels of avenacins per unit area of root surface, especially at the root tips.

As a preliminary test of whether avenacins were released from root surfaces, we examined whether fungal growth was supported on the paper toweling used to produce oat roots for extraction. Inhibition of fungal growth was evident adjacent to the root zone; this area also exhibited blue fluorescence under UV light, indicative of avenacins and consistent with an earlier report.⁵⁴

To determine whether avenacins were present in exudates from growing roots, varieties Saia, Pennuda, and Porter were grown in a greenhouse root growth system for up to 6 weeks. A greenhouse growth system for root exudate collection^{55,56} was set up in triplicate for each variety; exudate collections were twice weekly via elution of individual chambers onto Amberlite XAD-4TM resin, which was subsequently flushed with 100 ml of distilled water to remove polar materials, then flushed with 100 ml of methanol to elute avenacins. The methanol solutions of exudates for each oat variety were pooled, evaporated to dryness, and yields recorded. Components of exudates from weeks 1 through 6 were separated by TLC and assessed for biological activity (Figure 20.4). Root exudates collected from

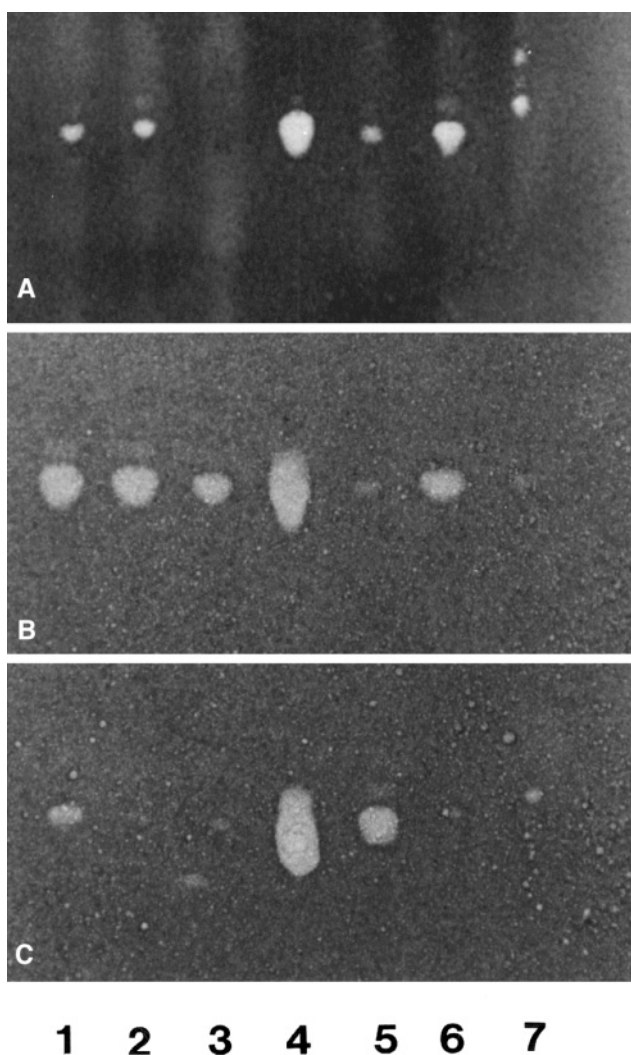


FIGURE 20.4

TLC bioassay of root exudates collected at weeks 1 through 6. Exudate samples were concentrated to 20 mg/ml, then separated via TLC using chloroform/methanol/H₂O, 13:6:1) and dried. Plate was then sprayed with 25 ml of *B. sorokiniana* spore suspension (4.0 to 6.0×10^5 spores/ml) in potato dextrose broth containing 0.1% agar and 0.1% Tween 80 and incubated in a moist container in the dark at 25°C. Inhibition was scored within 24 h. (A) Week 1 samples: lane 1, Saia; lane 2, Porter; lane 3, Pennuda; lane 4, avenacin standard; Week 2 samples: lane 5, Saia; lane 6, Porter; lane 7, Pennuda. (B) Week 3 and week 4 samples loaded as in A. (C) Week 5 and week 6 samples loaded as in A.

week 2 through 4 from varieties Saia, Porter, and Pennudam produced TLC spots that co-eluted with avenacin standards A-1 and B-1. Week 1 exudate from Pennuda did not produce an inhibition zone, although exudates from both Saia and Porter produced detectable inhibition zones. By week 3, exudates from all three varieties displayed strong inhibition zones at R_fs equal to those of avenacin A-1 and B-1. By weeks 5 and 6, only slight inhibition was seen with exudates from oat varieties Pennuda and Porter. Saia exudates, however, still produced a large fluorescent spot and a strong inhibition zone at the R_fs of A-1 and B-1 through week 6.

Saponins, such as avenacins, may be important determinants of resistance and suppressive activity in crops due to their widespread occurrence in nature and reported antimicrobial and antihelminthic activities.^{57,58} Although saponins are biologically active compounds, a clear correlation between saponin content and cultivar resistance has yet to be established, as in the case of alfalfa resistance to downy mildew.^{59,60} Our study indicates that the amount of saponin present in a sample may be extensively hydrolyzed during extraction. Earlier studies also are difficult to interpret because resistance may be linked to concentration or release of particular active saponins during the infection process rather than total saponin content.⁶¹ We were able to detect avenacins in root exudates up to 6 weeks past germination in the oat variety Saia; other oat variety exudates contained avenacins up to 4 weeks after germination. The slow release of avenacins into the rhizosphere over an extended time should provide some measure of antimicrobial and/or antihelminthic protection for the plant as well as transfer suppressive activity to the soil, both during growth and from residues. Varietal differences in avenacins, the expression of avenacins along root surfaces, and the release of avenacins from the root zone during plant development, and perhaps during infections, may all contribute to overall plant resistance and suppression of fungal soil pathogens and nematodes in oats and related plants.

20.4 Summary

The goal of our research is to complement the development of practical pest control strategies that minimize or eliminate chemical pesticides by discovery of novel, naturally occurring pesticidal chemistries. Both plants and fungi offer new opportunities as source materials in this search effort. Since much of the secondary metabolism of fungal pathogens of invertebrates is unknown, there is great hope for successes in this endeavor, as in the case of the novel destruxins from *Aschersonia*. Likewise, plants (such as oats) that produce compounds whose roles in disease or pest resistance are poorly understood, may represent untapped repositories of novel, biologically active compounds to control soilborne pests and diseases in an environmentally rational manner.

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Recent Advances in Saponins Used in Foods, Agriculture, and Medicine

George R. Waller

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ABSTRACT The variation of secondary metabolites produced by plants is enormous and differs greatly with the stages of growth and development. Saponins are being carefully examined because of their biological effects on humans, plants, and animals. Saponins are localized in plant organelles that have a high turnover rate, which implies that they are metabolically active as well as sequestered from the remaining parts of the cell. The biological activity is found in agriculture, forestry, natural and developed ecological systems, and may provide, in part, an explanation of the reason for plant survival in a hostile world. The advantage of saponins to the plant producing them is that they may function as protecting agents, growth regulators, and allelochemicals. Some saponins have cardiac activity, hemolytic activity, activity as fish poisons, cholesterol-reducing ability, bitterness, activity as sweeteners, cosmetics, herbs, nonalcoholic beverages, and growth-regulating effects on

crops. Chemical identification using NMR and mass spectrometry as well as a brief description of the types of saponins is described. Their use in plant drugs, folk medicines, etc. has generated great interest in the chemical characterization of these molecules.

21.1 Introduction

The extraordinary technological advances made in saponin research in recent years that have been created and developed by scientific establishments throughout the world present intellectually challenging problems which are becoming more solvable using modern techniques. How many of us could have imagined a decade ago what has been accomplished and is now done routinely in the chemical, biological, agricultural, and pharmaceutical laboratories today. Advanced techniques in NMR spectroscopy, mass spectrometry, x-ray crystallography, use of enzymes, various chromatographic procedures, and new bioassays have been developed. The advances in these areas have been truly phenomenal. On the other hand we have not progressed nearly so much elucidating the structures of saponins and adopting these techniques coupled with molecular biology. It is our hope that finding a saponin that can inhibit a disease or plant traits (e.g., resistance to a virus, tumor, microorganism, or insects) can be isolated to a specific gene and this will become routine during the 21st century. The advances made in the understanding of traditional medicine are legendary, but the search for their use as modern medicines is just beginning.¹⁻³

Saponins are a class of natural products that are structurally constructed of aglycones (triterpene or steroidal) and sugars [pentose(s), hexose(s), and/or uronic acid(s)]. An appropriate hydrolysis of saponins yield sugars and aglycone; however, hydrolysis does not necessarily produce the genuine aglycone. Saponins are biological detergents because of glycosylation of the hydrophobic aglycone and when agitated in water form a soapy lather that gives rise to the name of the group of compounds. This unique ability to cause foaming has been used by mankind throughout the centuries for making cleaning solutions, and indeed it can act as an aid in identification of plant extracts. Triterpenoid, steroid, and steroid alkaloid glycosides are widely spread throughout the plant kingdom, and several have been found in marine animals and plants.

Table 21.1 describes most of the biological activities of saponins which are behaviors found in certain saponins rather than in all members of this chemical family. From the biological viewpoint saponins have a diverse group of properties, some deleterious but many beneficial. Their use in plant drugs, folk medicines, etc. has generated great interest in the chemical characterization of these molecules. This has been evident in Asia (particularly Japan and China), where the literature on the isolation, purification, separation, structural elucidation, and biological activity of saponins attests to the skill of natural products biochemists and chemists.⁴ The industrial demand for saponins is increasing and, with more attention being given to this field, it offers a renewable resource that provides a stable raw material with a practical and dependable supply.

Saponins are widely distributed in dicotyledonous plants species and also in monocots. They occur in foods (e.g., beans, peanuts, oats, green peppers, asparagus, garlic, onions, spinach, tomatoes, and potatoes) and animal feed (alfalfa and clover), as well as in some marine organisms. Saponins are present in numerous herbal remedies, e.g., ginseng, quilla, horse-chestnut, and beans of various types. Saponin content in plants depends upon many

TABLE 21.1

Agricultural, Biological, Medical and Pharmacological Properties of Saponins

Adaptogenic activity	Diuretic activity
Adrenocorticotrophic system effects	Enzyme activities
Allelochemical	Glucose transport system effects
Analgesic activity	Expectorant activity
Antianging activity	Fish and arrow poisons
Antiexudative activity	Folk medicine
Antifeeding activity	Growth regulators in plants
Antifungal activity	Hemolytic activity
Antiinflammatory	Hypoglycemic activity
Antileishmanial activity	Immunomodulatory activity
Antimicrobial activity	Insecticidal activity
Antioedematous activity	Molluscosidal activity
Antiprotozoal properties	Nutrition
Antitumor activity/cytotoxic activity	Human
Antiulcer activity	Animals
Antiviral activity	Plant drugs
Bitterness	Reduction of alcoholism
Capillary fragility effects	Sedative activity
Cardiovascular activity	Shark repellents
Cholesterol reducing ability	Spermicidal and contraceptive activities
Cosmetic activity	Sweeteners

factors such as the cultivar, the age, physiological state, and geographical location. The same species may vary in saponin composition and quantity when it is grown in different places. Several authors have reviewed the occurrence and distribution of the triterpene saponins, steroid saponins, and the steroid alkaloid glycosides from plants and some marine organisms.^{4,5}

Saponins are localized in organelles that have a high turnover rate, which implies that they are not only metabolically active but they may be important regulatory substances in the development of an organism. The primary action of saponins upon cells is to cause a general increase in the permeability of the plasma membrane;⁴ whether or not this action leads toward changing the auxin activity is not known. The biosynthesis of saponins has been the subject of a small amount of research activity, and mostly it is based on the classical biosynthetic pathways involving squalene-2,3-epoxide which is cyclized to give cholesterol and the aglycones of saponins. Enzymes catalyzing the glycosylation of the saponins have been isolated, but much remains to be done before an understanding of the biosynthesis and biodegradation are intimately known and understood.^{2,3}

The nomenclature and stereochemistry of saponins has been difficult to determine. *Mono-desmosidic* saponins have a single sugar chain which is normally attached to carbon atom-3 (C-3) of the triterpene or steroid nucleus. *Bidesmosidic* saponins have two sugar chains frequently attached at C-3 through an ether linkage, and one attached through an ester linkage at C-28 (triterpene glycosides) or an ether linkage at C-26 (furostanol saponins). *Tridesmosidic* saponins have three sugar chains and are rarely found. Significant other variations in the saponin structure will be noted in this chapter. *Bidesmosides* appear to be primarily a transport form and when the plant is damaged they can be rapidly converted by enzymes into *monodesmosides* which tend to be more active.⁶ It seems that the advantage of saponins to the plant producing them is that they may function as protecting agents, growth regulators, and allelochemicals.

TABLE 21.2

Methanol Extracts from Natural Medicines Action on Blood Ethanol Concentration

	Dose (mg/kg, <i>p.o.</i>)	Ethanol Concentration in Blood (mg/ml, 1 h)
<i>Control</i>		0.72 ± 0.03
<i>Aralia elata</i> (Japanese Angelica tree, bark)	400	0.07 ± 0.04 ^b
<i>Sapindus mukurossi</i> (Japanese Soapnut tree, pericarps)	400	0.27 ± 0.09 ^a
<i>Aesculus hippocastanum</i> (Horse Chestnut tree, seeds)	400	0.25 ± 0.01 ^b
<i>Camellia japonica</i> (Camellia, seeds)	400	0.30 ± 0.03 ^b
<i>Polygala senega</i> (Senegae radix)	400	0.01 ± 0.01 ^b
<i>Thea sinensis</i> (seeds)	400	0.05 ± 0.01 ^b
<i>Platycodi radix</i>	400	0.30 ± 0.09 ^a
<i>Bupleuri radix</i>	400	0.35 ± 0.04 ^a

Note: The tested samples dissolved in water (5 ml/kg) were orally administered to male Wistar rats at each dose 1 h before oral administration of 20% aq. ethanol (5 ml/kg). Blood (0.5 ml) was collected from the carotid at 1 h after ethanol administration. The plasma ethanol was assayed by the enzyme method (blood alcohol test "BMV").

^a $p < 0.05$

^b $p < 0.01$

Source: Yoshikawa, M. and Yamahara, J., in *Advances in Experimental Medicine and Biology*, 404, Plenum Press, New York, 1995. With permission.

21.2 Reduction of Alcoholism

Alcoholism is a major health problem in the world that causes as much trouble physiologically as it does socially. Excessive consumption of ethanol profoundly affects nearly every organ in the body, particularly the endocrine system, heart, central nervous system, immune system, and liver. In order to relieve ethanol toxicity in acute alcohol ingestion, several methods using accelerators of ethanol metabolism (e.g., clofibrate, methyl γ -linolenate, ginseng extract) and the sequestering of acetaldehyde (e.g., *D*-penicillamine, *L*-cysteine) have been reported.⁷ In addition, dehydrogenate inhibitors such as cyanamide and disulfiram have been used clinically for chronic alcoholics. It is assumed that dehydrogenase inhibitors force alcoholics to quit drinking based on the fear of unpleasant reaction elicited after ethanol intake, but these drugs also are reported to show many strong side effects.

Screening for biologically active principles that are contained in traditional medicines yields several products that contain inhibitors of ethanol absorption as shown in Table 21.2. By monitoring the inhibitory effect on ethanol absorption in the rat, new active triterpene oligoglycosides were characterized from *Aralia elata* (elatosides), *Aesculus hippocastanum* (escins), *Camellia japonica* (camelliasaponins), and *Polygala senega* var. *latifolia* (senegasaponins) (Figure 21.1). Structural requirements for the activity was done and it was found that the active saponins can be classified into the following three types of structures: (1) oleanene-28-oic acid 3-*O*-monodesmosides such as elatosides, (2) acylated polyhydroxyoleanene 3-*O*-monodesmosides such as escins and camelliasaponins, and (3) oleanene acylated bis-desmosides such as senegasaponins. Only the first classification will be discussed.

Research on the characterization of inhibitors of alcohol absorption in natural medicines and their structure-activity relationships shows interesting results. The bark and root cortex of *Aralia elata* (Japanese angelica tree, Araliaceae) has been used in Japanese and

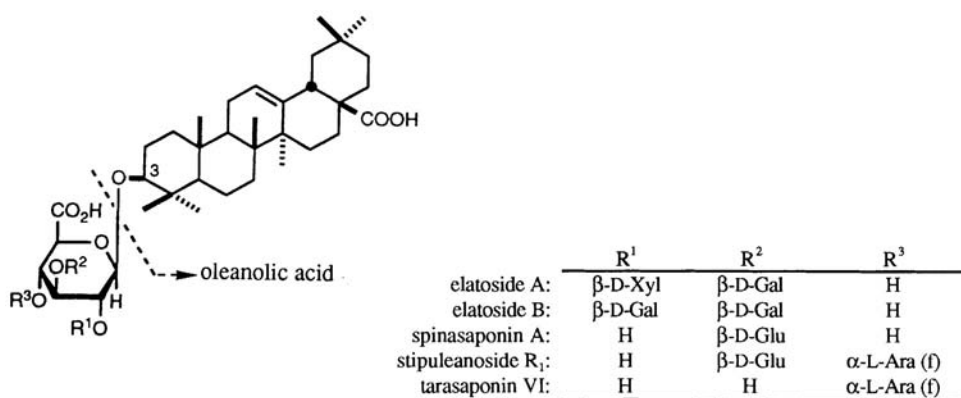


FIGURE 21.1

Structures of some saponin constituents from *Aralia elata*. (From Yoshikawa, M. and Yamahara, J., *Advances in Experimental Medicine and Biology*, 404, Plenum Press, New York, 1995. With permission.)

Chinese traditional medicines as a tonic, antidiabetic, and antiarthritic agent. The young shoot of this medicinal plant, which is commonly called “Taranome” in Japanese, has been known as a garnish for food in Japanese-style dishes. Yoshikawa and Yamahara⁷ found that the methanol extracts of the bark, root cortex, and young shoots of this plant have an inhibitory effect on ethanol absorption. The methanol extracts were subjected to bioassay-guided separation procedures which provide oleanene-type triterpene oligoglycosides named elatosides with several known oligoglycosides (Figure 21.1). Inhibitory effects of oleanolic acid and its glycosides on ethanol absorption are summarized in Table 21.3. Among the compounds tested, oleanolic acid 3-O-monodesmosides showed potent inhibitory activity on ethanol absorption with elatoside A possessing the highest activity. These

TABLE 21.3

Oleanolic Acid 3-O-Monodesmosides: Effects on Blood Ethanol Concentration

	Dose (mg/kg, p.o.)	n	Ethanol Concentration in blood (mg/ml)		
			1 h	2 h	3 h
Control		10	0.61	0.18	0.05
3-O-monodesmosides					
Elatoside A	25	4	0.11	0.13	0.02
	50	4	0.01	0.04	0.01
	100	5	0.00	0.00	0.00
Elatoside B	25	5	0.56	0.19	0.01
	50	5	0.50	0.19	0.02
	100	5	0.25	0.18	0.02
Spinasaponin A	25	5	0.26	0.20	0.03
	50	5	0.03	0.04	0.02
	100	4	0.03	0.02	0.01
Stipuleanoside R ₁	25	5	0.42	0.21	0.03
	50	4	0.34	0.18	0.01
	100	5	0.08	0.09	0.00
Tarasaponin IV	25	5	0.21	0.11	0.00
	50	5	0.02	0.03	0.00
	100	5	0.00	0.02	0.01

Source: Yoshikawa, M. and Yamahara, J., in *Advances in Experimental Medicine and Biology*, 404, Plenum Press, New York, 1995. With permission.

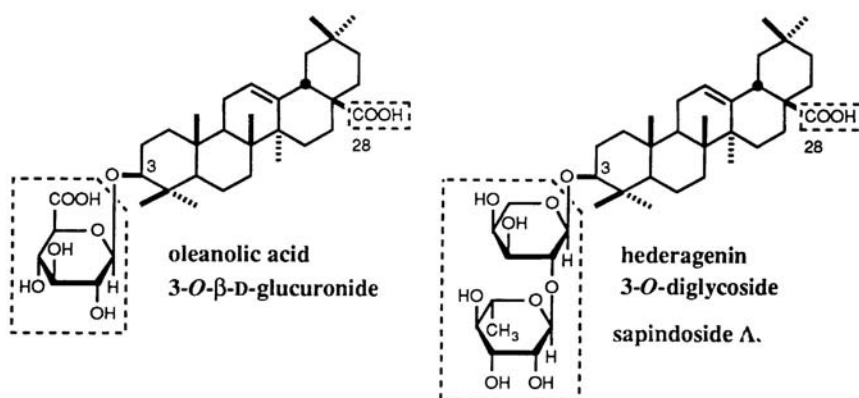


FIGURE 21.2

Structural requirements for inhibitory activity on ethanol absorption. The glycoside linkage is required at C-3 and the carboxylic acid group must be free at C-28. (From Yoshikawa, M. and Yamahara, J., *Advances in Experimental Medicine and Biology*, 404, Plenum Press, New York, 1995. With permission.)

authors have obtained oleanolic acid glycosides from various natural medicines such as *Panax japonicus* (rhizoma, Araliaceae), *Beta vulgaris* (root, Chenopodiaceae), and *Kochia scoparia* (fruit, Chenopodiaceae), and examined their effects on ethanol concentration in the blood. A comparison of the inhibitory activity for oleanolic acid oligoglycosides led them to the following structure–activity relationships (Figure 21.2): (1) the 3-O-glycoside moiety is essential to the inhibitory activity of ethanol absorption, and (2) the presence of the 28-ester glycoside moiety significantly reduces the activity. Consequently, the authors concluded that the oleanolic acid 3-O-glucuronide structure was required for the inhibitory activity of ethanol absorption. These saponins decrease the ethanol concentration in the blood by inhibiting absorption across the cell membranes of the digestive tract.

21.3 Allelopathy

21.3.1 Growth Promotion

Examination of soya beans, peanuts, peas, chickpeas, and several varieties of beans showed that soyasaponin VI was present in large quantities despite the fact that soyasaponin I was previously reported as the major compound.⁸⁻¹² Soyasaponin VI readily decomposes into soyasaponin I in acidic or basic pH or by simply standing in alcoholic solutions (Figure 21.3). Thus, soyasaponin I is, at least in part, an artifact made from soyasaponin VI. This compound was simultaneously isolated in two Japanese and one French laboratory at approximately the same time. Solutions of 3 mM of soyasaponin VI stimulates the growth of cells in lettuce roots (*Lactuca sativa* L. cv. Grand Rapids) to around 190% of the control (Figure 21.4).¹³ The same laboratory¹⁴ showed that the cortical cells of the lettuce roots are the site of action (Figure 21.5) that produces the acceleration of growth. The concentration of soyasaponin VI is 2 to 3 mM in the root tips of 7-day-old pea seedlings (*Pisum sativum* L.), whereas the concentration of soyasaponin VI in the nonmeristematic tissue is around 0.2 to 0.3 mM (10-fold decrease). The entire molecule of soyasaponin VI is required for maximum stimulation of lettuce, whereas, only a 10 to 15% growth stimulation was found

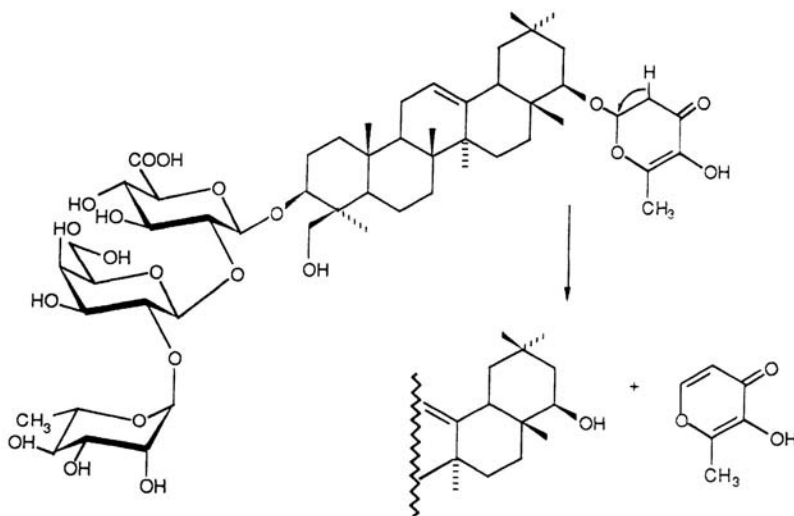


FIGURE 21.3

Soyasaponin VI and degradation products: soyasaponin I and maltol. (From Massiot, G. et al., *J. Nat. Prod.*, 55, 1339, 1992. With permission.)

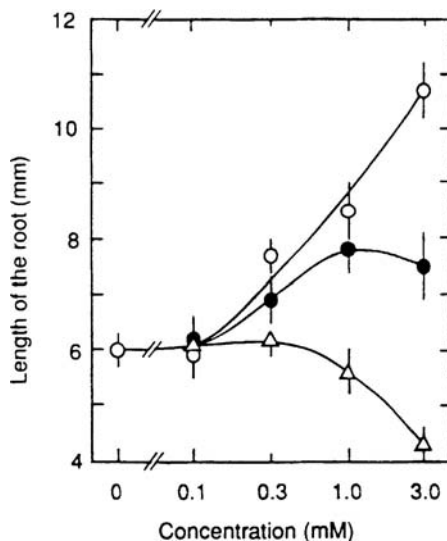


FIGURE 21.4

The effect of soyasaponin I-●, maltol-Δ, and soyasaponin VI-○ on the growth of lettuce roots (*Lactuca sativa*). (From Tsurumi, S. and Tsujino, Y., *Physiol. Plant.*, 93, 785, 1995. With permission.)

for soyasaponin I, and maltol caused a decrease in growth rate when higher than 0.1 mM concentration. The lower rate of growth enhancement (10 to 15%) of soyasaponin I supports the findings of Waller, et al.¹⁵⁻¹⁷

The growth stimulation induced by soyasaponin VI was highest in lettuce, followed by chrysanthemum, leaf mustard, timothy, Italian ryegrass, white clover, salt green, alfalfa, milk vetch, and Japanese hornwort. The assay with lettuce roots was conducted under red light and two species of Compositae, lettuce and chrysanthemum, were very sensitive to soyasaponin VI, while burdock in the same family was not. Such disunity within a family

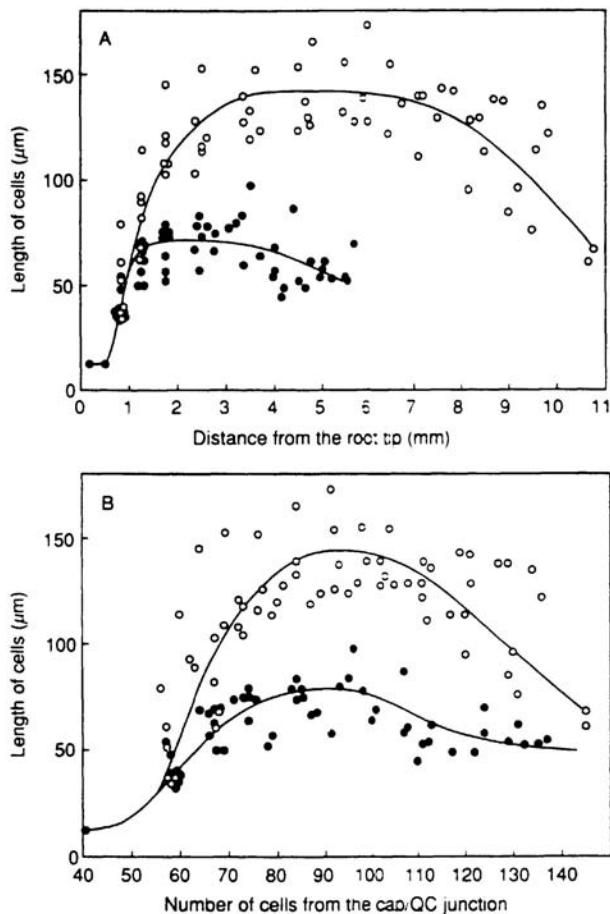


FIGURE 21.5

Lengths of meristematic and cortical cells of lettuce roots incubated with (○) or without (●) 3 mM soyasaponin VI for 24 h. (A) Roots were divided into several zones and the mean length of 10 cells in each zone is plotted against the distance of the midpoint of the zone from the root tip. The length of a zone was about 0.3 mm in the meristem, 0.5 mm in the elongating zone and 1 mm in the basal part. Six roots were measured after treatment with soyasaponin VI and eight roots were measured as controls. (B) Data are replotted against the number of cells from the junction between the cap and quiescent center (the cap/QC junction). (From Tsurumi, S. and Wada, S., *Plant Cell Physiol.*, 36, 925, 1995. With permission.)

with respect to the growth response to soyasaponin I also was observed in Cruciferae, Umbelliferae, and Gramineae. Among the concentrations of soyasaponin VI tested, 3.0 mM was most effective in six species; however, 1.0 mM was most effective for leaf mustard and 0.3 mM for timothy, Italian ryegrass, and salt green. The final length of cells in the mature cortical portions of the roots of chrysanthemum, leaf mustard, white clover, and timothy were increased by soyasaponin VI, indicating that the soyasaponin VI-induced stimulation of growth in the roots of these plants involved the acceleration of cell elongation.

21.3.2 Growth Inhibiting

Nohara, et al.¹⁸ isolated a saponin from *Heloniosis japonica*, a member of the liliaceous plants, and obtained a novel compound named helojaposide (Figure 21.6.) which showed a significant inhibition action at 10 ppm for the root growth of rice. Waller, et al.¹⁵⁻¹⁷ showed

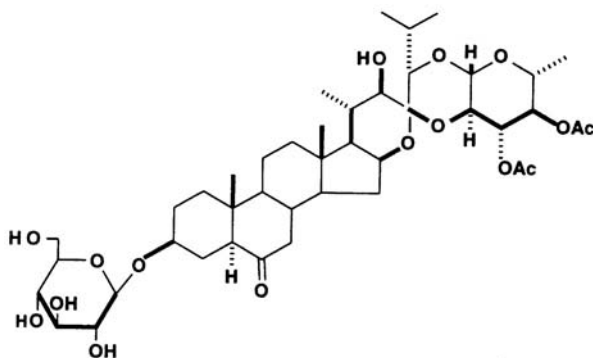


FIGURE 21.6

Helojoposide from *Heloniosis japonica*. (From Nohara, T., Yahara, S., and Kinjo, J., *Advances in Experimental Medicine and Biology*, 404, Plenum Press, New York, 1995. With permission.)

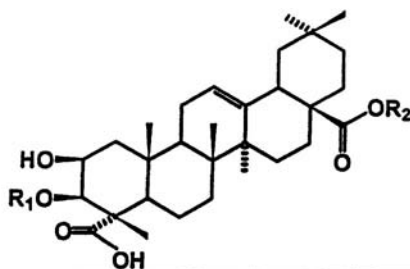
that soyasaponin I, III, and a new saponin inhibited the growth of mungbeans and lettuce growing for 72 h.

Alfalfa saponins (Figure 21.7) have been shown to possess high allelopathic potential against plants, fungi, and microorganisms; however, they remain to be implicated in the autotoxicity of alfalfa plants growing in the fields.¹⁹⁻²⁴ There is a problem when the alfalfa is planted immediately following alfalfa since the soil is toxic to alfalfa, corn, or other crops; the relationship of saponins from alfalfa has been made, but evidence has not been conclusive. After 1 year the soil recovers from its toxicity and no problems remain. Figure 21.7 shows some structures of different medicagenic acid glycosides, most of which have shown inhibitory action. The alfalfa plant also contains hederagenin, soyasapogenol, and zanhic acid glycosides which are less inhibiting, but structures are not shown. The problem of alfalfa saponins being excreted into the soil has not been fully shown,²⁰ and the degree of accumulation in the soil-humus complex cannot be clearly estimated; however, 2 to 24% of saponins were recovered from the humic acid in a range of soils in the laboratory.²⁵

21.4 Cancer Chemoprevention by Saponins from Ginseng

The chemical carcinogenesis mechanism has been explained by either a two-stage theory or a multi-stage theory which consists of initiation, promotion, and progression stages. In these stages, the promotion stage is a long-term and reversible reaction, and the development of antitumor promoters has been regarded as the most effective method for the chemoprevention of cancer.

Konoshima et al.^{26,27} carried out primary screening tests of the saponins from ginseng utilizing a short-term *in vitro* assay on the Epstein-Barr virus early antigen (EBA-EA) activation as shown in Figure 21.8. Raji cells carrying EBV genome were incubated in a medium containing *n*-butyric acid, TPA, and various amounts of test compound. They made use of the inhibitory effects on EBV-EA activation induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) which is known to be a strong promoter. Smears were made from the cell suspension and the EBA-EA inducing cells were stained using an indirect immunofluorescence technique. Damarane-saponin (ginsenosides) were isolated from several *Panax* plants and biological studies on antitumor-promoters, using the primary screening test of the extracts, were completed on four crude drugs. As shown in Table 21.4, the extract of *Panax notoginseng*



	R ₁	R ₂	Plant Part
I	Glu	H	Roots
II	Glu	Glu	Roots
III	Glu(1→4)Glu	H	Roots
IV	Glu(1→6)Glu(1→3)Glu	H	Roots
V	Rha(1→6)Glu(1→2)Glu	H	Flowers
VI	Glu	Xyl(1→4)Rha(1→2)Ara	Roots, Tops
VII	Glu(1→2)Glu	Xyl(1→4)Rha(1→2)Ara	Roots, Tops
VIII	Glu	Rha(1→2)Ara	Roots
IX	Glu(1→2)Glu(1→2)Glu	Glu	Roots
X	Rha(1→3)GluA	H	Roots
XI	GluA	Xyl(1→4)Rha(1→2)Ara	Roots, Tops
XII	Gal(1→2)Glu	Glu	Roots
XIII	Rha(1→2)Glu(1→2)Glu	Glu	Roots
XIV	H	Xyl(1→4)Rha(1→2)Ara	Tops
XV	GluA	Rha(1→2)Ara	Tops

FIGURE 21.7

Medicagenic acid glycosides identified in alfalfa. (From Oleszek, W., *Advances in Experimental Medicine and Biology*, Plenum Press, New York, 1996. With permission.)

exhibited significant inhibitory effects on EBV-EA activation (100% inhibition of activation at 500 µg/ml, more than 90%, 65%, and 45% inhibitions at 100 µg, 50 µg/ml, and even at 10 µg/ml). The extract of *Panax ginseng* (steamed ginseng, commonly known as red ginseng) also exhibited the inhibitory effect at high concentration (500 µg and 100 µg/ml). Only weak inhibitory effects resulted from either *Panax japonica* or *Panax ginseng* (white ginseng). On the bases of these results, the details of the anti-tumor-promoting activity of *P. notoginseng* and its constituents were investigated for their cancer chemopreventive activity.

P. notoginseng is distributed in limited parts of China (Yunnan and Kweichow) and is recently cultivated in Yunnan. The root of this plant, called Sanchi-Ginseng, is one of the famous Chinese medicines used mainly as a hemostatic drug and the treatment of hepatitis.

Five dammarane-saponins (ginsenoside-Rb₁, -Rb₂, -Rd, -Re, and -Rg₁) were isolated as major neutral saponins from the root of *P. notoginseng* together with other minor saponins (Figure 21.9). Furthermore, an acetylene derivative, panaxytriol, which had been obtained from red ginseng but not from white ginseng, also was isolated and identified. These five

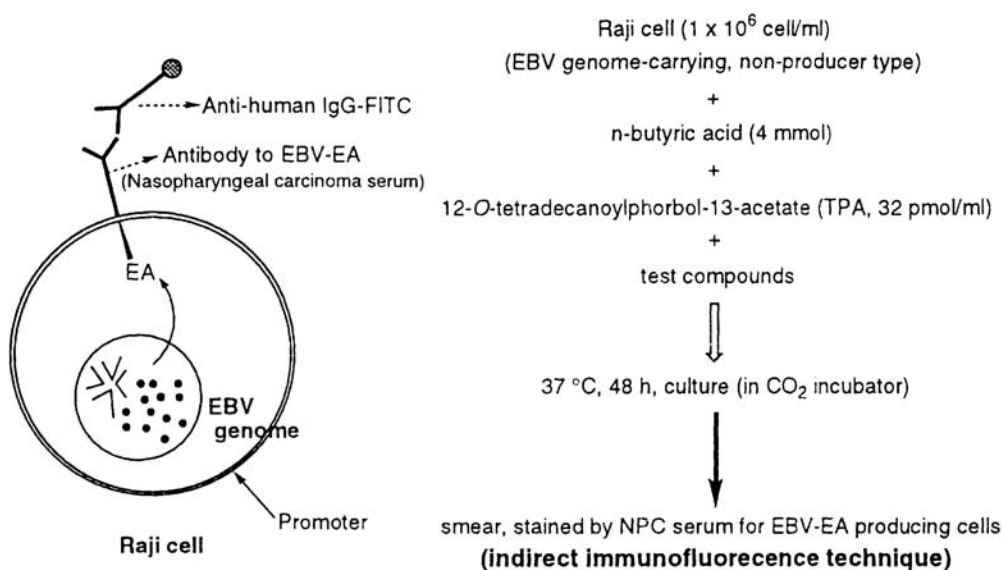


FIGURE 21.8

Method of synergistic assay using Epstein-Barr virus early antigen. (From Konoshima, T., *Advances in Experimental Medicine and Biology*, 404, Plenum Press, New York, 1995. With permission.)

TABLE 21.4

Percentages of EBV-EA Induction in Presence of Extracts of *Panax* Plants with Respect to Positive Control (100%)

Sample	Concentration ($\mu\text{g/ml}$) ^a			
	500	100	50	10
<i>Panax notoginseng</i>	0.0 ^b (60) ^c	7.6 (70)	33.4 (>80)	53.8 (>80)
<i>Panax ginseng</i> (white)	38.7 (70)	79.6 (>80)	100.0 (>80)	100.0 (>80)
<i>Panax ginseng</i> (red)	13.5 (60)	22.4 (>80)	75.3 (>80)	100.0 (>80)
<i>Panax japonica</i>	83.2 (50)	100.0 (>80)	100.0 (>80)	100.0 (>80)

^a mg/ml, TPA (20 ng = 32 p mol).

^b Values represent relative percentages to the positive control.

^c Values in parentheses are viability percentages of Raji cells.

Source: Konoshima, T., in *Advances in Experimental Medicine and Biology*, 404, Plenum Press, New York, 1995. With permission.

saponins and acetylenes were assayed in the EBV-EA assay and results are shown in Table 21.5. Acetylenes showed significant inhibitory effects (more than 80% inhibition of activity at 1×10^2 mol ratio/TPA), but have strong cytotoxicities on Raji cells (0% viability of Raji cells at 1×10^2 mol ratio/TPA, and less than 30% viability at 5×10^2 mol ratio/TPA). On the other hand, ginsenoside-Rg₁ exhibited strongest inhibitory effects (100% inhibition of activation at 2.5×10^3 mol ratio/TPA, and more than 85%, 65%, and 35% inhibitions at 1×10^3 , 5×10^2 , and 1×10^2 mol ratio/TPA) of these five saponins.

Tanaka's laboratory,²⁸ reporting on the analysis of saponins of ginsengs, found that the content of ginsenoside-Rg₁ in the root of *P. notoginseng* was more than 10 times that of other *Panax* plants. Ginsenoside-Rg₁ has inhibitory effects on EBV-EA activation (see Table 21.5) and was observed to strongly enhance the inhibitory effect of panaxytriol. The significant antitumor activity of the crude extract of *P. notoginseng* is exhibited by the combination of ginsenoside-Rg₁ with panaxytriol in mice studies. The inhibitory activities, evaluated by both

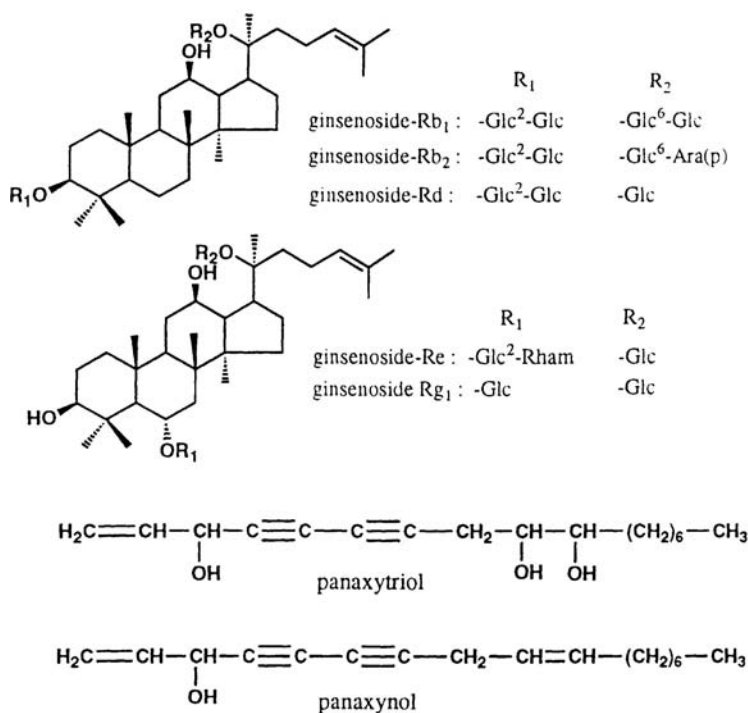


FIGURE 21.9

Saponins and acetylenes from *Panax notoginseng*. (From Konoshima, T., *Advances in Experimental Medicine and Biology*, 404, Plenum Press, New York, 1995. With permission.)

TABLE 21.5

Percentages of EBV-EA Induction in Presence of Ginsenosides and Acetylenes with Respect to Positive Control (100%)

Sample	Concentration (mol ratio, compound/TPA)				
	2.5×10^3	1×10^3	5×10^2	1×10^2	1×10
Ginsenoside Rb ₁	0.0 ^a (>80) ^b	20.1 (>80)	41.7 (>80)	71.8 (>80)	100.0 (>80)
Ginsenoside Rb ₂	0.0 (>80)	22.6 (>80)	48.3 (>80)	78.5 (>80)	100.0 (>80)
Ginsenoside Rd	0.0 (>80)	17.6 (>80)	38.0 (>80)	67.4 (>80)	94.8 (>80)
Ginsenoside Re	0.0 (>80)	18.9 (>80)	40.7 (>80)	69.3 (>80)	94.4 (>80)
Ginsenoside Rg ₁	0.0 (>80)	12.4 (>80)	32.5 (>80)	63.6 (>80)	91.0 (>80)
	Concentration (mol ratio, compound/TPA)				
	1×10	1×10^b	5×10	1×10	$\times 1$
Panaxytriol	— ^c (0)	— (0)	— (0)	0.0 (20)	64.9 (>80)
Panaxynol	— (0)	— (0)	0.0 (30)	23.3 (60)	84.5 (60)

^a Values represent relative percentages to the positive control value (100%).

^b Values in parentheses are viability percentages of Raji cells.

^c Not detected.

Source: Konoshima, T., in *Advances in Experimental Medicine and Biology*, 404, Plenum Press, New York, 1995. With permission.

rate (%) of papilloma-bearing mice (A) and average number of papillomas per mouse (B), were compared with those of a positive control. In the positive control, more than 90% and 100% of mice bore papillomas at 9 and 10 weeks of promotion, respectively. More than

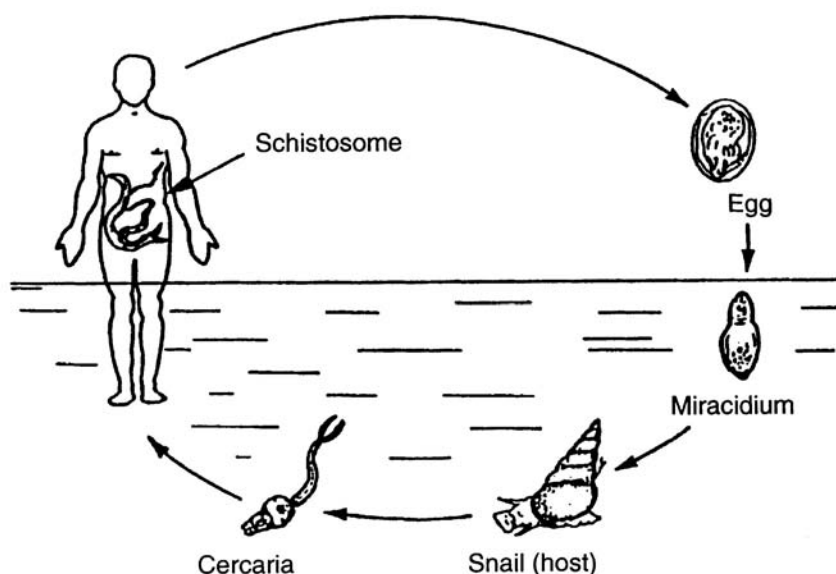


FIGURE 21.10

Life cycle of *Schistosoma* species. (From Hostettman, H. et al., *Advances in Experimental Medicine and Biology*, 404, Plenum Press, New York, 1995. With permission.)

10 papillomas were formed per mouse at 20 weeks of promotion. When ginsenoside-Rg₁ was applied continuously before each TPA treatment, it remarkably delayed the formation of papillomas in mouse skin and reduced the number of papillomas per mouse (about 10% and 30% of mice bore papillomas at 9 and 12 weeks of promotion, respectively; 80% of mice bore papillomas even at 20 weeks; and less than 7% papillomas were formed per mouse at 20 weeks of promotion). These inhibitory effects of ginsenoside-Rg₁ were similar to those of glycyrrhetic acid which has been known as a strong antitumor-promoter. To summarize the structure/activity of the ginsenosides isolated from *Panax notoginseng*, Rg₁ was clearly the most effective and it had only a glucose substituted on position C-3 and C-27, whereas the less active ginsenosides had additional sugar linkages at either carbon atoms 1→6 or 1→2.

21.5 Molluscicidal and Antifungal Saponins

The parasitic disease “schistosomiasis” is a major health problem in many tropical countries with over 300 million people being infected. There are two European laboratories that have been quite active in investigating molluscicidal activity of saponins.^{29,30} Freshwater snails act as intermediate hosts for the parasite and because of that, the infection is transmitted among humans on water contact, mainly in rural areas with minimal domestic water supply and poor sanitary facilities (Figure 21.10). The eradication of the disease appears to be impossible with the available resources and, therefore, an immediate concern is control of morbidity. The prevalence of schistosomiasis remains constant, largely because population growth and development of manmade water resources has continued. Treatment of water bodies with molluscicidal compounds to reduce the number of transmitting snails is an important element among integrated strategies for morbidity control. The interest in studying plant material containing molluscicidal compounds is based on producing a local supply of molluscicides in the endemic areas at low cost and by simple technologies.

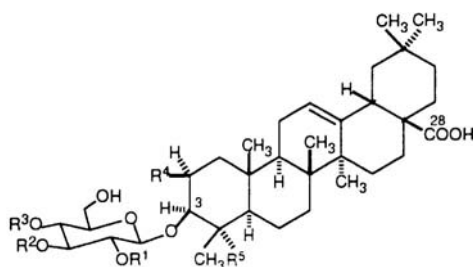


FIGURE 21.11

Monodesmosidic Saponins (R^1 -H, R^2 - α -L-Rha-(1 \rightarrow 2)- β -D-Gal, R^3 -H, R^4 -H, R^5 -CH₃) isolated from the aqueous berry extracts of the Ethiopian strain E44 and the Zimbabwean strain MS1 of *Phytolacca dodecandra*. (From Thillborg, S.T. et al., *Advances in Experimental Medicine and Biology*, 404, Plenum Press, New York, 1995. With permission.)

	R ₁	R ₂	Molluscicidal activity (mg/L) ^{a)}
1	H	H	3.0
2	Glc	H	25.0
3	Glc	Glc	inactive

a) Against *Biomphalaria glabrata* snails.

FIGURE 21.12

Bioactive molluscicidal saponins from *Swartzia madagascariensis* fruits. (From Hostettman, H. et al., *Advances in Experimental Medicine and Biology*, Plenum Press, New York, 1995. With permission.)

The unripe berries from the Endod plant, *Phytolacca dodecandra*, have a high content of saponins and have been used for generations in Ethiopia as a detergent for traditional laundering at river banks. This plant seems to have a toxic effect on snails. The characterization of the saponins from *P. dodecandra* berries showed that the main contribution to the molluscicidal activity is provided by a saponin that has a single glycoside chain attached at position C-3 (Figure 21.11). Other saponins which have an additional sugar chain attached at position C-28 (bidesmosidic saponins) are not active.

The fruits of *Swartzia madagascariensis* (Leguminosae), have been used for controlling populations of schistosomiasis-transmitting snails. Phytochemical investigation of this plant, using modern separation techniques for isolation of the active constituents,³¹ has shown that saponins are responsible for the molluscicidal activity. These glycosides have varying activities depending on the structure, with the most active being the monodesmoside 1 (Figure 21.12). Because of wide distribution of this tree and its high content of the molluscicidal activity of the saponins, field trials with the fruits of *S. madagascariensis* were undertaken in Tanzania. Water extracts of the pods exhibited significant molluscicidal activity against *Bulinus globosus* snails at dilutions of up to 100 mg ground pods per liter and were effective at killing snail populations in infected sites. Furthermore, half-lives of the saponins were short (12 to 24 h), thus reducing the risk of toxicity to humans.

The increasing incidence of mycoses due to the AIDS epidemic and to the use of immunosuppressive drugs has led to new approaches in the search for novel antifungal agents. There are few antifungal preparations currently indicated for the treatment of systemic mycoses and their efficacy is rather limited. Promising new compounds also are needed for agricultural use.

TABLE 21.6

Spermicidal Activity of Saponins 1-4 on Human Spermatozoa

Compound	Concentration (mg/ml)	Motility (%)
1	1	0
	0.10	0
	0.05	23
	0.01	36
2	1	20
3	1	0
4	0.10	5
	1 ^a	0
	0.50	5
	0.10	100
NP-9 (positive control)	1	0
	0.50	0
	0.25	20
	0.10	39
PS (blank control)		>80

^a Broken sperm was observed.Source: Yang, C.R. and Li, X.-C., in *Advances in Experimental Medicine and Biology*, 404, Plenum Press, New York, 1995. With permission.

21.6 Spermicidal Activity

Chong-Ren Yang's Phytochemical Laboratory in Kunming, China has been a leader in plant glycoside isolation and characterization of biological activity for the past 2 decades. Due to special geographic characteristics and complex climatic patterns in southwest China, Yunnan province is well known for its abundant plant species. Among the 30,000 seed plants in China, this province has more than 18,000 species. There are many minority peoples residing in this region of China who have formed a considerable depth and knowledge on valuable ethnopharmaceutical medicines. This background for discovering biologically active compounds from plant secondary metabolites is perhaps the premier of the world. Yang and Li³² selected folkloric plants in southwest China to study the medicinal uses of their steroid and triterpenoid saponins that have known antifungal, antitumor, and spermicidal activity. These polar glycosides occur in traditional Chinese medicines such as ginseng (*Panax ginseng*), South China ginseng (*P. notoginseng*), licorice (*Glycyrrhiza uralensis*), etc. The spermicidal activity of *Oreosolen wattii* Hook (Scrophulariaceae), a Tibetan traditional drug growing in high mountain areas of more than 4000 m, showed a strong inhibitory activity toward human spermatozoa.

Steroid and triterpenoid saponins were tested for inhibitory effects on human spermatozoa *in vitro* by means of a modified Sander-Cramer method. These saponins were divided into three main skeleton types: monodesmosides of spirostanes, bisdesmosides of spirostanes, and furostanes. The results from testing *Oreosolen wattii* Hook are summarized in Table 21.6 and structures are shown in Figure 21.13.³³ A known spermicidal agent, nonoxynol-9 (NP-9) at concentrations of 0.10-1.0 mg/ml, was used as the standard. The direct *in vitro* spermicidal activities of saponins **1-4** were tested using different concentrations (Table 21.6), with results indicating that all the saponins had sperm antimotility activity at a concentration of 1 mg/ml, and sperm decomposition in the case of buddlejasaponin Ia (**4**) at 1 mg/ml. The sequence of spermicidal activities from high to low was mimengoside A (**1**), buddlejasaponin I (**3**), NP-9, buddlejasaponin Ia (**4**), and mimengoside B (**2**). At a concentration of 0.01 mg/ml, saponin **1**

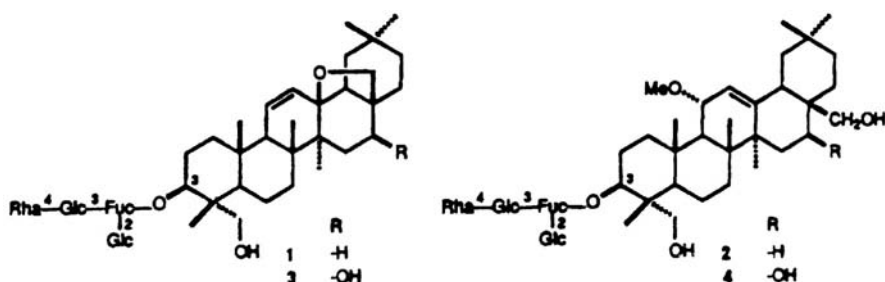


FIGURE 21.13

Structures of spermicidal saponins from *Oreosolen watti*: (1) mimengoside A, (2) mimengoside B, (3) buddlejasaponin I, (4) buddlejasaponin Ia. (From Yu, G.-P. et al., *Acta Botanica Yunnanica*, 18, 229, 1996. With permission.)

had the same effect as that of NP-9 at 0.1 mg/ml. Saponin 1 and 3 possessed an epoxy group between C-13 and C-28 positions which may be the reason for their high activities. Compared to 1, saponin 3 possesses an additional hydroxyl group at C-16 position and was less active than 1. Similarity was observed for saponin 2 and 4. It is worthwhile to note that mimengoside A (1) and buddlejasaponin I (3) have stronger activity than the positive control NP-9.

21.7 Studies on QS-21: A Unique Immunological Adjuvant from *Quillaja saponaria*

QS-21 (Figure 21.14) is an acylated triterpene glycoside isolated from the bark of the South American tree *Quillaja saponaria* Molina.^{34,35} It is a potent immunological adjuvant that has been shown to enhance antigen-specific antibody titers to a wide variety of T-dependent

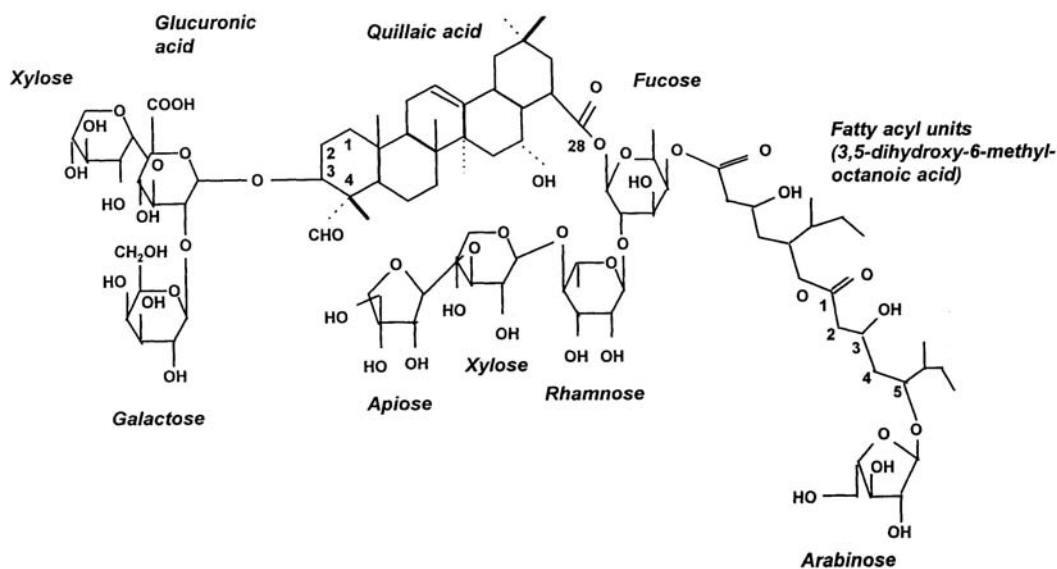


FIGURE 21.14

Structure of QS-21 from *Quillaja saponaria* molina. (From Kensil, C.R. et al., *Advances in Experimental Medicine and Biology*, 404, Plenum Press, New York, 1995. With permission.)

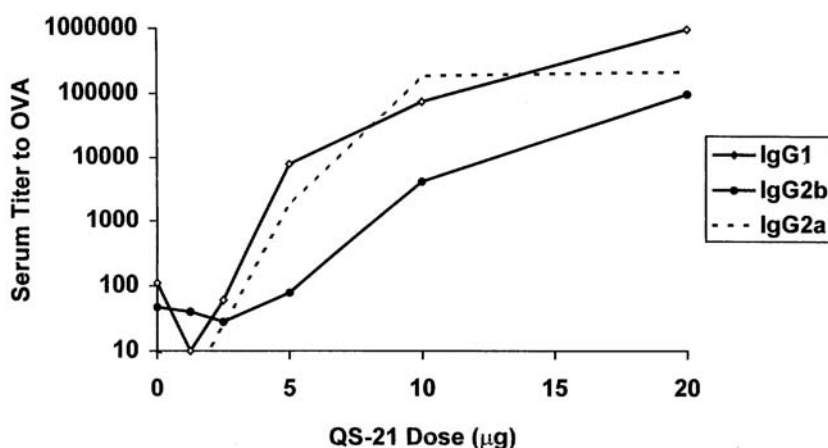


FIGURE 21.15

Effects of QS-21 adjuvant on IgG antibody response to ovalbumin: (A) 10 C57BL/6 mice per group, (B) subcutaneous immunization with 25 µg ovalbumin + QS-21 (day 1 to 15), (C) EIA analysis of antibody response to ovalbumin at day 29. (From Kensil, C.R. et al., *Advances in Experimental Medicine and Biology*, Plenum Press, New York, 1995. With permission.)

and T-independent antigens (reviewed by Kensil³⁶). An example of enhancement of antibody titers to hen egg albumin (ovalbumin, also known as OVA) is shown in Figure 21.15. QS-21 adjuvanted subunit antigen vaccines also stimulate a CD8+ T cell-mediated cytotoxic T lymphocyte (CTL) response against target cells expressing that antigen on Class I major histocompatibility antigen. This may be due in part to the acylation of the *Quillaja* saponins. The deacylsaponin of QS-21 (designated DS-1 and produced by mild alkaline hydrolysis of QS-21) was shown to be ineffective as an adjuvant for inducing CTL responses to OVA.³⁷

QS-21 also has been tested in clinical trials. Recently, QS-21 was shown to stimulate antigen-specific IgM and IgG responses in a Phase I clinical study of a melanoma immunotherapeutic vaccine.³⁸ This vaccine is currently in Phase III trials. QS-21 also has been shown to be useful in a prototype for a malaria vaccine. *Plasmodium falciparum* (malaria) causes more than 2 million deaths annually. There is currently no effective vaccine to prevent it. The candidate vaccines against malaria are poorly immunogenic and thus have been ineffective in preventing infection. However, Stoute et al.³⁹ developed a vaccine based upon the circumsporozoite protein of *Plasmodium falciparum* that incorporates adjuvants to enhance the immune response. The antigen consists of a hybrid in which the circumsporozoite protein fused to hepatitis B surface antigen (HBsAg) is expressed together with unfused HBsAg. Three adjuvant formulations of this antigen were evaluated in an unblinded trial in 46 subjects who had never been exposed to malaria.³⁹ Two of the vaccine formulations were highly immunogenic, inducing high IgG titers to the immunizing antigen and immunofluorescence (IFA) titers against sporozoites. Twenty-two vaccinated subjects and six unimmunized controls underwent a challenge consisting of bites from mosquitos infected with *P. falciparum*. Malaria developed in all six control subjects, seven of eight subjects who received vaccine 1 (formulated in monophosphoryl lipid A (MPL) and aluminum hydroxide (alum)), five of seven subjects who received vaccine 2 (formulated in QA-21 + MPL + oil-in-water emulsion), a result that was statistically different from the other vaccines. It was concluded³⁹ that a recombinant vaccine based on fusion of the circumsporozoite protein and HBsAg plus a potent adjuvant that includes the saponin QS-21 can protect against experimental challenge with *P. falciparum* sporozoites (Table 21.7).

TABLE 21.7

Phase I Trial of a Malaria Vaccine: Recombinant Circumsporozoite Antigen + Adjuvants

	Vaccine 1 (MPL + Alum)	Vaccine 2 (oil-in-water)	Vaccine 3 (QS-21 + MPL + oil-in-water)
Total IgG (μg/ml)	7.00	52.63	52.98
IgG1 (μg/ml)	1.55	12.65	15.54
IgG2 (μg/ml)	2.13	4.19	8.20
IFA	654	4755	6400
Protection/challenge (Control 0/6 subjects)	1/7 subj.	2/7 subj.	6/7 subj.

Source: Stoute, J.A. et al., *N.E. J. Med.*, 336, 86, 1997. With permission.

21.8 Cholestane Glycosides from *Ornithogalum Saundersiae* and their Potent Cytotoxic Activity on Various Malignant Tumor Cells

Ornithogalum saundersiae (Liliaceae) is a perennial plant native to Natal, Swaziland. Mimaki and Sashida⁴⁰ found that the methanol extract of *Ornithogalum saundersiae* bulbs exhibited extremely potent cytotoxic activity toward human promyelocytic leukemia HL-60 cells with an LC₅₀ value of 0.031 μg/ml. Bioassay-guided fractionation of the MeOH extract led to the isolation of a series of seven cholestane glycosides with potent cytotoxic activities toward HL-60 cells and human T-lymphocytic leukemia MOLT-4 cells. Their cytotoxic activity indicated that they possessed an acetyl and an aromatic acyl group linked to the saccharide moiety, which on HL-60 and MOLT-4 cells were more effective than those of the clinically applied anticancer agents: etoposide, adriamycin (ADM), and methotrexate. More detailed examination of the main saponin constituent (Figure 21.16) showed potent cytotoxic activities against various malignant tumor cells: (1) mouse mastocarcinoma, (2) human pulmonary adenocarcinoma, and (3) human pulmonary large cell carcinoma which includes adriamycin-resistant mouse leukemia (P388) and camptothecin resistant P388.

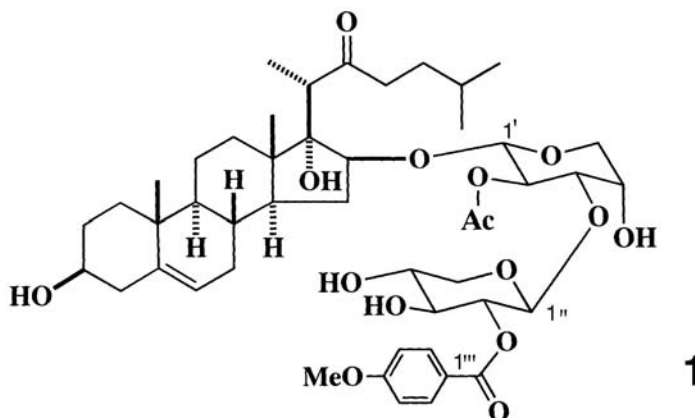


FIGURE 21.16

Structure of the strongly antitumor saponin isolated from *Ornithogalum saundersiae*. (From Tsurumi, S. and Wada, S., *Plant Cell Physiol.*, 36, 925, 1995. With permission.)

TABLE 21.8

Cytostatic Activities of **1** and Clinically Applied Anticancer Agents On Various Malignant Tumor Cells

Malignant Cells	IC ₅₀ (μg/ml) 1	MMC	ADM	CDDP	CPT	TAX
CCD-19Lu	1.5	2.0	2.0	10	2	2
P388	0.00013	0.01	0.003	0.05	0.005	0.01
P388/ADM	0.00077					
P388/CPT	0.00010					
FM3A	0.00016					
A-549	0.00068					
Lu-65	0.00020					
Lu-99	0.00020	0.01	0.002	0.001	0.001	0.002
RERF-LC-AI	0.00026					
CCRF-CEM	0.00016	0.02	0.01	0.005	0.005	0.001

Note: CCD-19Lu (human normal pulmonary cell adenocarcinoma)
 P388 (mouse leukemia carcinoma)
 P388/ADM (adriamycin-resistance P388 carcinoma)
 P388/CPT (camptothecin-resistant P388 squamous cell)
 FM3A (mouse mastrocarcinoma)
 CCRF-CEM (human leukemia)
 A-549 (human adenocarcinoma)
 Lu-65 (human pulmonary large cell)
 Lu-99 (human pulmonary large cell)
 RERF-LC-AI (human pulmonary carcinoma)

Source: Mimaki, Y., Kuroda, M., and Sashida, Y., in *Advances in Experimental Medicine and Biology*, 404, Plenum Press, New York, 1995. With permission.

The cytostatic activity of the compound shown in Figure 21.16 is 10 to 100 times more potent than those of the clinically applied anticancer agent (Table 21.8): (1) mitomycin C, (2) adriamycin, (3) cisplatin, (4) Camptothecin (CPT), and (5) taxol (TAX). This compound from *O. saundersiae* has little toxicity to normal human pulmonary cells. *In vivo* evaluation showed that it was remarkably effective for mouse P388 (increased life span: 59%) by a one-time administration of 0.01 mg/kg. Thus, this compound could be expected to be a new anticancer agent from a higher plant next to camptothecin and taxol.

21.9 Saponins Effect on Glucose Transport System (Diabetes)

Glucose is an important nutrient for many types of cells, such as the brain, which are entirely dependent upon this sugar as an energy source. Since glucose is a hydrophilic molecule, its uptake into cells across the hydrophobic core of the plasma needs some assistance. In mammals, where complex homeostatic mechanisms keep the blood glucose levels roughly constant, most cells contain a passive facilitated diffusion system for sugar uptake, called the glucose transporter. The structure consists of a protein of about 500 amino acid residues which are localized on the surface of the cell membranes in many tissues and organs. In other words, most cells contain passive facilitated diffusion systems for sugar uptake. The transporters which are responsible for facilitated diffusion of glucose of the mammalian tissues are collectively called the GLUT family. Active sodium-linked sugar transporters are found in certain locations, such as the kidney and small intestine, where transepithelial transport against a concentration gradient is required.

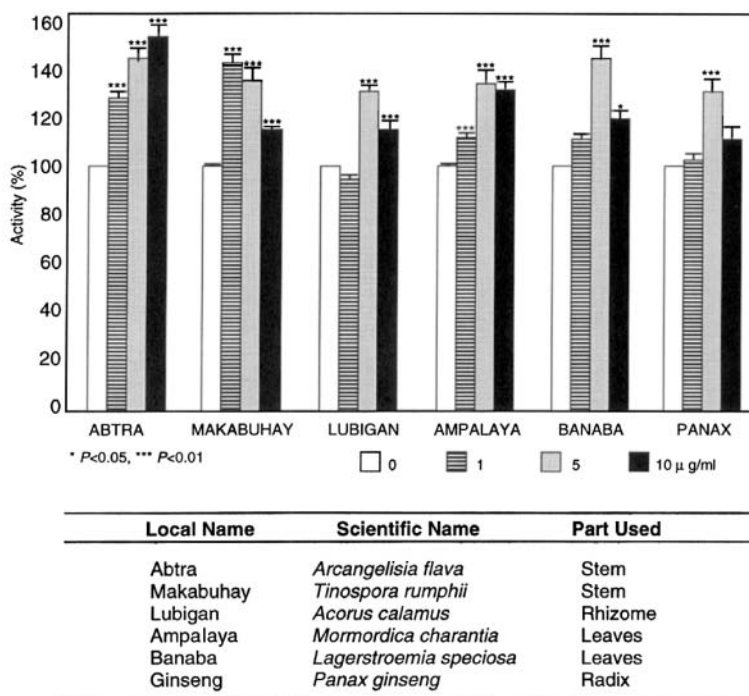


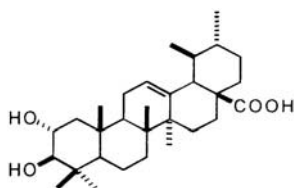
FIGURE 21.17

Effects of MeOH extracts from the medicinal plants on the uptake of 2-DG by EAT cells. (From Yamasaki, K., *Advances in Experimental Medicine and Biology*, 404, Plenum Press, New York, 1995. With permission.)

Modification of the activity of glucose transport would cause several physiological effects. A few compounds have been known to affect glucose transport activity; a forskolin, a diterpene isolated from an Indian plant *Coleus forskolii* (Labiatae), phloretin; a dihydrochalcone of the Rosaceae; and cytochalasin B, one of the mycotoxins. On the other hand, no other agent able to increase glucose transporter activity is known except insulin, a pancreatic hormone that regulates blood sugar level intrinsically.

Yamasaki⁴¹ used Ehrlich ascites tumor (EAT) cells and sheep erythrocytes to measure glucose transport activity. These EAT cells are known to contain a glucose transporter (very probably GLUT 1) and they can be easily obtained and used for an experimental system without the need for a complex procedure to separate cells, which might injure the cellular membranes. Glucose transport in EAT cells was measured by sheep erythrocytes using 2-deoxy-D-glucose (2-DG) which could not be metabolized further although it was transported and phosphorylated by the same process as D-glucose. Using the established bioassay system they proceeded to analyze 23 methanol extracts for medicinal plants selected for their antidiabetic properties on glucose transport activity. Six samples accelerated 2-DG uptake (Figure 21.17) and five samples reduced it significantly while the other one was inactive. No stimulating agent of glucose transport activity has been reported except insulin. Among the plants exhibiting a positive effect, *Lagerstroemia speciosa* and *Momordica charantia* were locally used as antidiabetic agents in the Philippines. The hypoglycemic effect of *Tinospora cordifolia* (syn. *T. rumphii*) had recently been reported. Preliminary results of the effect of ginseng extracts also have been reported.

These screening results led them into a study of the active principles of these glucose transport stimulating plants. The active compound found in leaves of *Lagerstroemia speciosa*



corosolic acid

FIGURE 21.18

Corosolic acid isolated from *Lagerstroemia speciosa* that showed a significant increase in 2-deoxy-D-glucose. (From Yamasaki, K., *Advances in Experimental Medicine and Biology*, 404, Plenum Press, New York, 1995. With permission.)

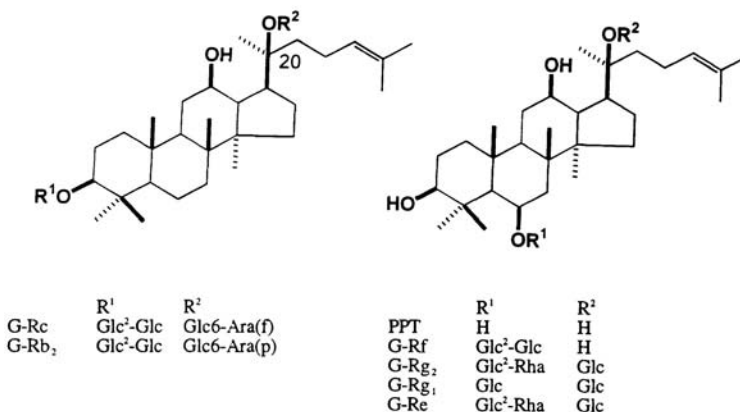


FIGURE 21.19

Ginseng saponins which induce glucose transport stimulatory effects. (From Yamasaki, K., *Advances in Experimental Medicine and Biology*, 404, Plenum Press, New York, 1995. With permission.)

(crepe myrtle) used for treatment of diabetes was found to be corosolic acid, the most active species tested and its structure is shown in Figure 21.18. It caused a 28% increase in 2-DG uptake at a concentration of 1 μ M.

Using sheep erythrocytes, the effect of MeOH on 2-DG was measured on extracts of white ginseng (*Panax ginseng*). *P. ginseng* showed potent stimulatory activity against 2-DG uptake. The stimulatory effect was significantly reached at 100 μ g/ml of the extract and gave an increase of 11% above basal activity. The extract was then fractionated into ethyl acetate-soluble and water-soluble fractions and the ginsenosides were characterized. The structure/activity relationship of ginsenosides were conducted and the effects on 2-DG transport at various concentrations (0.1 to 10 μ M) were measured. The chemical structures of examined agents and the results of measurement of 2-DG uptake by sheep erythrocytes are shown in Figure 21.19 and Table 21.9, respectively. Ginsenosides Rc, Rb₂, Rf, Rg₂, Rg₁, Re, and PPT are concluded to be the active components of ginseng extract which induce the glucose transport stimulatory effect and determines the structure/activity. A preliminary analysis of the structure/activity relationship indicates that small or no sugar moieties on the triterpenoid saponin show increasing 2-DG uptake, and protopanaxatriol (PPT) showed the highest activity.

TABLE 21.9

Effect of Ginseng Saponins and Related Compounds on 2-DG Uptake by Sheep Erythrocytes Against Control Value 100.0 ± 2.8

Compound	Concentration (μM)		
	0.1	1	10
G-Rc	106.0 ± 4.7	112.2 ± 4.6^b	117.3 ± 5.0^c
G-Rb ₂	108.9 ± 4.7	112.4 ± 6.6	117.0 ± 4.7^c
G-Rb ₁	103.1 ± 9.6	124.3 ± 5.3^c	101.5 ± 3.6
PPT	128.8 ± 8.4^c	121.1 ± 5.3^c	109.3 ± 8.6
G-Rf	106.1 ± 7.1	115.9 ± 5.8^b	111.9 ± 5.0^a
G-Rg ₂	118.3 ± 5.2^c	118.1 ± 5.2^c	113.1 ± 5.3^b
G-Rg ₁	98.2 ± 6.3	117.1 ± 6.0^b	110.6 ± 7.3
G-Re	100.1 ± 4.0	102.0 ± 4.3	116.7 ± 6.0^b

Note: Each value shows the mean of quadruplicate determinations from three separate experiments. PPT = protopanaxatriol

^a $p < 0.05$, ^b $p < 0.025$, ^c $p < 0.01$

Source: Yamasaki, K., in *Advances in Experimental Medicine and Biology*, 404, Plenum Press, New York, 1995. With permission.

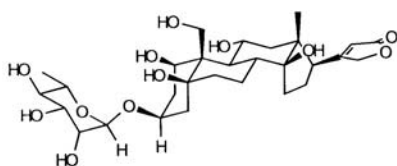
21.10 Cardiotonic Drugs

Nakanishi et al.⁴² isolated and characterized from the plants *Digitalis purpurea* and *Strophanthus gratus* several cardiotonic factors that have polyhydroxylated C₁₇-steroids with an α,β -unsaturated γ -lactone attached to C-17 (β) with various sugars attached to C-3. The cardiotonic effects of these cardenolides are shown through their inhibitory action against Na⁺/K⁺- in the presence of ATPase, which is the enzymatic expression of the cellular sodium pump. The requirements for bioactivity are

1. The C-3 and C-17 configurations must be β .
2. A/B ring junctures can be either *cis* or *trans*.
3. The C/D ring juncture must be *cis*.
4. All bioactive cardenolides identified so far have a 14 β -OH.
5. 3-Epidigitoxigenin is inactive.
6. Structure and number of sugars at C-3 are not restricted to monosaccharides.
7. C-19 can be a methyl group; CHO as in strophanthidin or CH₂OH as in ouabagenin.
8. C-11 can be α -OH as in ouabagenin.
9. One active cardenolide has an 11,12-epoxide.

Their experimental evidence suggests the presence of an endogenous ouabain-like factor which is involved in regulation of renal sodium excretion, and in the pathogenesis of the disease hypertension which has stimulated worldwide research for a number of years. The isolation from human plasma of a ouabain-like compound ("OLC") which is indistinguishable from ouabain, and the characterization of a ouabain *isomer* (i.e., not ouabain) from bovine hypothalamus which was called "HIF" (hypothalamic inhibitory factor), suggests that OLC and HIF are most possibly identical and that both are not ouabain (Figure 21.20).

Nakanishi's⁴² group, working with 31 μg of OLC from human plasma (300 liters) and antibodies, concluded that OLC was ouabain on the basis of their identical molecular formula,



Ouabain

(*Strophanthus gratus* and other plants)

Purification: affinity chromat. / HPLC

HIF (Hypothalamic Inhibitory Factor)
1 μ g from 5 Kg of bovine hypothalamus

OLC (Ouabain-Like Compound)
31 μ g from 300 Liters human plasma

Facts

- (i) **All three Ouabain, OLC and HIF have:**
identical molecular weight (ion-spray LC / MS)
identical retention time (HPLC)
identical sugar: α -L-Rhamnose
(glycosidic linkage/L-config. : naringinase, chiral GC/MS)
- (ii) **HIF exhibits different bioactivity from ouabain**

FIGURE 21.20

Comparisons of OLC and HIF from mammals and ouabain from plants. (From Nakanishi, K. et al., *Advances in Experimental Medicine and Biology*, 404, Plenum Press, New York, 1995. With permission.)

peracetate MS Profiles, HPLC retention times, and physiological assay. Except for the subtle difference in bioactivity, the chemical and physical data on ouabain and HIF were very similar, if not identical. The sugar moiety of HIF was shown to be α -L-rhamnose since there was a 100-fold loss of bioactivity of the entire molecule upon treatment with naringinase, an enzyme specific for this sugar and linkage. The sugar resulting from acid cleavage was from the L-series according to chiral GC/MS of the silyl derivative. Because of the extremely limited amount available, ca. 1 μ g at the most, a method based on exciton-coupled circular dichroism (CD) was used. The 2-naphthoates of sugars was known to proceed in high yield at the picomole level or less to give fluorescent derivatives with characteristic CD. Conditions were selected by Nakanishi's group to take 300 ng aliquots of ouabain so that pentanaphthoates would be formed maximally rather than mixtures of pentanaphthoates. Thus, 400 ng and 300 ng of ouabain and HIF, respectively, were naphthoylated and the reaction mixtures were analyzed by HPLC with fluorescence detection (Figure 21.21).

HPLC analysis showed that I (ouabain pentanaphthoate) and III (HIF pentanaphthoate) were clearly different (Figure 21.21). The circular dichroism (CD) was more dramatic for several reasons. When five naphthoate groups interact through space, one would expect the coupling would give rise to a bisignate CD, which is the case with ouabain. However, with HIF the couplings between the five naphthoates cancel out and yield a CD spectrum with no clearcut Cotton effect, which was unexpected. This canceling effect reveals a most unusual spatial arrangement of the naphthoates which is of immense diagnostic value for use in structural studies. Rhamnose 2',3',4'-trinaphthoate itself has an intense positive split CD and this large positive value has to be nullified by through-space coupling with two naphthoates on the aglycone skeleton. The same naphtholyation procedure was performed with 400 ng of the 1 μ g of OLC left at Upjohn. Here again, the pentanaphthoates of ouabain and OLC (II) did not co-elute on HPLC; moreover, OLC and HIF pentanaphthoates II and III not only co-eluted but the CD of OLC pentanaphthoate II also showed no Cotton effects. Although the sugar moiety in OLC has not been characterized, these results show that in all likelihood it is α -L-rhamnose.

What is the structure of the endogenous mammalian cardiogenic factor? The search for characterization of this challenging and important compound existing in exceptionally minuscule quantities continues. If the aglycone is the same as that of ouabain, HIF must have the rhamnose attached to any one of the hydroxyls other than 3-OH.

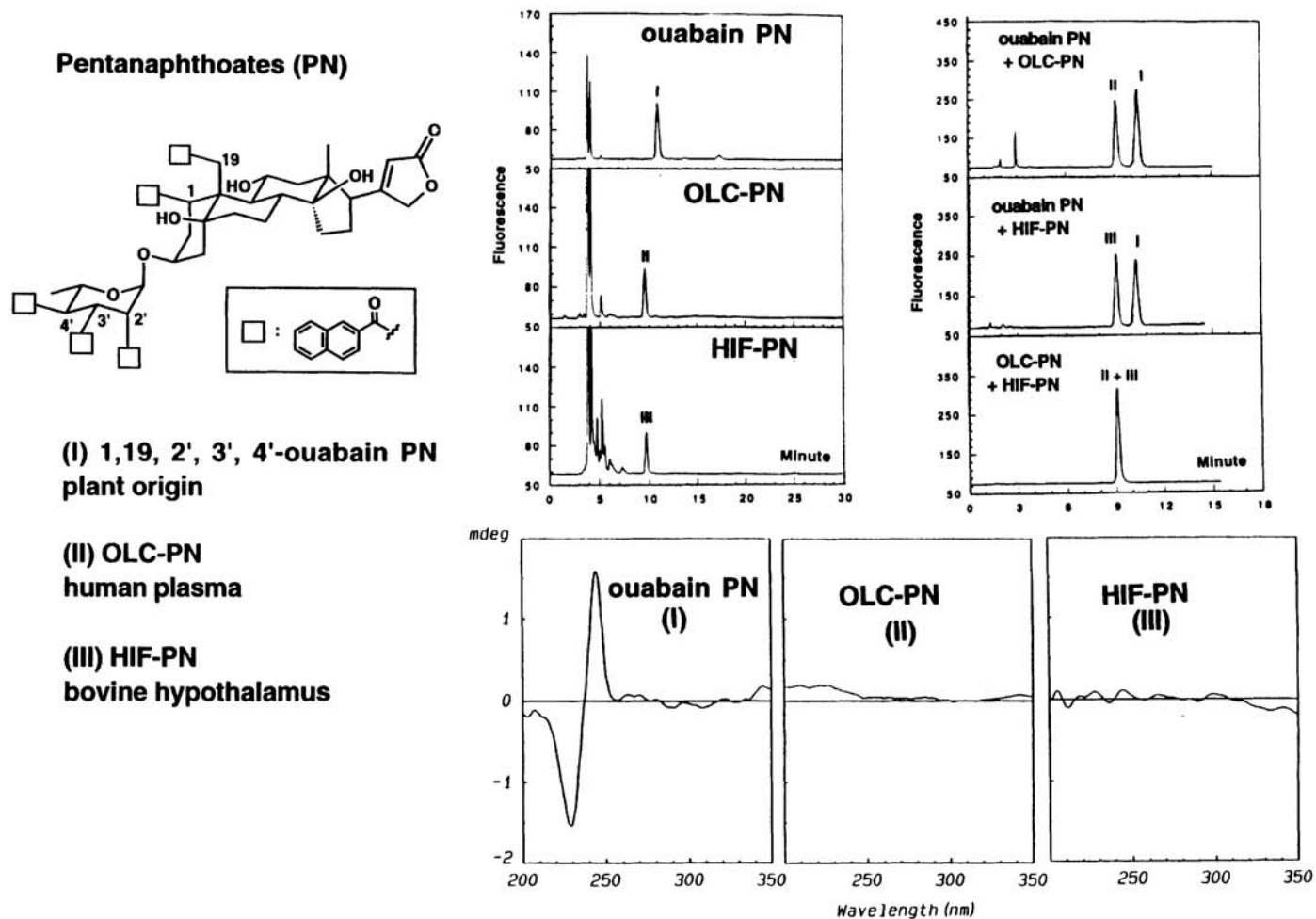


FIGURE 21.21
Fluorescence-detected HPLC profiles of ouabain, OLC, and HIF naphthoylation reaction mixture, and coinjected mixture of pentanaphthoates. (Bottom right) Circular dichromism of ouabain, OLC, and HIF pentanaphthoate in acetonitrile. (From Nakanishi, K. et al., *Advances in Experimental Medicine and Biology*, 404, Plenum Press, New York, 1995. With permission.)

21.11 Foods and Nutrition

21.11.1 Hypercholesterolemia

21.11.1.1 Alfalfa Saponins

There is abundant evidence that saponins interact with sterols in the gastrointestinal tract in a way which might prove beneficial to humans. Elevated plasma cholesterol is a significant risk factor in the etiology of cardiovascular disease. The hypocholesterolemic effect of some alfalfa saponins has been of clinical interest and has led some to propose that they might provide a useful means of dietary management of plasma cholesterol in man.^{4,5,43-46} This assumes that alfalfa seeds decrease plasma cholesterol level in the human body. Molgaard et al.⁴⁷ gave 40 g of heat-prepared alfalfa seeds three times daily at mealtimes for 8 weeks with an unchanged diet. Maximal decreases in 15 patients with hyperlipoproteinemia were 26% for cholesterol and 30% for low density lipoprotein. After the treatment ceased, all lipoprotein concentrations returned to pretreatment levels. When the saponins were extracted, alfalfa lost its hypocholesterolemic effect,⁴⁸ thus the conclusions of Molgaard et al.⁴⁷ were that the alfalfa seed saponins were the active component.

The effects of isolated, crude alfalfa-top saponins on cholesterol absorption in rats were investigated. Doses of noncrystalline saponins (5, 10, and 20 mg) were given to each animal by intragastric administration together with radioactive labeled cholesterol and the extent of cholesterol absorption was determined by analysis of plasma radioactivity and subsequent appearance of fecal sterols. A progressive inhibition of cholesterol absorption with increasing alfalfa saponins was observed.⁴⁵ Experimentally reduced hypercholesterolemia brought about by alfalfa saponins leads to changes in systemic cholesterol metabolism that beneficially alters the progress of cardiovascular disease.⁴⁶ Diets containing 1.0 to 1.2% alfalfa saponins or 40% alfalfa seeds reduced both plasma cholesterol and the concentration of cholesterol in aortic tissue and the liver without evidence of hemolysis or other toxic symptoms.⁴⁴ The possibility that such an effect may be of benefit to man is suggested by work with primates. A semipurified diet containing 1.0% isolated alfalfa-root saponins or 0.6% alfalfa-top saponins⁴⁸ fed to monkeys (*Macacos fascicularis*) led to reduced cholesterol absorption and reduced plasma response to a cholesterol-supplemented diet. Regression of aortic and coronary atherosclerosis in monkeys occurred when alfalfa saponins were in their diet.⁴³ Alfalfa saponins appear to be nontoxic at the levels necessary to produce a physiological response; however, the mechanism by which they interfere with cholesterol absorption has not been determined.^{4,46}

21.11.1.2 Other Saponin Sources

The accumulated evidence shows that saponins from dietary sources confer protection from experimentally induced hypercholesterolemia (e.g., with the inclusion of cholesterol and sodium cholate) in animal diets. There is no evidence to permit the setting of guidelines recommending the quantities of saponins in the diets of humans. However, Ridout et al.⁴⁹ provides some estimates of daily saponins in the United Kingdom in Table 21.10. The mean daily intake is only 14.6 mg, but in vegetarian and Asian households, it is 100 to 200 mg. These latter levels are comparable with the daily intakes that were experimentally effective in reducing plasma cholesterol concentrations.⁵⁰ Vegetarians are at least risk from cardiovascular disease.⁵⁰ From these data, daily intakes of 100 to 200 mg are likely to be effective and at the same time available. Saponins differ in their ability to interact with cholesterol and bile acids such as ginseng, alfalfa, *Yucca*, and *Quillaja* saponins. Obviously

TABLE 21.10

Mean Daily Intake of Saponins (mg Per Person) in Different Subpopulations in the U.K.

Population Studied	Dietary Intake (mg/d)	Main Sources
All U.K. households (7193)	14.6	Baked beans, lentils, peas
All U.K., omnivore (17)	13.3	Baked beans, lentils, chickpeas
All U.K., vegetarian (12)	109.9	Soya, baked beans, lentils
All U.K., children (22)	12.6	Baked beans, lentils, kidney beans
Male caucasian (2)	9.6	Baked beans, peas, soya
Male West Indian (11)	46.9	Kidney beans, baked beans, blackeye peas
Male Asian (15)	167.7	Kidney beans, guar beans, chickpeas
Male Asian vegetarian (10)	213.4	Kidney beans, guar beans, chickpeas

Source: Ridout, C.L. et al., *Food Sci. Nutri.*, 42F, 111, 1988. With permission.

saponins from different plant sources will differ in their ability to lower plasma cholesterol. Saponin toxicity is very low when administered to mammals.^{4,5} Thus, the saponins present naturally in foods would be relatively nontoxic. Saponin-containing foods, such as alfalfa, soyabean, and mungbean sprouts, could contribute to the cholesterol-lowering diets and increase the longevity of the individual's lifespan.

21.11.1.3 Cooked Chickpeas and Lentils

Ruiz et al.^{4,51} provided quantitative results using intact saponins from chickpeas and lentils on soaking and cooking. The fate of soyasaponin VI (the major saponin, a first publication showing the DDMP saponin) in the seed matrix was shown to be stable after soaking, but unstable after cooking (Table 21.11). The conversion of soyasaponin VI to soyasaponin I upon cooking and leaching of both saponins and subsequent reduction of the saponin level were found in the seed (Table 21.11). This suggests that the important biological and pharmacological properties ascribed to maltol (DDMP) combined with soyasaponin I (that yields soyasaponin VI^{52,53}) may be partially lost as a result of legume processing. The work of Ruiz et al.⁵¹ represents an important accomplishment in relating the relevant structural bioactivity function to using processed foods in human and animal health. (See Section 21.3, Allelopathy)

21.12 Agricultural Usage of Saponins

The total amount of saponins and their products of export–import trade occurring on a worldwide basis is not well known. However, a report given by Balandrin⁵⁴ showed that American ginseng root (*Panax quinquefolius*, L.) accounted for tens of million dollars in exports per annum in the 1980s. More than 60 million pounds of licorice root (*Glycyrrhiza glabra* L.) and 350,000 lbs of licorice extract were imported into the U.S. annually in the 1970s. Much of the licorice used is in the form of glycyrrhizin which is a medicine, sweetener, and flavor enhancer in cigarettes. The aglycone, glycyrrhetic acid, is used in cosmetics, the monoglucuronide of glycyrrhetic acid is around 940 times as sweet as sucrose, and it also serves as an inhibitor of carcinogenesis.⁵⁵⁻⁵⁷ Of interest is the 11-oxo-12,13-dehydro group of glycyrrhizin (Figure 21.22) which is responsible for the adrenocortico mimetic effects.⁵⁸

TABLE 21.11

Saponin Content^a of Chickpeas and Lentils Before and After Soaking Plus Cooking Treatment

Saponin Content (mg/kg of dry weight)								
Cultiver and Treatment	Seeds			Cooking Solution			% Saponin Leaching	Total Saponin Content of Seeds and Solution
	Soyasaponin I	Soyasaponin VI	Total	Soyasaponin I	Soyasaponin VI	Total		
<i>C. arielinum</i> pardon unprocessed								
30 min of cooking	Nd ^b	752 ± 14	752 ± 14					752 ± 14 ^a
60 min of cooking	407 ± 17	339 ± 13	746 ± 24	10 ± 1	5 ± 0	15 ± 1	2	763 ± 24 ^a
90 min of cooking	513 ± 18	227 ± 15	740 ± 30	18 ± 2	ND	18 ± 1	2	758 ± 30 ^a
120 min of cooking	585 ± 15	141 ± 4	726 ± 13	23 ± 2	ND	23 ± 2	3	749 ± 13 ^a
	640 ± 8	83 ± 7	723 ± 7	30 ± 2	ND	30 ± 2	4	753 ± 10 ^a
<i>L. culinaris</i> Magda 20 unprocessed	ND	703 ± 14	703 ± 14					703 ± 14
30 min of cooking	105 ± 11	319 ± 9	424 ± 10	27 ± 0	40 ± 6	67 ± 4	14	491 ± 13
60 min of cooking	176 ± 14	276 ± 7	452 ± 12	35 ± 1	29 ± 1	64 ± 0	12	516 ± 12
90 min of cooking	191 ± 10	228 ± 19	419 ± 29	44 ± 1	25 ± 1	69 ± 0	14	488 ± 30
120 min of cooking	233 ± 11	200 ± 13	433 ± 12	48 ± 0	22 ± 0	70 ± 0	14	503 ± 12

^a Values are mean of four determinations ± standard deviation. The same superscripts in the same column indicate no significant differences (family error rate = 0.05).

^b Not detectable.

Source: Ruiz, R.G. et al., *J. Agric. Food Chem.*, 44, 1526, 1996. With permission.

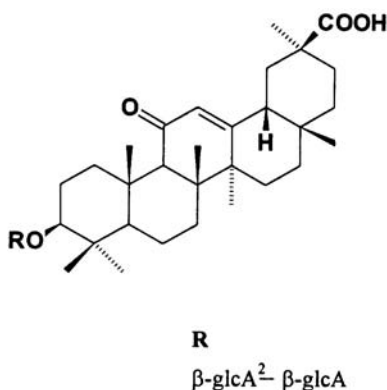


FIGURE 21.22

Glycyrrhizin from *Glycyrrhiza glabra* L. (From Kenelly, E.J. et al., *Advances in Experimental Medicine and Biology*, Plenum Press, New York, 1996. With permission.)

The *Yucca* and *Quillija* saponins are used in the U.S. in cola drinks, root beer, main foam agent for crushed ice, emulsifier, emollient, mining operations, etc. and in animal feed.^{59,60} Saponins are added to the diet of chickens and swine for ammonia control because of their effectiveness in blocking urease activity.^{59,60} Their use as food additives indicate that they are effective in promoting digestion of grasses by ruminants. *Medicago* saponins and solanaceous glycoalkaloids have been used to control numerous classes of insects.^{1,61} Glycoalkaloids are marginally toxic to humans at 1 to 2 mg/kg body weight, severely so at 4 to 5 mg/kg, and higher doses may be lethal.^{62,63} They are formed in tomatoes and potatoes, two of the most frequently used vegetables in the world. In South America in the Andes Mountains, they have to be processed to remove the glycoalkaloids from their diet before they are consumed by humans.⁶²

From Moldava (formerly part of the Soviet Union) it has been reported that commercial products, Moldstim[®] and Pavstim[®], are made from alcoholic extraction of seeds from hot peppers (*Capsicum annum* L.) and from digitalis leaves, (*Digitalis purpurea* L.), respectively.^{64,65} These products have been registered by Moldava's State Chemical Commission and put in large scale use in agriculture as plant growth regulators and to reduce the incidence of pathological diseases. Saponins are used as a means of controlling pathogenesis of root rots in cereal crops. Ecostem[®] is derived from tomatoes (*Lycopersicon esculentum* Mill; also registered in the former Soviet Union)^{64,65} and possesses significant antiviral activity *in vivo* and *in vitro*. Ecostem is almost as active as the interferons of a few years ago. Kintia⁶⁵⁻⁶⁷ reported on triterpenoid and steroid saponins that are used commercially for antifungus and antibacterial activity, antioxidative, contraceptive, hypercholesteremia, antitumor, and allelopathic activities as well as plant growth promoting activity. This report mentions approximately 150 steroid saponins type compounds from 23 plants (Table 21.12). The saponin-detoxifying enzymes have been studied in detail for plant pathogens of oat and tomato.⁶⁸ The possibility of the use of saponins that could be controlled by phytopathogenic fungi and then converted to nontoxic compounds has a promising future.

TABLE 21.12

Plant Sources Used for Isolating Steroidal Glycosides with their Saponins and Aglycones

Plant Species	Aglycone ^a	Saponins
1. <i>Agave americana</i> L. (Agavaceae)	Hegogenin	Agavosides
2. <i>Allium cepa</i> L. (Alliaceae)	Diosgenin	Alliumosides
3. <i>Allium narcissiflorum</i> Wills (Alliaceae)	Diosgenin	Alliumosides
4. <i>Asparagus officinalis</i> L. (Asparagaceae)	Sarsasapogenin	Asparagoides
5. <i>Atropa belladonna</i> L. (Solanaceae)	Diosgenin + Gitogenin	Atroposides
6. <i>Beshorneria yuccoides</i> L. (Liliaceae)	Tigogenin	Beshornosides
7. <i>Capsicum annuum</i> L. (Solanaceae)	Tigogenin + Gitogenin + Diosgenin	Capsicosides
8. <i>Datura stramonium</i> (Solanaceae)	Diosgenin	Daturosides
9. <i>Digitalis purpurea</i> L. (Scrophulariaceae)	Gitogenin	Purpureagitoside + F-gitonins
10. <i>Funkia ovata</i> Spr. (Liliaceae)	Diosgenin	Funkiosides
11. <i>Lilia henri</i> L. (Liliaceae)	Isonarthogenin	Lilioglycosides
12. <i>Lilium regale</i> (Liliaceae)	Isonarthogenin + Diosgenin	Lilioglycosides
13. <i>Lycopersicon esculentum</i> Mill. (Solanaceae)	Neotigogenin	Tomatosides
14. <i>Melilotus officinalis</i> (Leguminosae)	Diosgenin	Melilotosides
15. <i>Nicotiana rustica</i> (Solanaceae)	Tigogenin	Nicotianosides
16. <i>Nicotiana tabacum</i> L. (Solanaceae)	Tigogenin	Nicotianosides
17. <i>Petunia hybrida</i> L. (Solanaceae)	Tigogenin + Gitogenin	Petuniosides
18. <i>Polygonatum latifolium</i> (Liliaceae)	Diosgenin	Polygonatoside + Protopolygonatoside
19. <i>Solanum melongena</i> L. (Solanaceae)	Diosgenin + Tigogenin	Melongosides
20. <i>Solanum tuberosum</i> L. (Solanaceae)	Yamogenin + Diosgenin	Tuberosides
21. <i>Tribulus terrestris</i> L. (Zygophyllaceae)	Diosgenin	Tribulosides
22. <i>Trigonella foenum graecum</i> (Leguminosae)	Diosgenin	Trigonellosides
23. <i>Yucca filamentosa</i> L. (Liliaceae)	Sarsasapogenin	Yuccosides

^a After hydrolysis of saponins.Source: Kintia, P.K., *Zaschita Rasteniy* (Plant Protection), 3, 4, 1992. With permission.

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Phytochemicals: Implications for Long-Duration Space Missions

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22.1 Introduction

Phytochemicals are naturally occurring compounds produced by plants. These compounds are essential for normal growth and development and can be specific for a given plant species or cultivar. Phytochemicals also are essential components of human nutrition and include carbohydrates, lipids, proteins, fiber, and vitamins. Secondary phytochemicals, such as polyphenols and flavanoids, provide human nutritional and health benefits. Phytochemicals associated with flavor and aroma play a significant, yet currently unquantified, psychological role in human mental health. In addition to the phytochemicals contained within plants, releasing of volatile and soluble phytochemicals into the environment

occurs during growth and development. Examples of volatile phytochemicals include the fragrance of flowers and the aroma of freshly cut grass. Examples of soluble phytochemicals include allelopathic substances such as pyrethrins.

The objective of this paper is to discuss the role and implications of phytochemicals in the development of a biological life support system for long-duration space missions. Specifically, an overview of phytochemical products that affect atmospheric composition will be presented, and implications to crew health and safety will be discussed. The significance of biologically active organic materials supplied to a hydroponic nutrient delivery system for plant growth also will be described.

22.2 Bioregenerative Life Support Systems

NASA's Advanced Life Support (ALS) program is evaluating the use of plants to regenerate the atmosphere, purify water, and produce food during long-duration space missions such as a lunar or Mars base or a Mars transit vehicle. The use of plants and other biological methods (in contrast to chemical or physical methods) for recycling materials used to sustain a crew are referred to as bioregenerative life support systems (BLSS).¹

In a BLSS, plants will regenerate the atmosphere by converting carbon dioxide (CO₂) into oxygen (O₂) through the process of photosynthesis. During normal growth and development, plants produce biogenic volatile compounds. On Earth volatile phytochemicals are typically present in trace amounts and do not accumulate in the atmosphere. However, in the closed space habitat of a long-duration mission (1 to 10 years or longer), these compounds have the potential to accumulate in the environment. The Breadboard Project at John F. Kennedy Space Center (KSC) in Florida has been evaluating crop production in a closed environment for several years.² As a component of these tests, the production of volatile phytochemicals from candidate crops (i.e., soybean, wheat, potato, tomato, rice, and lettuce) has been monitored throughout growth and development. These experiments have identified approximately 100 different volatile phytochemicals that have the potential to accumulate in the atmosphere.³⁻⁵

Currently, the full range of biological activity of most of these volatile phytochemicals is not known. Based on current toxicological data and engineering models, the concentration of a volatile phytochemical required to induce a plant response (<0.1 $\mu\text{mol mol}^{-1}$) is typically several hundred times lower than the concentration considered likely to pose any direct risk to crew health (typically >1 mmol mol^{-1}). The experimental data suggests that volatile phytochemicals can be managed for a limited impact on a BLSS's ability to purify water, remove CO₂, generate O₂, and produce food. This research will help identify the essential role that these trace volatiles have in growth and development of the different plant species.

22.3 Volatile Phytochemicals in BLSS

22.3.1 Sources of Volatile Phytochemicals

Atmospheric monitoring of Space Shuttle missions has identified over 250 organic compounds in the cabin atmosphere of the Space Shuttle.⁶ Most of these compounds are found

in trace amounts and are well below spacecraft maximum allowable concentration (SMAC) limits for airborne contaminants.⁷ In addition, a number of unidentified compounds have been detected at or near the detection limit of GC/MS analysis.

One of the first long-duration human-rated bioregenerative testbed studies using higher plants was the BIOS-3 study conducted in 1977 by the Department of Biophysics, L.V. Kirenskii Institute of Physics, in Krasnoyorsk, Russia.^{11,12} This study documented volatile organic compounds (VOCs) in a human habitat in an atmosphere being regenerated by higher plants continuously for 4 months. The volatiles detected during BIOS-3 were primarily associated with manmade materials (e.g., solvents, glues) and not of biological origin. During the 4-month test, the “readily oxidizable” substances fluctuated around a low, consistent concentration of 7.8 mg O₂ equivalent m⁻³ and the “difficult to oxidize” compounds were at a concentration of 30.1 mg O₂ equivalent m⁻³.^{11,12} The BIOS-3 facility had no physical or chemical atmospheric regeneration system to maintain these concentrations, suggesting an interaction with the plants used for bioregenerative life support.¹² Incorporation of a catalytic furnace during a subsequent monitoring period reduced the concentration of carbon monoxide, but had limited effect on aldehydes, alcohols, and mercaptans.¹¹

A limited number of studies to monitor VOC production in closed plant chambers have been reported. Zlotopolsk’ii and Smolenskaja⁸ monitored volatiles in the atmosphere of closed chambers used for plant growth. They detected acetone, ethanol, methanol, toluene, acetaldehyde, ethylacetate, methylethylketone, and cyclohexane. Charron et al.⁹ reported on the production of hexenal, hexenol, and hexenyl-acetate from lettuce grown in a closed chamber. Wheeler et al.¹⁰ detailed the developmental production of ethylene by soybean, lettuce, wheat, and potato in the biomass production chamber (BPC) at KSC.

Recently, trace atmospheric components were monitored twice a week at NASA’s Johnson Space Center in conjunction with tests integrating BLSS and human test subjects.^{13,14} A number of compounds were reported in all samples at trace concentrations. The background compounds included solvents, Freon 113, siloxanes, and silenes. Tetrahydrofuran and ethylbenzene were detected as transients in the chamber. Several VOCs were detected; all of them were at concentrations <0.1 ppm with the exception of methanol and isopropyl alcohol. The only volatile phytochemical reported was ethylene which reached concentrations of 0.9 mg m⁻³ which is sufficient to affect growth and development of plants,^{15,16} but does not pose any health risk to the crew.⁷

22.3.2 Classification of Volatile Phytochemicals

Within a closed environment, VOCs can be classified based on whether the compounds are anthropogenic or biogenic.³ Anthropogenic compounds originate from manmade sources, such as construction materials, solvents, or physical/chemical processes. Biogenic compounds originate from biological systems including animals, plants, and microbes. This categorization is useful in distinguishing between volatiles that can be partially controlled through pretreatment (off-gassing of materials)¹⁷ and those expected to be produced by the plant or human subsystems. In addition to identifying the source of the volatiles, this classification provides a means of estimating the relative impact of the physical and biological systems on total atmospheric VOC load.¹⁸

Anthropogenic chemicals include substances such as refrigerants (e.g., Freon), plasticizers (e.g., siloxanes), and solvents (e.g., acetone). Other sources include by-products of physical/chemical life support technologies (e.g., catalytic conversion) and volatiles produced directly by the crew (e.g., food preparation). Biological systems also are sources of nonorganic compounds, such as ammonia and N₂O that result from the microbial breakdown of nitrogenous compounds.¹⁷

As a result of the biological and mechanical processes inherent with a long-duration space mission, the atmosphere will consist of several trace compounds, in addition to N₂, O₂, and CO₂. The composition of the atmosphere will change as a consequence of the activities associated with a particular stage of the mission. For example, the production of volatiles from plants is highly dependent upon stage of development. The aroma of ripening strawberries being but one example.^{15,16} In addition, hardware malfunctions can reduce the efficiency of the atmospheric regeneration or waste processing subsystems which may increase atmospheric complexity. Routine repair and maintenance operations, as well as unscheduled malfunctions of hardware, may result in periods with increased VOC loads being produced.

22.3.3 Guidelines for Volatile Phytochemicals in BLSS

To date, NASA has considered these trace compounds as atmospheric contaminants and has established guidelines for maximum concentrations in the atmosphere.¹⁷ The primary document establishing these guidelines are the spacecraft maximum allowable concentrations (SMACs).⁷ The SMACs are “those atmospheric concentrations that would be unlikely to cause discomfort, impairment, illness, or injury to crew members upon continuous exposure for a 7-day period.” The limits also serve as interim 30-day SMAC levels for extended Space Shuttle missions. The primary sources for SMAC values are the American Conference of Governmental Industrial Hygienist (ACGIH) and the U.S. Occupation Safety and Health Administration (OSHA).^{7,17} For compounds where insufficient information exists to establish a SMAC value, a default of 0.1 mg m⁻³ has been adopted.

Underlying this classification approach is a philosophy that all compounds, other than those specified for a given air mixture, should be regarded as contaminants and thus are undesirable.¹⁸ A deficiency in the approach of classifying all compounds, other than the primary life support gases as contaminants, is that many of these compounds may be essential to the effective and efficient functioning of a long-duration space mission. For example, ethylene is a naturally occurring volatile plant hormone that has been implicated in all aspects of plant growth.^{15,16}

A less well understood but perhaps equally significant role will be the interaction of volatiles and human psychological well being. It is well known that the presence of plant material has a calming effect on humans and that fragrance is an extremely powerful psychological trigger.¹⁹ It has been noted that human sense of smell is greatly diminished during spaceflight conditions, and that many of the “pleasurable” effects associated with certain activities such as eating are reduced.²⁰

A diversity of biological specimens (including plants) has been maintained on the Russian MIR space station during the past decade.²¹ Almost without exception, the cosmonauts report developing “attachments” to these specimens and provide a high level of technical support to these experiments (A. Mashinsky, personal communication). The theoretical and physiological underpinnings of these observations are not well understood, but are suggestive of a interaction between plant development and crew performance.

22.3.4 Interactions Between Volatile Phytochemicals and Microflora

A final area of interest is the maintenance of a stable microbial community within the facility. Because of the complexity, duration, and biological diversity of a long-duration space mission, it is simply not feasible to maintain a sterile environment.²² In fact, a case can be

made that sterility is not desirable.²³ However, there is always a concern that a pathogen could obtain a foothold and negatively influence performance of both a crew and life support system.²⁴ Several compounds that have been identified in the atmosphere of the BPC are known to have bactericidal and/or fungicidal activity, including benzaldehyde, hexenal, and nonanol.²⁵⁻²⁷ These compounds have been formulated for commercial use to preserve fruits and vegetables.²⁸

The concentrations observed in NASA's large-scale test-beds are significantly lower than those required for post-harvest control of diseases, but it is reasonable to assume that under conditions of closure, with minimum contamination pressure, the concentrations could become sufficient to prevent the uncontrolled growth of specific organisms.

22.3.5 Ethylene and BLSS

Ethylene (C₂H₄) is a volatile aliphatic hydrocarbon that is a natural product of plant metabolism.^{15,16} Ethylene is considered to be an essential plant hormone and has been the subject of intensive study. Several excellent reviews of the metabolism, physiology, and molecular biology of ethylene exist, and will not be covered here. It is well documented that ethylene is produced by healthy as well as diseased or senescent plants and interacts with physiological processes in numerous and complex ways. A cursory list of the plant growth and development processes involving ethylene include leaf abscission, fruit ripening, reduction in stem length, delay of flowering, inhibition of terminal shoot growth, inhibition of primary root growth, promotion of adventitious root growth, leaf epinasty, and gravitropism.^{15,16} Recently, ethylene has been implicated in the failure of wheat to flower onboard the Russian space station MIR (F. Salisbury, personal communication), and in poor growth of soybean in test canisters on the U.S. Space Shuttle. (C. Brown, personal communication).

To evaluate the impact of ethylene on a long-duration space mission, acceptable exposure limits for particular plant species need to be established. Current exposure limits established by NASA (294 $\mu\text{mol mol}^{-1}$)^{7,29} are clearly unsuitable for a plant-based bioregenerative system (Table 22.1). The highest published concentrations observed during Breadboard testing at KSC in the BPC was 0.34 $\mu\text{mol mol}^{-1}$ ¹⁰ although concentrations observed during wheat tests at JSC have exceeded 0.5 $\mu\text{mol mol}^{-1}$.¹³ Once limits are established, management systems can be designed into the mission. The management can be based on monitoring and control of C₂H₄ concentrations through physical (combustion), chemical (K permanganate traps), or horticultural (planting, harvesting schedules) means.^{1,10,29}

TABLE 22.1

Typical Concentrations ($\mu\text{mol mol}^{-1}$) of Ethylene Required to Induce Biological Response in Plant and Animal Systems

Biological Response	Exposure	$\mu\text{mol mol}^{-1}$
FID detection limit ¹⁰	NA	0.02
Plant threshold response ^{15,16}	Chronic	0.05
Plant threshold response ^{15,16}	Periodic	0.10
Plant ½ max response ^{15,16}	Periodic	1.00
Plant maximum response ^{15,16}	Periodic	10.00
Odor threshold, human ³⁰	Periodic	290.00
SMAC, human ^{7,20}	Chronic	294.00
Lethal response, mouse ³⁰	Periodic	950,000.00
Workplace limits ⁷	Chronic	No limit

TABLE 22.2

Relative Concentration ($\mu\text{g m}^{-3}$) of Biogenic Volatiles Detected in the Atmosphere of NASA's Biomass Production Chamber

Class	Compound ^z	Tomato	Soybean	Wheat	Rice	Lettuce	Potato
Alcohol	Butanol, 1-	—	—	<10	—	—	—
	Ethyl-1-hexanol, 2-	<10	—	<10	<10	<10	10–100
Aldehyde	Benzaldehyde	10–100	>100	10–100	<10	<10	<10
	Butanal	—	—	—	<10	<10	—
	Heptanal	>100	<10	<10	—	—	—
	Hexanal	>100	<10	<10	<10	—	—
	Nonanal	>100	<10	<10	<10	<10	—
Ester	Hexen-1-ol acetate, 2-	>100	<10	—	—	—	—
Ether	Ethylfuran, 2-	<10	10–100	—	10–100	—	—
	Furan	—	—	<10	—	<10	—
	Methylfuran, 2-	10–100	—	<10	—	<10	—
	Methylfuran, 3-	—	—	<10	—	<10	—
Hydrocarbon	Tetrahydrofuran	>100	10–100	—	—	—	—
	Ethylene	>100	10–100	10–100	10–100	10–100	10–100
Ketone	Butanone, 2-	—	>100	<10	—	<10	—
Sulfide	Carbon disulfide	<10	<10	<10	10–100	<10	<10
	Dimethyl sulfide	—	—	—	10–100	<10	<10
	Tetramethylthiorea	—	—	<10	—	—	<10
	Thiobismethane	—	—	<10	10–100	<10	<10
Terpene	Isoprene	10–100	—	<10	<10	<10	—
	Limonene	10–100	<10	—	<10	—	—
	Ocimene	<10	<10	—	—	—	—
	Pinene, α -	>100	10–100	—	<10	<10	—
	Pinene, β -	10–100	10–100	—	—	<10	—
	Terpinene, α -	>100	<10	—	—	—	—
	Terpinene, γ -	10–100	<10	—	—	—	—
Urea	Tetramethylurea	—	—	<10	—	<10	10–100

Note: The concentrations $\mu\text{g m}^{-3}$ are the average concentration of times detected. Only compounds identified in three or more samplings are included in the table.

22.3.6 Identification of Volatile Phytochemicals

In addition to ethylene, plants produce several VOCs which may accumulate in the atmosphere (Table 22.2). Unlike ethylene, the biological activity of most biogenic volatile compounds has not been the subject of extensive investigation. Although the production of volatile compounds is well documented in the literature,^{26,31,32} their biological roles are not typically defined. Some progress is being made regarding VOCs released from plant foliage in response to insect feeding, which then serve as chemical cues to attract natural predators of the insects.^{33–35} These results clearly indicate that there is a connection between biogenic VOC production and insect behavior.

One of the most common biogenic volatiles is isoprene, a basic chemical structure from which a number of terpenes are derived. The production of isoprene is well documented,^{31,32} and is known to react with the atmosphere. The production of isoprene has been correlated to temperature and is suggested to provide tolerance to high temperature.³⁶ Isoprene demonstrates bacteriocidal and fungicidal functions in bioassays.^{26,28} However, the role of isoprene in growth and development of plants is not known. Larger terpenes (e.g., limonene) are often present in the atmosphere and appear to be associated with specific developmental events (e.g., flowering).^{32,37–39} The list of compounds in Table 22.2 is by

TABLE 22.3

Effect of Removal of Abscised Leaves from the BPC
on Atmospheric Concentration of Biogenic
Volatile Phytochemicals

	Before (-8 Days)	After (+3 Days)
Day after planting	57	69
Biogenic VOCs ($\mu\text{g m}^{-3}$)	434	2700
Ethylene ($\mu\text{g m}^{-3}$)	11.5	9.8

no means exhaustive, but serves to indicate that several different classes of compounds are produced and that this production is dependent upon a given species.

22.3.7 Horticultural Practices and Volatile Phytochemical Production

In addition to normal development, horticultural practices can alter concentrations of biogenic compounds in the atmosphere. In one example, the total concentration of biogenic compounds increased six-fold following removal of abscised leaves from the chamber (Table 22.3). This routine horticultural operation involved the collection of senescent leaves which resulted in significant disruption of cellular structure and apparent release of volatiles into the atmosphere. Constituent analysis indicated that the increase was associated primarily with terpene production. The effect was not associated with natural senescence processes as suggested by the lack of change in ethylene concentration in the BPC.

22.3.8 Control of Volatile Phytochemicals

Because of the potential impact of volatiles on crew health and performance during a long-duration space mission, various control system have been applied. One approach has been the use of plants to remove formaldehyde from the atmosphere.⁴⁰ Subsequent work has indicated that the detoxification is associated with the passive removal of reactive compounds on the soil matrix and subsequent degradation by the resident microbial community (Darlington and Dixon, personal communication).

Passive filtering through activated carbon is a method to reduce the VOC load.^{8,33,41} This approach is effective at reducing concentrations of medium-to-high molecular weight reactive compounds such as siloxanes, but is ineffective at reducing concentrations of low molecular weight hydrocarbons such as ethylene.^{15,16,41} Results at KSC indicated that concentrations of total VOCs could be reduced from 25 to 50% with the use of activated carbon filters.^{4,41} A limitation of the approach is a requirement for replacement, recharge, and removal of the filters. An analysis of the filtering system indicated that the charcoal was most effective at reducing the concentration of siloxanes and sulfides, and much less effective on furans and chlorinated hydrocarbons.^{4,41}

Atmospheric filtering of the BPC at KSC generally has not had a significant effect on final crop yield. A possible explanation is that the effects of volatiles on plant growth, development, and morphology have resulted in environmental changes that would mitigate a yield effect. For example, elongation of internodes of wheat will result in a higher light level at the top of the canopy in a closed chamber, thus increasing photosynthetic rate. As a result, delays in anthesis associated with filtering will be compensated for by an increased rate of carbon assimilation in the plants.

22.4 Soluble Phytochemicals and Bioregenerative Life Support Systems

In a BLSS, water and nutrients will have to be supplied to plants to maintain life support functions. Because delivery costs associated with long-duration space missions are high, much effort has focused on the use of recirculating nutrient film techniques for hydroponic nutrient delivery systems.^{8,42,43} Hydroponic systems are a reliable and effective means of providing both nutrients and water to plants.

As with biogenic volatiles, there has been little attention given to the effects of soluble organics in the hydroponic nutrient solution as would affect BLSS performance. Marschner⁴⁴ suggests that low molecular weight organic solutes (e.g., organic acids) may contribute to anaerobic conditions around the root zone by providing substrates for microbial respiration, but do not affect plant growth *per se*. It is further suggested that by maintaining adequate O₂ concentrations in the rhizosphere will prevent adverse effects on plant growth. Mackowiak et al.^{45,46} found that microbial degradation of soluble organics from tissue leachate eliminated a growth inhibition associated with untreated materials. This observation indicates that biologically active organics are in the leachate solution.

22.4.1 Sources of Soluble Phytochemicals in the Hydroponic Nutrient Delivery System

The physical hardware used to support a hydroponic nutrient delivery system is a potential source of organic compounds. Potential sources of contamination include pipes, pumps, valves, and trays that deliver and contain the nutrient solution.^{22,42} However, the concentration of solubilized volatile organic compounds in a hydroponic nutrient delivery systems is low (B. Peterson, personal communication), suggesting minimal contamination.

In addition to hardware supporting plant growth, there are other potential sources of soluble organics that can be introduced into the hydroponic nutrient delivery system including root exudates, microbial exudates,⁴⁷ and root debris.^{48,49} Less obvious sources are organics released into the solution from leaching processes used to recycle nutrients from inedible biomass back to the hydroponic solution.⁵⁰⁻⁵³

To reduce resupply requirements, it will be desirable to recycle inorganic nutrients from inedible plant material (roots, shoots, stems).⁴⁶ Several technologies for recovery and recycling nutrients including ashing, leaching, aerobic bioreactors, anaerobic bioreactors, and composters, have been proposed¹ and tested.^{41,54,55} Each of these processes results in the extraction of organic compounds that are subsequently oxidized. However, the oxidation is not complete^{41,54-56} and with recirculation and continuous replenishment, recalcitrant organics may accumulate in the nutrient delivery solutions over time.^{45,46}

22.4.2 Identification of Soluble Phytochemicals in the Hydroponic Nutrient Delivery System

Although extensive data exists on the effects of cropping systems on the concentration of inorganic compounds in the recirculating nutrient solution, there is limited information on the exact composition of organic compounds that occur in the solution. In general, total

TABLE 22.4

Summary of Long-Duration Tests Conducted by NASA to Evaluate the Addition of Recycled Nutrients to a Recirculating Hydroponic Nutrient Delivery System

Crop	Cultivar	Duration	Location	Test Bed	Notes
Wheat	Veery 10	84 days	KSC	BPC	Batch
Wheat	Apogee	120 days	JSC	LSSSIF	Staggered batch
Potato	Norland	105 days	KSC	BPC	Batch
Potato	Norland	418 days	KSC	BPC	Staggered batch
Lettuce	Waldmann's Green	30 days	KSC	BPC	Batch

organic carbon (TOC) remains at a level $<50 \text{ mg l}^{-1}$ because microbial action rapidly degrades most compounds.^{45,56} The TOC, however, increases with time and recalcitrant materials accumulate in the nutrient solution. The recalcitrant compounds impart a dark pigmentation to the solution suggestive of tannic or humic materials.

In certain studies, HPLC analysis of the nutrient solution containing recycled nutrients did not detect chlorogenic, caffeic, or benzoic acids,^{51,57} phytotoxic phenols commonly detected in leaf tissue.^{26,58} This suggests that soluble phenolic compounds extracted from leaf tissue are either oxidized or polymerized during nutrient recovery processing. Based on UV analysis, the concentration of soluble UV-absorbing compounds increased in the nutrient solutions during certain experiments.^{45,46,59}

22.4.3 Effects of Soluble Phytochemicals on Plant Growth

Seedling bioassays using leached material have consistently indicated that growth of wheat roots is reduced.⁵⁹ The reduction in root growth does not occur if the leachate is allowed to "age" for 72 h prior to initiating the bioassay. This suggests that labile phytotoxic compounds in the leachate are readily oxidized and can be managed in a BLSS. Aerobic bioreactors have been developed to recover nutrients from inedible biomass and remove readily volatile carbon materials.^{42,55} The effluent from these reactors has been used to grow several crops in several long-duration tests of BLSS systems without negative yield effects (Table 22.4).

In another study, when potatoes were grown in a staggered batch production system using a recirculating nutrient solution, a buildup of a stable compound(s) resulted in early induction of tubers on Norland potato.⁴⁹ The presence of this compound resulted in the tuber initiation occurring approximately 1 week earlier than when grown in fresh nutrient solution.⁶⁰ Associated with this induction was significant reduction in shoot length (Figure 22.1). This reallocation of carbon resulted in small plants but higher harvest indices of production. The compound could be removed from the nutrient solution using activated carbon filters. However, filtering control solutions resulted in a delay in tuber formation.⁶⁰ The presence of this naturally occurring biogenic compound provides an opportunity to manage potatoes in a closed environment to achieve greater efficiency of production.

However, the presence of this compound requires active management of the plant in order to optimize canopy development. As a consequence, it will be necessary to develop appropriate monitoring and control mechanisms for biologically active compounds in the nutrient solution. As with the VOCs, it appears that current exposure and concentration guidelines used by NASA that are based on human health considerations are not appropriate for plant systems.



FIGURE 22.1

Effect of tuber-inducing factor on growth of potatoes in NFT production under a staggered batch planting (21-day harvest cycles) management system. (DAP = days after planting.)

22.5 Conclusions

- Phytochemicals are inherent components of a bioregenerative life support system utilized on long-duration space mission.
- Chronic exposure of plants to low concentrations of bioactive volatile compounds alter growth, development, and morphology of plants being considered for ALS applications. Atmospheric concentrations limits for volatile phytochemicals need to be established that do not result in reductions in productivity.
- Biologically active organic compounds accumulate in the nutrient solution of hydroponically grown plants. There is little information on the composition of these compounds from different crops. These compounds need to be identified and concentration limits for soluble phytochemicals need to be controlled to a concentration that will not result in reductions in productivity.
- Research on biological activity of naturally occurring production of phytochemicals in a bioregenerative life support system is needed to determine what impacts they have on plant productivity and to develop effective means of managing these compounds on long-duration space missions.

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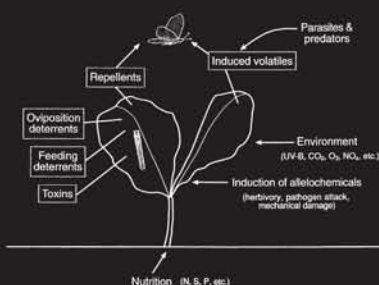
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BIOLOGICALLY ACTIVE NATURAL PRODUCTS: Agrochemicals

A Companion Book to *Biologically Active
Natural Products: Pharmaceuticals*



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