

ACS SYMPOSIUM SERIES **325**

# **Ecology and Metabolism of Plant Lipids**

**Glenn Fuller, EDITOR**  
*U.S. Department of Agriculture*

**W. David Nes, EDITOR**  
*U.S. Department of Agriculture*

Developed from a symposium sponsored by  
the Division of Agricultural and Food Chemistry  
at the 189th Meeting  
of the American Chemical Society,  
Miami Beach, Florida,  
April 28–May 3, 1985



American Chemical Society, Washington, DC 1987



### Library of Congress Cataloging-in-Publication Data

Ecology and metabolism of plant lipids.  
(ACS symposium series, ISSN 0097-6156; 325)

"Developed from a symposium sponsored by the Division of Environmental and Food Chemistry at the 189th Meeting of the American Chemical Society, Miami Beach, Florida, April 28-May 3, 1985."

Includes bibliographies and indexes.

1. Plant lipids—Congresses. 2. Plant lipids—Metabolism—Congresses. 3. Botany—Ecology—Congresses.

I. Fuller, Glenn, 1929- . II. Nes, W. David, 1953- . III. American Chemical Society. Division of Agricultural and Food Chemistry. IV. American Chemical Society. V. Series.

QK898.L56E26 1987 581.19'247 86-26559  
ISBN 0-8412-1006-3

Copyright © 1987

American Chemical Society

All Rights Reserved. The appearance of the code at the bottom of the first page of each chapter in this volume indicates the copyright owner's consent that reprographic copies of the chapter may be made for personal or internal use or for the personal or internal use of specific clients. This consent is given on the condition, however, that the copier pay the stated per copy fee through the Copyright Clearance Center, Inc., 27 Congress Street, Salem, MA 01970, for copying beyond that permitted by Sections 107 or 108 of the U.S. Copyright Law. This consent does not extend to copying or transmission by any means—graphic or electronic—for any other purpose, such as for general distribution, for advertising or promotional purposes, for creating a new collective work, for resale, or for information storage and retrieval systems. The copying fee for each chapter is indicated in the code at the bottom of the first page of the chapter.

The citation of trade names and/or names of manufacturers in this publication is not to be construed as an endorsement or as approval by ACS of the commercial products or services referenced herein; nor should the mere reference herein to any drawing, specification, chemical process, or other data be regarded as a license or as a conveyance of any right or permission, to the holder, reader, or any other person or corporation, to manufacture, reproduce, use, or sell any patented invention or copyrighted work that may in any way be related thereto. Registered names, trademarks, etc., used in this publication, even without specific indication thereof, are not to be considered unprotected by law.

PRINTED IN THE UNITED STATES OF AMERICA

**American Chemical Society**  
**Library**

**1155 16th St., N.W.**

**Washington, D.C. 20036**

In Ecology and Metabolism of Plant Lipids. Fuller, G., et al.;  
ACS Symposium Series; American Chemical Society: Washington, DC, 1987.

# ACS Symposium Series

**M. Joan Comstock, *Series Editor***

## *Advisory Board*

**Harvey W. Blanch**  
University of California—Berkeley

**Alan Elzerman**  
Clemson University

**John W. Finley**  
Nabisco Brands, Inc.

**Marye Anne Fox**  
The University of Texas—Austin

**Martin L. Gorbaty**  
Exxon Research and Engineering Co.

**Roland F. Hirsch**  
U.S. Department of Energy

**Rudolph J. Marcus**  
Consultant, Computers &  
Chemistry Research

**Vincent D. McGinniss**  
Battelle Columbus Laboratories

**Donald E. Moreland**  
USDA, Agricultural Research Service

**W. H. Norton**  
J. T. Baker Chemical Company

**James C. Randall**  
Exxon Chemical Company

**W. D. Shults**  
Oak Ridge National Laboratory

**Geoffrey K. Smith**  
Rohm & Haas Co.

**Charles S. Tuesday**  
General Motors Research Laboratory

**Douglas B. Walters**  
National Institute of  
Environmental Health

**C. Grant Willson**  
IBM Research Department

## Foreword

The ACS SYMPOSIUM SERIES was founded in 1974 to provide a medium for publishing symposia quickly in book form. The format of the Series parallels that of the continuing ADVANCES IN CHEMISTRY SERIES except that, in order to save time, the papers are not typeset but are reproduced as they are submitted by the authors in camera-ready form. Papers are reviewed under the supervision of the Editors with the assistance of the Series Advisory Board and are selected to maintain the integrity of the symposia; however, verbatim reproductions of previously published papers are not accepted. Both reviews and reports of research are acceptable, because symposia may embrace both types of presentation.

# Preface

PLANTS PROVIDE APPROXIMATELY 90% of human caloric intake and a major part of protein in the diet. They are important as the ultimate source of nutrition for animal species because they have the unique capability of synthesizing proteins, carbohydrates, and fats from carbon dioxide, water, and inorganic chemicals by using sunlight as an energy source. Despite the importance of plants, knowledge of plant biochemistry has lagged behind that of mammalian biochemistry, although a renewed interest in photosynthetic processes and chemistry of plant enzymes is overcoming that lag. The study of plant lipids has also accelerated with increased understanding of the importance of plant lipids in cell regulatory functions. Now, investigators are establishing the importance of lipid interactions among different species as well as in the species themselves.

The symposium from which this book was developed was designed to review and present current research on the biosynthesis and metabolism of lipids in plants together with the chemistry and biochemistry of lipid interactions; however, some presentations reported input from disciplines other than chemistry. This 22-chapter volume presents a loose classification of the papers presented at the symposium into four sections: (1) Introduction, (2) Plant Lipid Metabolism and Plant-Plant Interactions, (3) Plant-Insect and Plant-Nematode Interactions, and (4) Plant-Microbial Interactions.

We still have much to learn about the lipid metabolism and ecology of higher plants. Especially intriguing is research concerning the genetics, mechanisms, and substrates involved in the formation of multiple double bonds in fatty acids. Results of research may allow us to tailor the triglyceride composition of vegetable oils. Further research on lipid interactions of plants with other organisms can show us how to enhance the resistance of plants to disease and insect infestation or to improve symbiotic relationships. Regulation of isopentenoid pathways may affect the control of vegetative and reproductive processes of both plants and the pathogens that attack them and may enhance the ability of crops to produce chemical raw materials. We hope that this volume will stimulate more interdisciplinary research that will help us understand and modify the reactions of plant lipids.

GLENN FULLER  
W. DAVID NES  
Western Regional Research Center  
Agricultural Research Service  
U.S. Department of Agriculture  
Albany, CA 94710

September 24, 1986

# Chapter 1

## Plant Lipids and Their Interactions

Glenn Fuller and W. David Nes

Western Regional Research Center, Agricultural Research Service, U.S. Department of  
Agriculture, Albany, CA 94710

Lipids are distinguished by their high solubility in non-polar organic solvents and their low solubility in water. Lipids can be classified as derivatives of fatty acids or other compounds not containing a fatty acid moiety. Quantitatively, the acyl glycerides, polar and non-polar, make up the bulk of plant lipids. These include the neutral triglycerides, found in seed storage lipids, and the polar acyl glycerides such as the phosphatides, glycolipids and sulfolipids. There are a variety of non-glyceride lipids which embrace waxes, sterols, terpenes and their derivatives, hydrocarbons, and even some phenolics. Many organisms do not synthesize all their required lipids *de novo*, but obtain them from other species. Some plants are able to synthesize lipids which modify behavior in and help to protect against pests and pathogenic organisms. Hence, a variety of interactions have evolved which are the subject matter of this volume.

The symposium leading to this book was designed to bring together scientists working in the field of interactions between various species based on lipid biochemistry. This area of research is important to agriculture because it can lead to biological control of beneficial or deleterious species. Many organisms have evolved requiring lipid nutrients from other organisms, e.g., certain insects do not synthesize cholesterol *de novo*, but they can either use plant steroids without modification or convert these steroids to cholesterol (Svoboda et al., Chap. 11). Other species have evolved protective compounds. Harborne (8) has classified the plant protective compounds as prohibitins, inhibitins (pre-infectious), post-inhibitins, and phytoalexins (post-infectious). A high proportion of these compounds are lipids, often functioning by changing membrane permeability in the invading species. Although there is overlap, the chapters of the book have been loosely grouped to cover general lipid metabolism and function, plant-plant

This chapter not subject to U.S. copyright.  
Published 1987, American Chemical Society

interactions, plant-insect interactions and plant-nematode interactions, and effects of microbes and plants on one another. Since the book is a symposium volume, there is heavy emphasis on plant-microbial relationships and somewhat less coverage of other areas. However, it is representative of current research in the field.

Lipids are one of the four major categories of compounds which are involved with growth and reproduction of crop plants and their pathogens, the others being carbohydrates, proteins and nucleic acids. Bioregulatory processes of lipids have been largely ignored. Acyl lipids and sterols were assumed to play a nonmetabolic role in the maintenance of cell membrane physico-chemical properties, while triglycerides were important in energy reserve and isopentenoid hormones influenced reproduction. However, later chapters of this volume show that lipids may have acted as ecological determinants of plant interactions with other organisms. Sterols have multiple non-metabolic roles during the life history of fungi and yeasts (W. R. Nes, Chapter 16), each of which is familially and temporarily expressed. In nature their influence is dependent on availability of sterols from the host plant in which structure and quantity of sterols are important to the host-pathogen relationship. Fatty acids or acyl lipids play physiological roles which are similarly related to their structures and their compartmentalization within the cell (Mudd, Chapter 2; Fuller and Stumpf, Chapter 4 ). The aim of this monograph is to attempt to cover for the first time this diverse area of research on lipid interactions. We have emphasized the sterol and fatty acid fields and plant interactions, rather than those of mammals since this reflects the editors' interest in these subjects. In addition, a few chapters are devoted to structure-occurrence and structure-biosynthesis of lipids since physiology is the basis for the interactions described.

Lipids are distinguished from other classes of biologically important compounds by the fact that they contain large non-polar moieties, which make them poorly soluble in aqueous media but soluble in organic solvents such as chloroform, alcohol, hexane or mixtures of these solvents. This characteristic enables one to extract lipids from fresh tissue; mixtures of chloroform - methanol or hexane - isopropanol are the most commonly used solvent systems. After extraction, the lipids may be separated by their chemical properties. The techniques of liquid chromatography, gas chromatography and thin-layer chromatography have been especially useful in separating classes of lipids for analytical purposes. It is now generally accepted that two major lipid biosynthetic pathways exist - the so-called isopentenoid and fatty acid pathways. While the two pathways have been assumed to be biochemically independent of one another, carbon-flow via the mevalonic acid shunt into the fatty acid pathway has been demonstrated in a crop plant and insect (2, 3). Because some lipids are very hydrophillic and remain in aqueous media, we sometimes group lipids according to their biosynthetic rather than chemical relationships.

Lipid Classes

Fatty acids and their derivatives. Fatty acids are characterized by the presence of a carboxylic acid function attached to a hydrocarbon chain. Because the biosynthesis of fatty acids involves the combination of a series of two-carbon fragments, the common fatty acids are unbranched chains with even numbers of carbon atoms. Many are saturated, but biochemical interest centers principally around the unsaturated fatty acids containing up to five double bonds. Common unsaturated acids have their double bonds in the cis-configuration rather than the thermodynamically more stable trans form. Multiple double bonds are usually methylene-interrupted, rather than conjugated. Table I indicates the common names and structures of some of the principal fatty acids found in plant and animal lipids. Though acyl glycerides of the relatively few acids listed make up the bulk of lipids in living organisms, small amounts of many other acids of unusual structure are found in nature. These include branched acids, long chain ( $>C_{18}$ ) saturated and unsaturated acids, ones with very high degrees of polyunsaturation (found in marine oils), epoxy and hydroxy fatty acids and acids with cyclopropane moieties in the chain. The chemistry and biochemistry of the fatty acids and their derived lipids have been reviewed by Gurr and James (4).

Table I. Major Fatty Acids in Plants and Animals

<u>Common Name</u>	<u>Chemical Structure</u>
<u>Saturated Acids</u>	
Lauric Acid	$CH_3(CH_2)_{10}COOH$
Myristic Acid	$CH_3(CH_2)_{12}COOH$
Palmitic Acid	$CH_3(CH_2)_{14}COOH$
Stearic Acid	$CH_3(CH_2)_{16}COOH$
<u>Unsaturated Acids</u>	
Oleic Acid	$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$ <u>cis</u>
Linoleic Acid	$CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_7COOH$ <u>cis</u> <u>cis</u>
$\alpha$ -Linolenic Acid	$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_7COOH$ <u>cis</u> <u>cis</u> <u>cis</u>



### Fatty Acid Derived Lipids

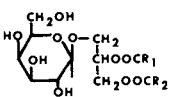
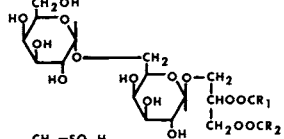
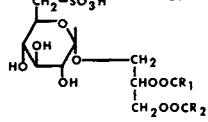
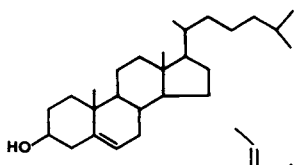
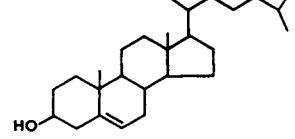
The fatty acids occur most commonly in nature as acyl glycerides (Table II). Triglycerides are the predominant neutral lipids in most living organisms. Triglycerides are the storage lipids in animal fat and in plant seeds, and because of their physical properties as well as high energy content, they are components of many food products. They are also the raw materials for making soaps and other surface active compounds. Animal triglycerides are made up of significant amounts of saturated fatty acids and thus tend to be solid at ambient temperature, while vegetable oils are usually liquids and have either shorter chain saturated acids or acids with higher polyunsaturation. Waxes have similar physical properties to those of triglycerides, but they occur as saturated acids esterified to long chain monohydric alcohols rather than to glycerol, and as minor components, long chain alcohols and alkanes.

Lipids with high levels of polyunsaturated fatty acids are considered desirable by many nutritionists because these lipids help to maintain low levels of blood cholesterol and favorable levels of serum high density lipoproteins. Many efforts are now directed at modification of the fatty acid composition in plants, especially the composition of seed oils (5). Genetic improvement of soybean oil is an especially desirable goal since the small amount of  $\alpha$ -linolenic acid present in the oil causes flavor instability.

Polar Lipids. The polar lipids (Table II) are extremely important to the life processes of living organisms, since glycerophospholipids are principal components of membranes. These membranes are for the most part lipid bilayers in which the nonpolar hydrocarbon tails point toward one another and the polar groups are on the outside, interacting with the aqueous phases inside and outside the region enclosed by the membrane. Various glycolipids, sterols, proteins, lipopolysaccharides and other compounds are also incorporated in the membranes and influence their selective properties. A significant proportion of cell enzymes are membrane-bound and hence are difficult to isolate and characterize. The biosynthesis and role of phospholipids has been reviewed by Mudd (6).

Although galactolipids (Table II) are found in the nervous systems of animals, they are present in very few other animal tissues. On the other hand, galactolipids and sulfolipids are prominent in green plants as important constituents of the chloroplast photosynthetic membranes. The galactolipid fatty acids of the chloroplast lamellae are highly polyunsaturated with  $\alpha$ -linolenic acid making up ca. 90% of the fatty acid content (7). Sulfolipids are also found in the photosynthetic tissues of the chloroplast. Sulfolipids are similar to the phospholipids in fatty acid composition, i.e., they contain significant amounts of palmitic, oleic and linoleic acids, as well as linolenic acid.

**TABLE II**  
**PRINCIPAL LIPID GROUPS**

<u>Name:</u>	<u>Structure:</u>
<b>A. Fatty Acids</b>	$\text{RCOOH}$
<b>B. Triglycerides</b>	$\begin{array}{c} \text{CH}_2\text{OOCR}_1 \\   \\ \text{R}_2\text{COOCH} \\   \\ \text{CH}_2\text{OOCR}_3 \end{array}$
<b>D. Waxes</b>	$\text{R}_1\text{COOCH}_2\text{R}_2$
<b>E. Glycerophospholipids</b>	$\begin{array}{c} \text{CH}_2\text{OOCR}_1 \\   \\ \text{R}_2\text{COOCH} \\   \\ \text{CH}_2\text{OP}-\text{OX} \\   \\ \text{O}^- \end{array}$
• Phosphatidyl choline	$\text{X} = \text{CH}_2\text{CH}_2\overset{\uparrow}{\text{N}}(\text{CH}_3)_3$
• Phosphatidyl ethanolamine	$\text{X} = \text{CH}_2\text{CH}_2\text{NH}_2$
• Phosphatidyl serine	$\text{X} = \text{CH}_2\overset{\uparrow}{\text{C}}\text{HNH}_2$   COOH
• Phosphatidyl glycerol	$\text{X} = \text{CH}_2\text{CHOHCH}_2\text{OH}$
<b>F. Sphingophospholipids</b>	$\begin{array}{c} \text{O} \\    \\ \text{CH}_3(\text{CH}_2)_{12}\text{CH} = \text{CHCH}-\text{CHCH}_2-\text{OPOX} \\   \quad   \\ \text{OH} \quad \text{NH} \\   \\ \text{COR} \end{array}$
<b>H. Galactolipids</b>	
• Monogalactosyl diacylglycerol (MGDG)	
• Digalactosyl diacylglycerol (DGDG)	
<b>H. Sulfolipids</b>	
• Plant Sulfolipid (Sulfoquinovosyl diacylglycerol)	
<b>J. Sterols</b>	
• Cholesterol	
• Fucosterol	

Lipids not derived from fatty acids

**Sterols.** Sterols and terpenes are both isopentenoid compounds. Like the fatty acids, their biosynthesis begins with acetate, which undergoes a series of reactions forming acetoacetate, hydroxymethylglutarate and finally mevalonate. Mevalonic acid is the precursor to all the isopentenoid compounds. Through a further series of reactions mevalonic acid is converted to isopentenyl pyro-



3R-Mevalonic acid

phosphate and then through successive condensations to squalene, a  $C_{30}$  open chain isopentenoid. In nonphotosynthetic organisms squalene forms an epoxide which cyclizes to lanosterol, a precursor of sterols (8). The principal sterol synthesized by mammals and red algae is cholesterol, from which a number of important steroidal hormones are derived. A sterol synthesized by one organism may not possess an analogous role in another organism in which it is synthesized. If present at all, cholesterol is only formed in minute amounts by crop plants; however, plants synthesize several important sterols, most of which are characterized by an alkyl or alkenyl group at the  $C_{24}$  position of the sterol side chain. The 24-alkylated sterols may be metabolized to hormones for which cholesterol cannot serve as a precursor, e.g., antheridiol. In addition to appearance as free sterols, these compounds are often found as esters or as glycosides. Steroidal alkaloids or azasteroids are nitrogen derivatives which may be important in the defense mechanisms of plants (9).

**Other isopentenoids.** Many lipids other than steroids are formed via the isopentenoid pathway. Terpenes and their derivatives are very important in interactions of plants with other organisms. Kúc and coworkers have proposed that fungal elicitors modify isopentenoid pathways in potato, shifting biosynthesis from triterpene alkaloids which are pre-infection inhibitors to sesquiterpene lactone stress metabolites (9). A variety of insect attractants, insect juvenile hormones, inhibitors and plant hormones are terpene derivatives.

**Other lipids.** Waxes are major lipids in a few organisms (e.g., jojoba, sperm whale). Cutins (condensation polymers of hydroxy fatty acids) are discussed in a later chapter (Kolattukudy et al., Chap. 10). Hydrocarbons other than isopentenoid compounds occur in a variety of species.

### Interactions of Lipids

The ecology of plants includes their interactions with beneficial and harmful organisms--human beings, animals, insects, bacteria, yeasts and fungi. In many of these interactions lipids are produced which are either required nutrients for other organisms or which are part of the defense mechanisms of plants. Isopentenoid compounds produced by one plant may be harmful to another (Elakovich, Chapter 7), while steroids may inhibit plant growth by exerting or modifying a regulatory function (Roddick, Chapter 18, W. D. Nes, Chapter 19). Some of the most interesting defenses of plants are those against insects, including physical barriers (Kolattukudy, Chapter 10) and regulatory compounds for insect development (Svoboda, Chapter 11, Thompson, Chapter 12). The interactions which have evolved in nature suggest protective strategies in which molecular biology and other biotechnological approaches may be used to protect crop plants. We expect to see a number of such solutions achieved from the types of work reported in this volume.

### Literature Cited

1. Harborne, J. B., "Introduction to Ecological Biochemistry", 2d Edition; Academic Press: London, 1982, p. 230.
2. Nes, W. D.; Bach, T. J. Proc. R. Soc. Lond. B225, 1975, p. 425-444.
3. Nes, W. D.; Campbell, B. C.; Stafford, A. E.; Haddon, W. F.; Benson, M. Biochem. Biophys. Res. Commun., 1982, 108, 1258-1263.
4. Gurr, M. I.; James, A. T., "Lipid Biochemistry: An Introduction", 3d Edition; Chapman and Hall: New York, 1980, Chap. 2.
5. Ratledge, C.; Dawson, P.; and Rattray, J., "Biotechnology for the Fats and Oils Industry"; American Oil Chemists Society, Champaign, IL., 1984.
6. Mudd, J. B. in "The Biochemistry of Plants, Vol. 4, Lipids: Structure and Function"; Stumpf, P. K., Ed.; Academic Press: New York, 1980; Chap. 9.
7. Harwood, J. L., Ibid., Chapter 1.
8. Nes, W. R.; McKean, M. L., "Biochemistry of Steroids and Other Isopentenoids"; University Park Press: Baltimore, 1977; Chapters 4-7.
9. Kúc, J.; Tjamos, E.; Bostock, R., in "Isopentenoids in Plants"; Nes, W. D., Fuller, G. and Tsai, L.-S., Eds.; Marcel Dekker: New York; 1984. pp. 103-123.

RECEIVED September 5, 1986

## Chapter 2

# Biosynthesis of Chloroplast Glycerolipids

J. B. Mudd, D. G. Bishop<sup>1</sup>, J. Sanchez, K. F. Kleppinger-Sparace, S. A. Sparace, J. Andrews, and S. Thomas

ARCO Plant Cell Research Institute, 6560 Trinity Court, Dublin, CA 94568

The glycerolipids of the chloroplast comprise monogalactosyldiacylglycerol (MGDG)\*, digalactosyldiacylglycerol (DGDG), sulfoquin-ovosyldiacylglycerol (SQDG), and phosphatidylglycerol (PG). PG is the only one of these found outside the chloroplast. While the synthesis of fatty acids is exclusively in the plastid of higher plants, the synthesis of the unique glycerolipids of the chloroplast requires contributions from the cytoplasmic compartment.

The fatty acids synthesized in the plastid are exported to the cytoplasmic compartment as acyl-CoAs generated by enzymic activity of the outer membrane of the plastid envelope. These acyl-CoAs are utilized in the synthesis of phospholipids in the mitochondria and the endoplasmic reticulum. The fatty acid specificity in the synthesis of phosphatidyl choline (PC) is such that palmitate (16:0) is never found at the sn-2 position. Thus the predominant molecular species are sn1-18, sn2-18 and sn1-16, sn2-18. These molecular species of PC supply the diacylglycerol (DAG) moiety for synthesis of MGDG, DGDG, and SQDG in the plastid. In some cases ("18:3 plants") the DAG from PC is the sole supplier of DAG moieties to the three glycolipids. The mechanism of transfer of the DAG from PC to the chloroplast moiety is unknown.

\*Abbreviations: ACP, acyl carrier protein; APS, adenosine-5'-phosphatosulfate; CDP-DG, cytidinediphosphate-diacylglycerol; DAG, diacylglycerol; DGDG, digalactosyldiacylglycerol; FdH<sub>2</sub>, ferredoxin (reduced); LPA, lysophosphatidic acid; MGDG, monogalactosyldiacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PG, phosphatidylglycerol; PGP, phosphatidylglycerolphosphate; PSSO<sub>3</sub><sup>-</sup>, protein-S-sulfonate; SQDG, sulfoquinovosyldiacylglycerol; UDP-SQ, uridinediphosphate-sulfoquinovose.

<sup>1</sup>Current address: CSIRO, Division of Food Research, North Ryde, New South Wales, Australia

0097-6156/87/0325-0010\$06.00/0  
© 1987 American Chemical Society

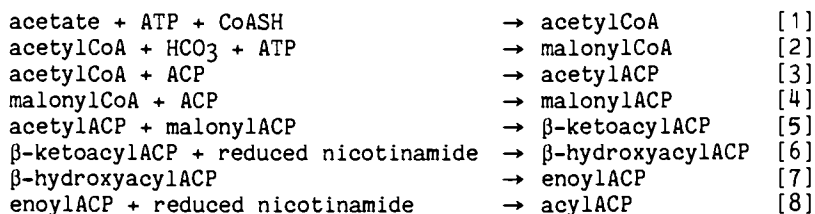
DAG is also synthesized in the plastid, but in this case the fatty acid specificity is such that 16 C acids are almost without exception found at the sn-2 position. Thus the predominant molecular species are sn1-18, sn2-16, and sn1-16, sn2-16. These molecular species are the sole supplier of DAG moieties in the synthesis of PG. In some cases ("16:3 plants") these molecular species contribute to the synthesis of MGDG, DGDG and SQDG.

Lipid biosynthesis in chloroplasts has been extensively studied for 25 years. We now have a good understanding of the synthesis of fatty acids and glycerolipids. Whereas fatty acid synthesis in higher plants is localized in the plastid, the synthesis of glycerolipids of the plastids depends to a large degree on enzymes in the cytoplasmic compartment.

This review attempts to emphasize recent developments in the study of glycerolipid metabolism in the chloroplast. Detailed current information may be found in the recently published proceedings of a symposium on Structure, Function and Metabolism of Plant Lipids (1).

### Fatty Acid Synthesis

The studies on fatty acid synthesis in higher plants over the last 25 years have led to a consensus about the individual reactions and their localization in the cell. This consensus is that the enzyme system for fatty acid synthesis is procaryotic in nature, that is the enzymes are soluble and separable, and that the system is localized entirely in the plastid. Thus the membranes of the mitochondria, the endoplasmic reticulum, the plasmalemma, the tonoplast, the nuclear membrane, and the Golgi apparatus all depend for their fatty acid components on the activities of the plastids. In outline the reactions of fatty acid synthesis may be summarized:



Although there is general agreement about the outline of the synthetic reactions, some questions may be raised on specific issues, such as the origin of the precursors for fatty acid synthesis and the origin of the reductants used.

Acetate has been used widely as a precursor for fatty acid synthesis by isolated chloroplasts largely as a matter of convenience and economy. Several studies have attempted to determine whether acetate is the physiological precursor.

Roughan *et al* (2) compared acetate, pyruvate and malonate as potential precursors and found that acetate was about three times better than pyruvate while malonate was not used at all. Consistent with this result is the report of Kuhn *et al* (3) who found that acetate concentration in plant tissue is in the order of mM and that acetate thiokinase is localized in the plastid. Nevertheless, alternative substrates can not be entirely excluded at this stage. Schulze-Siebert *et al* (4) have reported that when chloroplasts are incubated with bicarbonate, pyruvate accumulates in the chloroplast. Furthermore Williams and Randall (5) have reported that pyruvate dehydrogenase of pea chloroplasts has an activity of 6-9  $\mu\text{mol/h/mg}$  chlorophyll. It is therefore conceivable that the acetyl CoA used in the first steps of fatty acid synthesis is derived from pyruvate.

The question as to whether photosynthetically fixed carbon dioxide can directly give rise to precursors of fatty acid synthesis has also received considerable attention. Stitt and ap Rees (6) have reported that the pathway from 3-phosphoglyceric acid to pyruvate is incomplete in pea chloroplasts because of the absence of phosphoglyceric acid mutase. This result would suggest that the first product of photosynthesis leaves the chloroplast and the precursor of fatty acid synthesis (acetate or pyruvate) is eventually returned to the chloroplast. The result of Schulze-Siebert *et al* (4) with spinach chloroplasts appears to be consistent with the presence of the phosphoglyceric acid mutase in these chloroplasts. Furthermore the results of Journet and Douce (7) obtained by using plastids from cauliflower inflorescence, indicate that they are capable of the conversion of 3-phosphoglyceric acid to acetyl CoA.

The studies of the individual enzymes of fatty acid synthesis in higher plants has shown that the two reductive steps,  $\beta$ -ketoacyl ACP reductase and enoyl ACP reductase have different cofactor requirements. As a result the synthesis of fatty acids depends on the availability of both NADH and NADPH. While the provision of NADPH can be attributed to the photosynthetic reactions, the source of NADH in the chloroplast is less certain. Takahama *et al* (8) have demonstrated that the content of NADPH in the chloroplast is influenced by illumination as expected, but there is no such fluctuation of the oxidation state of NAD/NADH. The production of NADH to be utilized in fatty acid synthesis would therefore appear to depend on dark reactions. One possibility would be by the action of pyruvate dehydrogenase, which would generate not only the NADH required for reduction in fatty acid synthesis but also the precursor acetyl CoA.

Most studies of fatty acid synthesis by isolated chloroplasts are made under photosynthetic conditions. Illumination of the chloroplasts generates ATP and reductant necessary for the incorporation of acetate into the fatty acids. Other effects of illumination may influence fatty acid synthesis. For example the pH and the magnesium ion concentration of the stroma both rise when the chloroplast is illuminated. It should be noted that non-photosynthetic plastids are also assumed to be the sole site of fatty acid synthesis and they must have sources

of ATP and reductant alternative to photosynthetic mechanisms. Sauer and Heise (9) have addressed the problem of fatty acid synthesis by chloroplasts in the dark. They have used the dihydroxyacetone phosphate shuttle as first described by Werdan *et al* (10), which depends on the conversion of DHAP to glyceraldehyde-3-phosphate which is oxidized by GPDH, generating ATP and NADPH. Since DHAP is taken into the chloroplast by the phosphate translocator in exchange for phosphate it was necessary to include phosphate as a component of the shuttle to counteract the potential decrease of phosphate in the stroma. The original purpose of the DHAP shuttle was to obtain carbon dioxide incorporation into 3-PGA in the dark which required ATP but not reductant. It was therefore necessary to reoxidize the NADPH generated by the oxidation of phosphoglyceraldehyde otherwise the production of ATP would have been limited by the lack of NADP. The reoxidation of NADPH was accomplished by the addition of OAA which was reduced to malate in the stroma. In the experiments of Sauer and Heise (9) all three components of the DHAP shuttle were necessary to observe fatty acid synthesis in the dark. This is rather puzzling since the OAA would be expected to drain off NADPH which one would think is required for the reductive steps of fatty acid synthesis. Perhaps the components of the shuttle have other effects than those outlined above. The results of Browse *et al* (11) also bear on the question of fatty acid synthesis in the dark. They have reported rates of fatty acid synthesis by leaf discs of spinach kept in darkness which were 12-20% of the rates in the light.

Sauer and Heise (9) have also demonstrated that the synthesis of fatty acids in the dark is stimulated when an ionophore is used to increase the magnesium ion concentration in the stroma. This result indicates that the increase in stroma concentration of magnesium ion during illumination is favorable for fatty acid synthesis. The optimum pH for fatty acid synthesis is also achieved during illumination of the chloroplast.

### Fatty Acid Utilization

The long chain fatty acids synthesized by the chloroplast system are in the form of ACP derivatives. At any point in the fabrication of the chain there are four things that can happen to the acyl moiety: 1) the acyl ACP can be utilized in another cycle of elongation, 2) the acyl chain can be transferred to glycerol-3-phosphate, 3) the acyl moiety can be exported from the chloroplast to the cytoplasmic compartment, and 4) the acyl ACP can be desaturated. (Figure 1).

The fatty acid chain lengths synthesized by the chloroplast are primarily 16 and 18. This clearly implies that for acyl ACP of chain lengths less than 16 the predominant reaction is further elongation, and that for chain lengths of 18, elongation is rare. For 16:0 ACP, 18:0 ACP, and 18:1 ACP, both transfer to the cytoplasmic compartment and acylation of glycerol-3-phosphate are distinct possibilities. The concentration of ACP in the stroma has been determined to be 8  $\mu\text{M}$  [Ohlrogge *et al* (12)], so only small



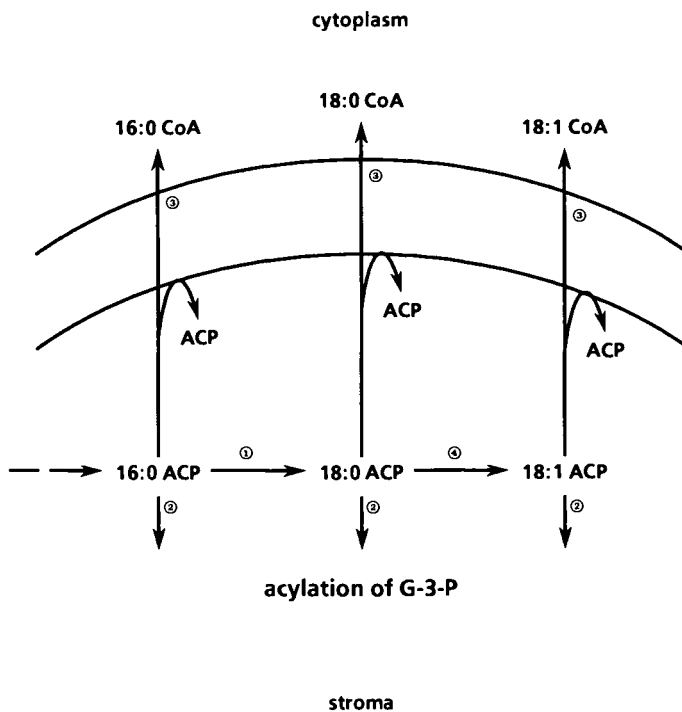


Figure 1. Utilization of acyl ACP. The acyl ACP generated by the fatty acid synthesising system can be elongated (reaction ①), transferred to glycerol-3-phosphate (reaction ②), desaturated (reaction ③), or exported to the cytoplasm (reaction ④).

steady state concentrations of acyl ACPs are possible. Soll and Roughan (13) have determined the concentrations of acyl ACPs during fatty acid synthesis by spinach chloroplasts. They accumulate to the level of 3 nmol/mg chlorophyll and 16:0 ACP, 18:0 ACP, and 18:1 ACP were represented at concentrations of 1.6  $\mu$ M, 0.99  $\mu$ M, and 2.08  $\mu$ M respectively.

Although the membranes of the chloroplast may comprise 75% of the membranes of the plant cell [Forde and Steer (14)], it should be noted that rather a large fraction of the fatty acids produced in the chloroplast must be processed in the cytoplasmic compartment before being returned to the chloroplast. In plants where the chloroplasts are relatively devoid of phosphatidic acid phosphatase and can therefore not provide DAG for the synthesis of glycolipids (MGDG, DGDG, SQDG) only the fatty acids that are found on the chloroplast PG have been used for the acylation of G-3-P in the chloroplast. In such plants ("18:3 plants"), 95% of the fatty acids synthesized in the chloroplast are transported to the cytoplasm. For the "16:3 plants" this figure is harder to calculate but is not less than 60%.

The export of the acyl moieties to the cytoplasm depends on the activities of both acyl-thioester esterases and acyl-thioester synthetases. Both Andrews and Keegstra (15) and Block *et al* (16) have reported that the acylCoA thioesterase is located in the inner membrane and the acylCoA synthetase is located in the outer membrane of the chloroplast. It is assumed that the physiological substrate for the thioesterase of the inner membrane is the ACP derivative. The combination of these two activities achieves the transport of newly synthesized fatty acids to the cytoplasm where, as the CoA derivatives, they are available for the synthesis of phospholipids in the mitochondria and endoplasmic reticulum.

#### Origin of the diacylglycerol moiety of chloroplast glycerolipids

By studying the kinetics of labeling of glycerolipids in pumpkin leaves, Roughan (17) concluded that the diacylglycerol moiety of MGDG originated in PC and that desaturation of the fatty acids of PC provided the unsaturated fatty acids found in the galactolipids. In a later study however, Roughan *et al* (18), using isolated spinach chloroplasts discovered that MGDG synthesised *in vitro* demonstrated successive desaturation to linoleate and to linolenate. This apparent contradiction eventually led to the recognition that in some plants the diacylglycerol moiety is derived entirely from the endoplasmic reticulum, whereas in others it is derived partly from the endoplasmic reticulum and partly from the synthesis of diacylglycerol in the plastid. When the diacylglycerol is obtained from the plastid it contains almost exclusively 16:0 initially at the sn-2 position. If this diacylglycerol is used in the synthesis of MGDG successive desaturations generate 16:3 at the sn-2 position. On the other hand if the diacylglycerol is derived from the endoplasmic reticulum 18 carbon fatty acids are found almost exclusively at the sn-2 position and after desaturation the MGDG contains 18:3 at the sn-2 position. The two groups have been called the "16:3

plants" and the "18:3 plants" respectively. This distinction was recognised by Jamieson and Reid (19) but the biochemistry is now understood. Gardiner and Roughan (20) have presented evidence that the crucial difference between 18:3 and 16:3 plants is in the presence of a more active phosphatidic acid phosphatase in the 16:3 plants. The consequence is that the phosphatidic acid synthesized in the plastid can give rise to those glycerolipids which use diacylglycerol as a precursor. In either the 16:3 or the 18:3 plants the synthesis of PG does not depend on the conversion of PA to DAG and it does not depend on the provision of DAG from the cytoplasmic compartment. Bishop *et al* (21) have analyzed the fatty acid distribution of glycerolipids from 16:3 and 18:3 plants to see what the origin of the DAG moiety is in the various plastid glycerolipids. Table I shows that the DAG moiety of PG is always synthesized in the plastid (procaryotic). MGDG shows the characteristic presence of 16:3 in MGDG indicating that the DAG moiety has come from the procaryotic pathway in spinach. In cucumber there is no 16:3 in the MGDG indicating that the DAG moiety came entirely from the cytoplasmic compartment (eucaryotic). Although it is proposed that the DAG moiety of MGDG comes from PC in 18:3 plants, it is clear that there is discrimination against molecular species that contain 16:0. Molecular species that contain 16:0 are utilized in the synthesis of DGDG, and in the synthesis of SQDG. The data presented by Bishop *et al* (21) demonstrate that SQDG is synthesized from the same DAG pool as the galactolipids even though there is selection of the molecular species in the synthesis of these three molecules. It is clear in the case of spinach that the DAG moiety of SQDG comes from both cytoplasmic and chloroplastic sources whereas the SQDG of cucumber (18:3) comes entirely from the cytoplasmic compartment.

#### Acylation of glycerol-3-phosphate

Following the demonstration by Douce and his colleagues that the acylation of glycerol-3-phosphate in the chloroplast is catalyzed by an enzyme of the stroma and the second acylation to form PA is catalyzed by an enzyme of the chloroplast envelope, further studies concentrated on the characterization of these reactions. Bertrams and Heinz (22) and Frentzen *et al* (23) have delineated the fatty acyl specificity for the acylation of glycerol-3-phosphate by the soluble enzyme from the chloroplast stroma. There was a strong preference for oleate over palmitate by the enzymes from spinach and pea. The specificity was the same both for acyl-CoAs and for acyl-ACPs although the latter is almost certainly the physiological substrate. This substrate specificity was reversed when the requirements for the second acylation reaction were studied, palmitate being almost exclusively used in the conversion of 1-oleyl-G-3-P to PA by enzymes localized on the chloroplast envelope. These specificities are entirely consistent with what we know of the fatty acid distribution of complex lipids of these two species. PG in both cases has oleate or desaturation products at position sn-1, and palmitate or desaturation products at position sn-2.

Table I. Positional distribution of fatty acids in leaf glycerolipids. The positional distribution was determined after digestion with *Rhizopus* lipase. The presence of 16 carbon fatty acids at position sn-2 indicates chloroplastic origin of the DAG. The presence of 16 carbon fatty acids at position sn-1 is indicative of cytoplasmic origin of the DAG.

[Data from Bishop *et al.* (21)]

		16:0	16:1 <sup>3t</sup>	16:3	18:0	18:2	18:3
<u>Spinach (16:3)</u>							
MGDG	sn-1	2	-	3	1	1	93
	sn-2	1	-	46	-	1	52
SQDG	sn-1	34	-	-	3	7	50
	sn-2	67	-	-	1	3	29
PG	sn-1	27	2	-	2	3	63
	sn-2	12	73	-	-	6	7
PC	sn-1	35	-	-	-	20	31
	sn-2	1	-	-	-	35	51
<u>Cucumber (18:3)</u>							
MGDG	sn-1	3	-	-	2	4	86
	sn-2	1	-	-	1	1	93
SQDG	sn-1	60	-	-	11	3	21
	sn-2	2	-	-	-	3	86
PG	sn-1	33	5	-	19	7	1
	sn-2	16	71	-	1	3	8
PC	sn-1	37	-	-	9	18	30
	sn-2	2	-	-	1	34	51

In spinach the PA synthesized in the chloroplast donates the DAG moiety in the synthesis of MGDG and in this molecule we find 18 carbon acids exclusively at position sn-1 and in some cases 16 carbon fatty acids at position sn-2.

Frentzen *et al* (23) have determined kinetic parameters for the G-3-P:acyl-ACP acyl transferase from spinach. The  $K_m$  for palmityl-ACP was 3.2  $\mu\text{M}$  and for oleyl-ACP it was 0.3  $\mu\text{M}$ . These figures may be compared with the steady state concentrations of 1.6  $\mu\text{M}$  and 2.08  $\mu\text{M}$  respectively determined by Soll and Roughan (13). Thus acylation with oleate should clearly be favored. The  $K_m$  for G-3-P was 3150  $\mu\text{M}$  in the presence of palmityl-ACP and 31  $\mu\text{M}$  in the presence of oleyl-ACP according to Frentzen *et al* (23), but Sauer and Heise (24) report the values to be 590  $\mu\text{M}$  and 290  $\mu\text{M}$  respectively. These values may be compared with stroma concentrations of G-3-P oscillating between 100  $\mu\text{M}$  in the light to 300  $\mu\text{M}$  in darkness. These values suggest a preference for oleate, though somewhat less if the data of Sauer and Heise (24) are used in the calculation.

### Galactolipid biosynthesis

The syntheses of MGDG and DGDG have been well studied for almost twenty years. The popularity of this area is no doubt due to the ease of assay and the ready availability of suitable substrate, radiolabelled UDP-galactose.

It is widely accepted that the synthesis of MGDG depends on the reaction of DAG and UDP-galactose. The synthesis of DGDG is more controversial. There has been no satisfactory demonstration of the reaction of MGDG with UDP-galactose. The alternative reaction described by van Besouw and Wintermans (25) is a dismutation reaction of two molecules of MGDG to generate one molecule of DGDG and one molecule of DAG. It is this reaction which probably gives rise to the considerable amounts of DAG in isolated chloroplasts and chloroplast envelopes. The physiological importance of this reaction is still not clear. The problem with both of the reactions already mentioned comes from considerations of the fatty acid composition and positional distribution. Table I illustrates the dilemma. In 16:3 plants the 16 carbon fatty acid is excluded from the DGDG, and in both 16:3 and in 18:3 plants 16:0 is much more represented in DGDG than in MGDG. If DGDG is formed from MGDG there must be strong discrimination against molecular species containing 16:3 and strong discrimination in favor of molecular species containing 16:0. If the dismutation reaction is operative, equally strong discriminations must apply.

It is notable that 18:3 and 16:3 plants yield chloroplasts that are significantly different in the characteristics of MGDG synthesis. Heinz and Roughan (26) have shown that whereas the chloroplasts from both 18:3 and 16:3 plants are capable of incorporating radiolabelled galactose from UDP-galactose, using the endogenous DAG pool, the chloroplasts from 16:3 plants are much more efficient in synthesizing MGDG from DAG which is synthesized from radioactive acetate. The implication is that the DAG synthesized *de novo* in the chloroplast is not accessible to

the galactolipid synthesizing equipment in the chloroplast from 18:3 plants. A further difference is that the chloroplasts from the 16:3 plants are capable of desaturating the 18 carbon fatty acids of MGDG while those from 18:3 plants are not.

### Biosynthesis of phosphatidylglycerol in chloroplasts

The synthesis of PG by isolated chloroplasts was reported by Mudd and deZacks (27). Before that report attempts to measure PG synthesis in isolated chloroplasts had failed and the consensus was forming that the PG of the chloroplast was actually synthesized in other organelles and transported to the plastid. Sparace and Mudd (28) degraded the PG synthesized by the isolated chloroplasts using various lipases to demonstrate that both glycerol moieties were labelled from radioactive G-3-P, indicating that *de novo* synthesis rather than an exchange reaction was taking place. PG labelled from radioactive acetate was labelled equally in both fatty acid moieties and the label at the sn-1 position was 18 carbon and the fatty acid at the sn-2 position was 16 carbon, consistent with fatty acid specificities determined for the acyl transferases determined by Frentzen *et al* (23). The fatty acid analysis of the radiolabelled PG showed that there had been desaturation of the 18 carbon fatty acid to 18:2 but there had been no desaturation of 16:0 to trans-3-hexadecenoic acid. The initial studies of PG synthesis in isolated chloroplasts showed strong inhibition by Triton X-100 and stimulation by CTP. These observations were understood in more detail as a result of the report of Andrews and Mudd (29) which showed that CDP-DG is an intermediate in the synthesis of PG and that the conversion of PA to CDP-DG is strongly inhibited by Triton X-100. In a subsequent paper Andrews and Mudd (30) have reported that the synthesis of PG is localized on the inner envelope membrane of the chloroplast. A summary of the relevant reaction is presented in Table II.

The synthesis of PG in chloroplasts has taken on particular significance because of the findings of Murata (31) showing a strong correlation between the fatty acid composition of PG and sensitivity to chilling. Chilling sensitive species have PG with a high proportion of molecular species with 16 carbon fatty acids at both positions sn-1 and sn-2. For example 65% of the molecular species of sweet potato PG are of this type whereas only 6% of the PG molecular species are of this type in spinach. (Spinach is resistant to chilling and sweet potato is sensitive.) This correlation is related to the phase behavior of the PGs from the chilling sensitive and chilling resistant plants [Murata and Yamaya (32)]. The observations of Murata have aroused a great deal of interest. Roughan (33) has reported the fatty acid compositions of PGs from a wide variety of plants and found Murata's generalization to hold with some exceptions such as solanaceous plants and C4 grasses.

The finding of plants with high proportions of molecular species with 16 carbon fatty acids at both sn-1 and sn-2 positions requires a revision of our conclusions regarding the fatty acid specificity of the G-3-P:acyl-ACP transacylase. We

must conclude that in the chilling sensitive species the fatty acid specificity is different. The fatty acid preferred for the first acylation must be 16 carbon in these cases.

### Biosynthesis of sulfoquinovosyldiacylglycerol

The biosynthesis of SQDG has not yet been elucidated. Davies *et al* (34) showed that cysteic acid is a suitable precursor for SQDG in *Euglena gracilis*, suggesting that the carbon skeleton and the sulfur moiety were incorporated together. This proposal required the postulation of a pathway which has been called the sulfolytic pathway being analogous to the glycolytic sequence. Harwood (35) concluded that the same pathway may exist in higher plants, but Mudd *et al* (36) found that neither cysteate nor cysteine is superior to sulfate as precursor for SQDG synthesis in spinach seedlings and concluded that any activity of cysteic acid or cysteine could be attributed to the conversion of these compounds to sulfate. Hoppe and Schwenn (37) have examined the synthesis of SQDG by *Chlamydomonas reinhardtii* and found that sulfate is a superior precursor to cysteic acid. The question remains: What are the intermediates between sulfate and SQDG?

Haas *et al* (38) described the synthesis of SQDG from radioactive sulfate by isolated chloroplasts. Separation of the labelled molecular species of SQDG showed that there was no change in distribution during the period from 5 minutes to 4.5 hr sampling times. One could interpret this result as showing not *de novo* synthesis but rather the incorporation of sulfate into pre-existing DAG acceptors. Kleppinger-Sparace *et al* (39) have also studied the synthesis of SQDG from radioactive sulfate by isolated chloroplasts. Rates of 0.7 nmol/hr/mg chlorophyll were observed. The reaction was stimulated by UTP, consistent with the possible involvement of an intermediate such as UDP-sulfoquinovose. The incorporation of sulfate was inhibited by the addition of UDP-galactose which was interpreted as the removal of DAG acceptors which otherwise would be available for the acceptance of sulfoquinovose. We would propose that the synthesis of SQDG proceeds by reactions as outlined in Figure 2.

The origin of the DAG moiety of SQDG has been clarified by the results of Bishop *et al* (21). The results, summarized in Table I, show that the final step of SQDG biosynthesis has access to the same pool of DAGs as galactolipid synthesis. Thus in 16:3 plants the DAG moiety can come from both the cytoplasm (eucaryotic) and the chloroplast (procaryotic) whereas in 18:3 plants the DAG moiety for SQDG synthesis comes only from the cytoplasmic compartment. The analyses of the fatty acid composition and positional distribution shows that there is considerable selectivity in the utilization of DAG molecular species for the synthesis of SQDG, MGDG and DGDG. The scheme of synthesis and utilization of DAG for SQDG synthesis is presented in Figure 3.

Table II. PG synthesis by chloroplast envelopes. Variation in components of the reaction mixtures causes the accumulation of specific intermediates. Mercuric ions inhibit PGP phosphatase. [Data from Andrews and Mudd (30)]

		acylACP	ACP	CTP	PPi	G-3-P	CMP	Pi					
		LPA → PA		PA → CDP-DG		CDP-DG → PGP		PGP → PG					
		% distribution of products											
Reaction mixture		PA	CDP-DG	PGP	PG								
①	5 min, $^{14}\text{C}$ -16:OACP+LPA	95	-	-	-								
②	①+10 min CTP, $\text{Mg}^{2+}$	5	75	2	12								
③	②+10 min G-3-P	3	5	3	85								
④	③+ $\text{Hg}^{2+}$	5	26	50	12								

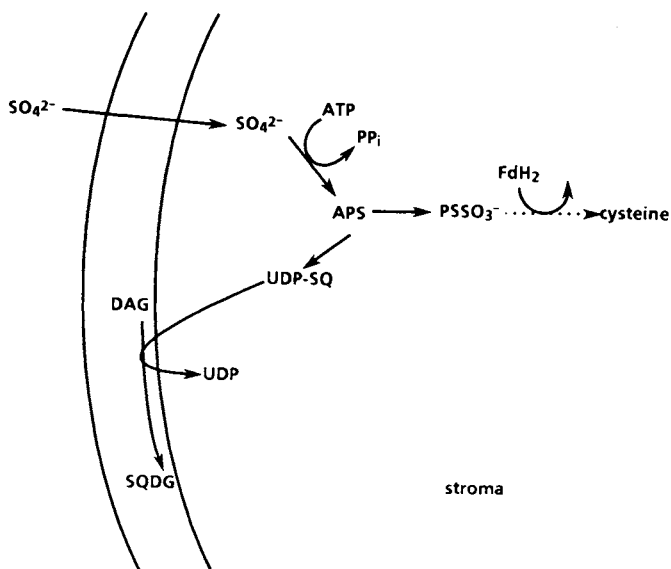


Figure 2. Incorporation of sulfate into SQDG. Sulfate is taken up by the chloroplast where it is activated to form APS. The sulfate can be 1) transferred to the carrier used in reductive steps leading to cysteine, 2) used in the formation of an intermediate ( $\text{UDP-SQ}$ ) which glycosylates DAG present in the envelope membrane, 3) used to form sulfate esters.



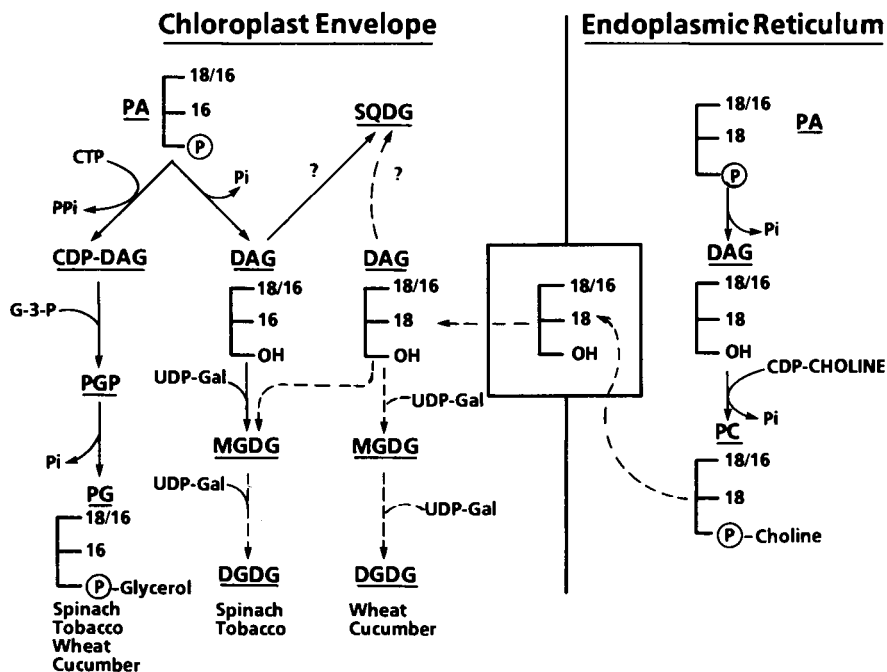


Figure 3. Origin of DAG moiety for SQDG synthesis. The DAG pool used in SQDG synthesis is the same pool as used in galactolipid synthesis. In 18:3 plants this pool is derived entirely from the cytoplasmic compartment. In 16:3 plants it is derived partly from the cytoplasm and partly from the chloroplast.

### Projections

Although the understanding of glycerolipid biosynthesis in the chloroplast is now quite comprehensive there are currently new areas of study opening. The use of mutants to study the importance of lipids in higher plants has now begun (40). Undoubtedly the molecular biology of the reactions of lipid biosynthesis will be studied in the next few years. Little is known about the regulation of glycerolipid synthesis in the chloroplast. It may be discovered that some enzymes of the biosynthetic pathway are activated by light because of conversion to an active sulfhydryl form. Thus it is likely that lipid biosynthesis in the chloroplast will be a fertile area for the next few years.

### Literature cited

1. "Structure, Function and Metabolism of Plant Lipids"; Siegenthaler, P.-A.; Eichenberger, W.; Eds.; Elsevier Science Publishers B.V.: Amsterdam, 1984.
2. Roughan, P.G.; Holland, R.; Slack, C.R. *Biochem. J.* 1978, 184, 565-569.
3. Kuhn, D.N.; Knauf, M.; Stumpf, P.K. *Arch. Biochem. Biophys.* 1981, 209:2, 441-450.
4. Schulze-Siebert, D.; Heineke, D.; Scharf, H.; Schultz, G. *Plant Physiol.* 1984, 76, 465-471.
5. Williams, M.; Randall, D.D. *Plant Physiol.* 1979, 64, 1099-1103.
6. Stitt, M.; ap Rees, T. *Phytochemistry* 1979, 18, 1905-1911.
7. Journet, E.-P.; Douce, R. *C.R. Acad. Sci. Paris* 1984, 298:III:13, 365-370.
8. Takahama, U.; Shimizu-Takahama, M.; Heber, U. *Biochim. Biophys. Acta* 1981, 637, 530-539.
9. Sauer, A.; Heise, K.-P. *Plant Physiol.* 1983, 73, 11-15.
10. Werdan, K.; Heldt, H.W.; Milovancev, M. *Biochim. Biophys. Acta* 1975, 396, 276-292.
11. Browse, J.; Roughan, P.G.; Slack, C.R. *Biochem. J.* 1981, 196, 347-354.
12. Ohlrogge, J.B.; Kuhn, D.N.; Stumpf, P.K. *Proc. Natl. Acad. Sci. USA* 1979, 76:3, 1194-1198.
13. Soll, J.; Roughan, G. *FEBS Letters* 1982, 146:1, 189-192.
14. Forde, J.; Steer, M.W. *J. Exp. Bot.* 1976, 27:101, 1137-1141.
15. Andrews, J.; Keegstra, K. *Plant Physiol.* 1983, 72, 735-740.
16. Block, M.A.; Dorne, A.-J.; Joyard, J.; Douce, R. *FEBS Letters* 1983, 153:2, 377-381.
17. Roughan, P.G. *Biochem. J.* 1970, 117, 1-8.
18. Roughan, P.G.; Mudd, J.B.; McManus, T.T.; Slack, C.R. *Biochem. J.* 1979, 184, 571-574.
19. Jamieson, G.R.; Reid, E.H. *Phytochemistry* 1971, 10, 1837-1843.
20. Gardiner, S.E.; Roughan, P.G. *Biochem. J.* 1983, 210, 949-952.
21. Bishop, D.G.; Sparace, S.A.; Mudd, J.B. *Arch. Biochem. Biophys.* 1985, 240, 851-858.

22. Bertrams, M.; Heinz, E. Plant Physiol. 1981, 68, 653-657.
23. Frentzen, M.; Heinz, E.; McKeon, T.A.; Stumpf, P.K. Eur. J. Biochem. 1983, 129, 629-636.
24. Sauer, A.; Heise, K.-P. Z. Naturforsch. 1984, 39c, 593-599.
25. van Besouw, A.; Wintermans, J.F.G.M. Biochim. Biophys. Acta 1978, 529, 44-53.
26. Heinz, E.; Roughan, P.G. Plant Physiol. 1983, 72, 273-279.
27. Mudd, J.B.; DeZacks, R. Arch. Biochem. Biophys. 1981, 209:2, 584-591.
28. Sparace, S.A.; Mudd, J.B. Plant Physiol. 1982, 70, 1260-1264.
29. Andrews, J.; Mudd, J.B. "Characterization of CDP-DG and PG Synthesis in Pea Chloroplast Envelope Membranes"; Siegenthaler, P.-A.; Eichenberger, W, Eds.; Elsevier Science Publishers B.V.: Amsterdam, 1984.
30. Andrews, J.; Mudd, J.B. Plant Physiol. 1985, 79, 259-265.
31. Murata, N. Plant Cell Physiol. 1983, 24:1, 81-86.
32. Murata, N.; Yamaya, J. Plant Physiol. 1984, 74, 1016-1024.
33. Roughan, P.G. Plant Physiol. 1985, 77, 740-746.
34. Davies, W.H.; Mercer, E.I.; Goodwin, T.W. Biochem. J. 1966, 98, 369-373.
35. Harwood, J.L. Biochim. Biophys. Acta 1975, 398, 224-230.
36. Mudd, J.B.; DeZacks, R; Smith, J. "Studies on the Biosynthesis of Sulfoquinovosyl Diacylglycerol in Higher Plants"; Mazliak, P.; Benveniste, P.; Costes, C.; Douce, R. Eds.; Elsevier/North-Holland Biomedical Press, 1980; pp. 57-66.
37. Hoppe, W.; Schwenn, J.D. Z. Naturforsch. 1981, 36c, 820-826.
38. Haas, R.; Siebertz, H.P.; Wrage, K.; Heinz, E. Planta 1980, 148, 238-244.
39. Kleppinger-Sparace, K.F.; Mudd, J.B.; Bishop, D.G.; Arch. Biochem. Biophys. 1985, 240, 859-865.
40. Browse, J.; McCourt, P.; Somerville, C.R. Science 1985, 227, 763-765.

RECEIVED January 10, 1986

## Chapter 3

# Gibberellins in Higher Plants: The Biosynthetic Pathway Leading to GA<sub>1</sub>

C. R. Spray and B. O. Phinney

Department of Biology, University of California, Los Angeles, CA 90024

The gibberellins (GAs) are a class of plant hormones that were first isolated as metabolites of the fungus, Gibberella fujikuroi. Thereafter they were found to be widespread among higher plants. The GAs have been shown to control a number of biological functions, including shoot elongation, fruit set and the de novo synthesis of  $\alpha$ -amylase. Recent advances in technology have provided methods leading to the identification of 72 GAs. Also biosynthetic studies have integrated these GAs into a limited number of pathways, all of which originate from the common diterpene precursor, GA<sub>12</sub>-aldehyde. In maize shoots, only a single pathway exists, which leads to the biologically active gibberellin, GA<sub>1</sub>. The availability of GA-deficient mutants (dwarf mutants) of maize has provided a unique opportunity to analyze the biosynthetic pathway at both the chemical and genetic level. The results have led to the conclusion that only one gibberellin (GA<sub>1</sub>) is active per se in the control of shoot elongation in maize. This unitary control is probably widespread among higher plants.

The sequence of events that led to the discovery of the gibberellins (GAs) has recently been reviewed by Phinney (1). While the GAs were first discovered as secondary metabolites of the fungus, Gibberella fujikuroi, they have now been shown to be present in most higher plants (both gymnosperms and angiosperms). Their recent identification from ferns and psilophytes (2,3) suggests that the GAs may be ubiquitous to the plant kingdom. Gibberellins have yet to be identified from liverworts, mosses, algae and bacteria.

Of the 72 gibberellins identified by gas chromatography-mass spectrometry (GC-MS), eleven are found only in the fungus, G. fujikuroi, forty seven in higher plants while fourteen additional GAs are common to both groups. The gibberellins are tetracyclic diterpene acids, having in common the carbon skeleton,

0097-6156/87/0325-0025\$06.00/0  
© 1987 American Chemical Society

ent-gibberellane. The gibberellins are then divided into two major groups, the ent-gibberellanes (the  $C_{20}$ -GAs) and the ent-20-norgibberellanes (the  $C_{19}$ -GAs) (see Figure 1). Within the two groups, the GAs differ from each other in a limited number of ways, e.g. the number and position of hydroxyl groups, the degree of unsaturation, and the oxidation state of carbon-19 and carbon-20. In order to simplify the gibberellin nomenclature, the trivial names  $GA_{1-n}$  are now used (4), with each newly characterized member being assigned the next available number.

In general, an individual plant species contains a limited number of gibberellins (usually less than fifteen); in many higher plants apparently only one of these,  $GA_1$ , is active per se in the control of shoot elongation; the other GAs of the shoot are active as biosynthetic precursors to  $GA_1$ , or biologically inactive as branch metabolites from the main pathway. The generalization, "one active GA for shoot elongation", originated from studies with maize (Zea mays) where dwarfing genes were shown to block specific steps in the early-13-hydroxylation pathway leading to  $GA_1$  (5-8). This generalization may be widespread among higher plants since dwarf mutants have been found to control steps of this same pathway in pea (9), rice (10) and tomato (11).

The absolute identification of the specific gibberellins native to a plant is central to the analysis of GA biosynthesis because the enzymes controlling specific steps in the pathway have been shown to be non-specific, i.e. non-native GAs are metabolized to non-native products when added to either the fungus or to the plant (12). Most of the definitive information on the identification of GAs from plants comes from studies using developing embryos and endosperm, which are relatively rich sources of GAs, with levels as high as 10 to 100 milligrams GA per kilogram fresh weight. In contrast, vegetative green shoots may contain less than 1 microgram GA per kilogram fresh weight (13). Such low levels have long frustrated plant physiologists because it is the shoot that shows dramatic elongation responses to applied gibberellins.

Much of the early work on the presence and levels of gibberellins in higher plants was based on bioassay data. While such bioassays are generally specific for the class of diterpenes, the gibberellins, they are essentially useless in determining kinds and levels of specific gibberellins.

In recent years physiological and biochemical research on plant hormones has been revolutionized by the use of high performance liquid chromatography (HPLC) as a key purification step, and capillary GC-MS, for the identification of specific GAs. Thus picogram levels of a particular GA can now be detected in a sample background of 1 milligram (14). As a result, endogenous GAs are being identified and quantified from the shoots of an increasing number of higher plants (e.g. 2,15-18). (For detailed discussions of the criteria for the unambiguous identification of gibberellins see references 14,19,20). A flow sheet summarizing the purification steps leading to the identification of endogenous GAs in maize is shown in Figure 2.

GC-MS is also critical to metabolic studies. For example, a plant can be fed a double labeled substrate (e.g. [ $^{13}C$ ,  $^3H$ ]- $GA_n$ ). The radioisotope ( $^3H$ ) can be used to track the substrate and metabolites during purification while the stable isotope ( $^{13}C$ )

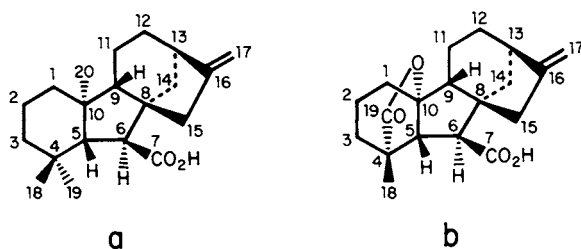


Figure 1. The carbon skeleton and numbering system for the gibberellins. All the known GAs are either C<sub>20</sub>-GAs (a), or C<sub>19</sub>-GAs (b), in which carbon-20 has been lost with the formation of a 19,10 $\gamma$  lactone. (For the structures of the 72 GAs see references [14](#),[57](#),[39](#)).

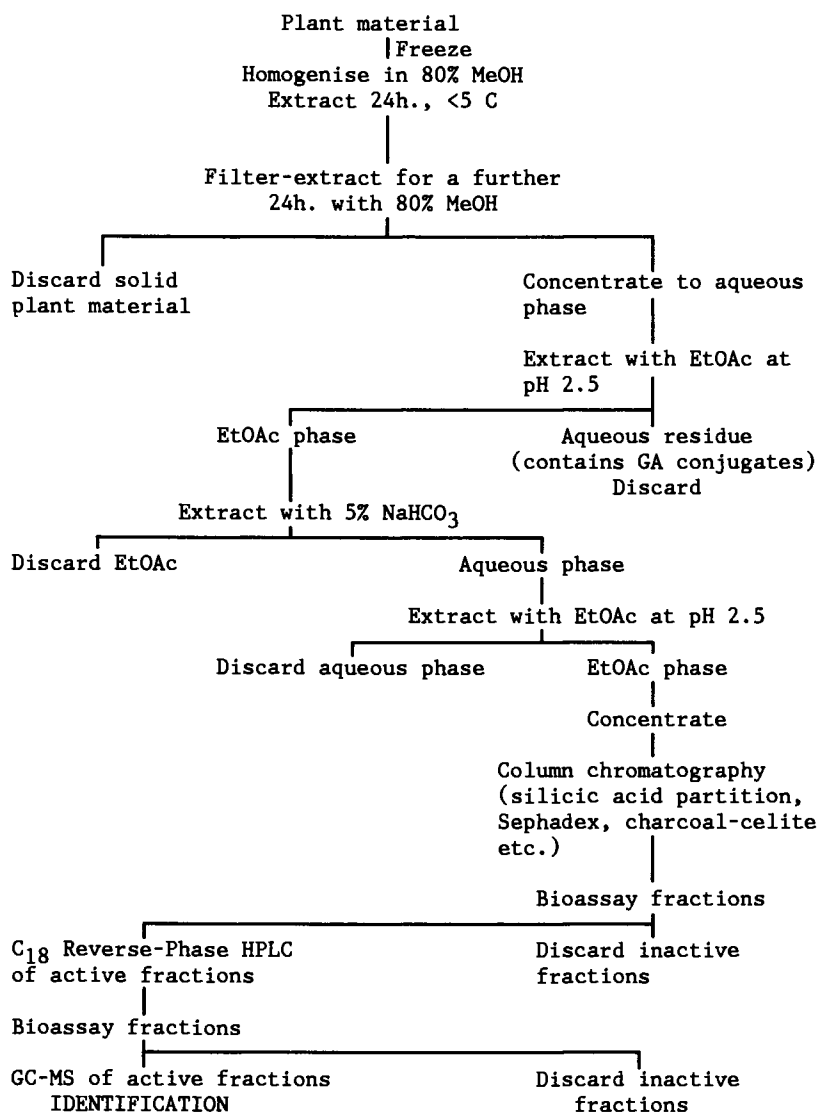


Figure 2. Flow sheet for the extraction and purification of GAs from plant material.

is used for identification in the mass spectrum (e.g. 7). It is also possible to use a single label, acting as both the radio and heavy isotope (e.g. [ $^{14}\text{C}$ ]), provided that sufficiently high levels of the isotope appear in the metabolites from the feed. In this approach, both the availability of the labeled substrates and GC-MS instrumentation of sufficient sensitivity are still limiting factors.

### The Biosynthesis of the Gibberellins

The gibberellin biosynthetic pathway can be divided into two major sections, the early pathway, from mevalonic acid (MVA) to  $\text{GA}_{12}$ -aldehyde (a single pathway), and the pathways subsequent to  $\text{GA}_{12}$ -aldehyde (at least four at this time).

#### The Early Pathway, MVA to $\text{GA}_{12}$ -aldehyde (Figure 3)

Gibberellin biosynthesis is initiated by the activation of MVA, followed by its conversion to isopentenylpyrophosphate (IPP). Stepwise condensation leads to the formation of trans-geranylpyrophosphate (GPP), trans-farnesylpyrophosphate (FPP) and trans-geranylgeranylpyrophosphate (GGPP).

GGPP is the first diterpene in the pathway; it undergoes ring closure (rings AB) to give copalylpyrophosphate (CPP); CPP then undergoes further cyclization (rings CD), to give the tetracyclic ent-kaurene. ent-Kaurene undergoes a series of oxidations at carbon-19, followed by ent-7 $\alpha$ -hydroxylation, to give ent-7 $\alpha$ -hydroxykaurenoic acid. Ring B is then contracted, with the extrusion of carbon-7, to give  $\text{GA}_{12}$ -aldehyde, the common precursor for all the known GAs.

There are a number of branches between MVA and ent-kaurene that lead to biologically important terpenes other than the gibberellins; for example, in plants, a branch at IPP leads to the side chain of cytokinins; a branch at FPP leads to squalene and the sterols, also abscisic acid; branches at GGPP lead to phytoene, fatty acid esters, chlorophyll esters and non-cyclic diterpenes; and branches at CPP lead to (-)-trachylobane, (+)-beyerene, (+)-sandaracopimaradiene (21) and ent-isokaurene (22). (For a review of the pathway to ent-kaurene see reference 23).

#### Pathways Subsequent to $\text{GA}_{12}$ -aldehyde

The remainder of this article will provide a brief review of selected details of pathways subsequent to  $\text{GA}_{12}$ -aldehyde, concentrating on the "early-13-hydroxylation" pathway and its role in the control of shoot growth in maize (Zea mays).

Gibberellin  $\text{A}_{12}$ -aldehyde undergoes a variety of stepwise oxidations and/or hydroxylations. The first step subsequent to  $\text{GA}_{12}$ -aldehyde defines a particular pathway (e.g. 3 $\beta$ -hydroxylation, or 13-hydroxylation, or 12 $\alpha$ -hydroxylation, etc). Any one plant species may have one or more pathways; and different organs of the same plant may have different pathways (19).

The pathways from  $\text{GA}_{12}$ -aldehyde have in common the loss of carbon-20 to give the biologically active  $\text{C}_{19}$ -GAs, and (in plants) 2 $\beta$ -hydroxylations to give biologically inactive GAs. In higher



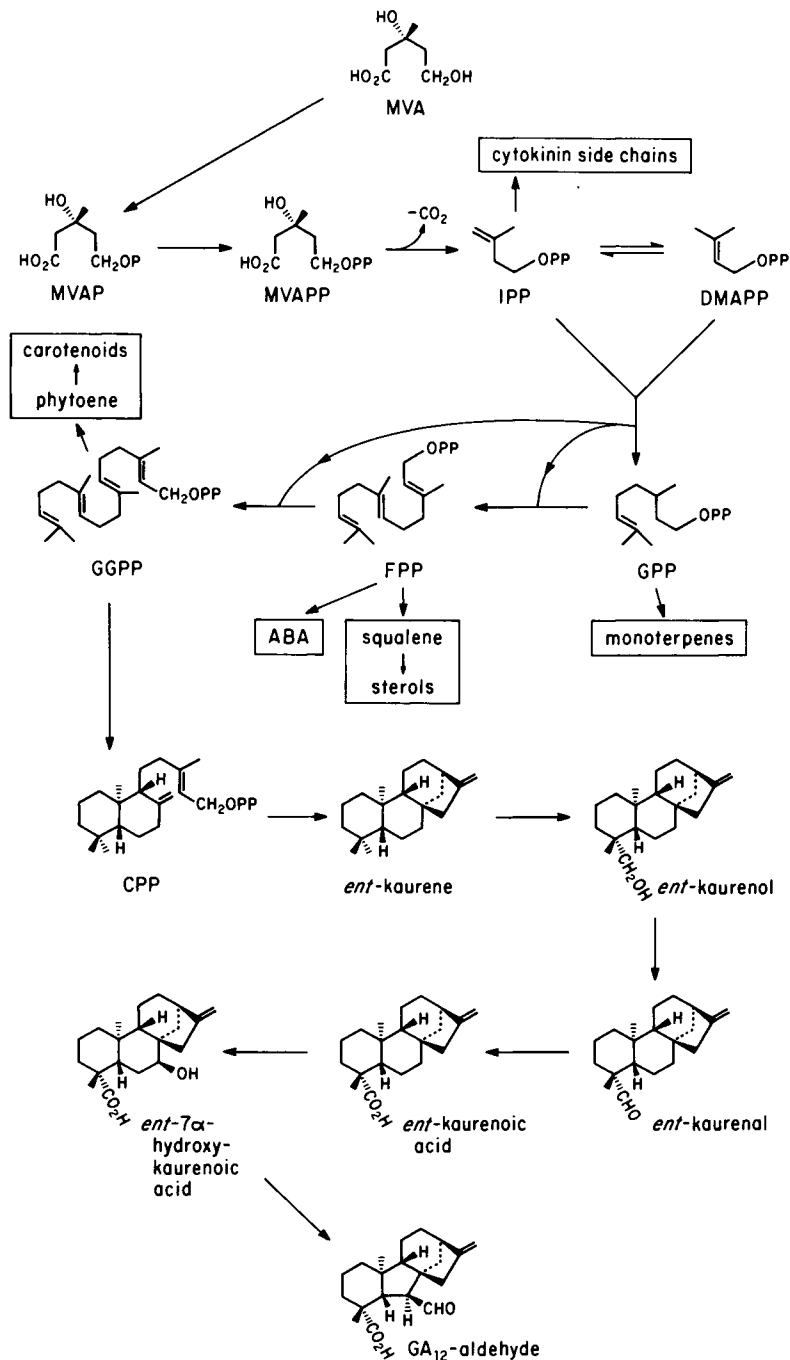


Figure 3. The gibberellin biosynthetic pathway, MVA to GA<sub>12</sub>-aldehyde. This portion of the pathway is the same for both the fungus, *Gibberella fujikuroi*, and higher plants.

plants, carbon-20 is apparently lost as the aldehyde, rather than as the carboxylic acid as would be expected by analogy with the sterols (24,25). The evidence for this loss comes from radiolabeled feeding studies using GAs with a carboxyl group at carbon-20. In a cell-free system from the endosperm of pumpkin (*Cucurbita maxima*) GA<sub>36</sub> (carbon-20 as the aldehyde) is readily metabolized to the C<sub>19</sub> gibberellin, GA<sub>4</sub>, whereas GA<sub>13</sub> (carbon-20 as the acid) is not metabolized (26). Likewise, in a cell-free system from immature embryos of pea (*Pisum sativum*) GA<sub>24</sub> (carbon-20 as the aldehyde) is metabolized to the C<sub>19</sub> gibberellin, GA<sub>9</sub>, while GA<sub>25</sub> (carbon-20 as the acid) is not metabolized (27). Interestingly, GA<sub>36</sub>, GA<sub>4</sub>, GA<sub>24</sub>, and GA<sub>9</sub> are biologically active (28,29) whereas GA<sub>13</sub> and GA<sub>25</sub> are inactive (28). These contrasting activities would be expected if the activity of the C<sub>20</sub>-GAs is dependent on their metabolism to C<sub>19</sub>-GAs. In general, C<sub>19</sub> gibberellins are biologically active (except for the 2β-hydroxylated GAs) and C<sub>20</sub> gibberellins inactive, or low in bioactivity.

In plants, a number of 2β-hydroxylated GAs have been identified, all of which are apparently inactive; many of these are C<sub>19</sub>-GAs (28,30); 2β-hydroxylation may thus be a general process for the deactivation of biologically active C<sub>19</sub>-GAs (30). It is interesting that 2β-hydroxylated GAs are absent in the fungus, *G. fujikuroi*. This absence may be related to the fact that the GAs have no known biological function in this organism; thus the fungus has no need for a deactivation system.

#### The Non-hydroxylation Pathway (Figure 4 - plants)

This pathway is present in both the fungus (*G. fujikuroi*) and in higher plants.

In the fungus, the pathway is initiated by the oxidation of carbon-7 of GA<sub>12</sub>-aldehyde to give GA<sub>12</sub>. Carbon-20 is then lost, followed by the formation of a 19,10 lactone to give the C<sub>19</sub> gibberellin, GA<sub>9</sub>. The precise steps leading to the loss of carbon-20 are not known. The obvious suggestion is oxidative demethylation, via the alcohol (GA<sub>15</sub> [opened lactone]), the aldehyde, (GA<sub>24</sub>) and the acid (GA<sub>25</sub>). These three GAs are naturally occurring in the fungus; however, when fed back to the fungus, they are not metabolized (31). Gibberellin A<sub>0</sub> is further metabolized, either by hydroxylations of carbon-16 (GA<sub>10</sub>), or carbon-2 with a stereochemistry (GA<sub>40</sub>); rearrangement of the lactone combined with epoxide formation at the 1,10 position to give GA<sub>11</sub>, or hydroxylation at carbon-13 to give GA<sub>20</sub> (32).

In plants, members of this pathway were identified from developing seeds of pea as early as 1974 (33). However, it was not until 1983 that the pathway itself was shown to be present (27).

The pathway (Figure 4) is initiated by the formation of GA<sub>12</sub> which is then oxidized sequentially at carbon-20 to the alcohol (GA<sub>15</sub> [opened lactone]) and to the aldehyde (GA<sub>24</sub>). Loss of carbon-20 combined with formation of a 19,10 lactone gives the bioactive gibberellin, GA<sub>9</sub>. In addition, the aldehyde of GA<sub>24</sub> may be further oxidized to the acid (GA<sub>25</sub>) which is apparently not further metabolized; also GA<sub>9</sub> may be hydroxylated at the 2β-position to give the biologically inactive GA<sub>51</sub>.

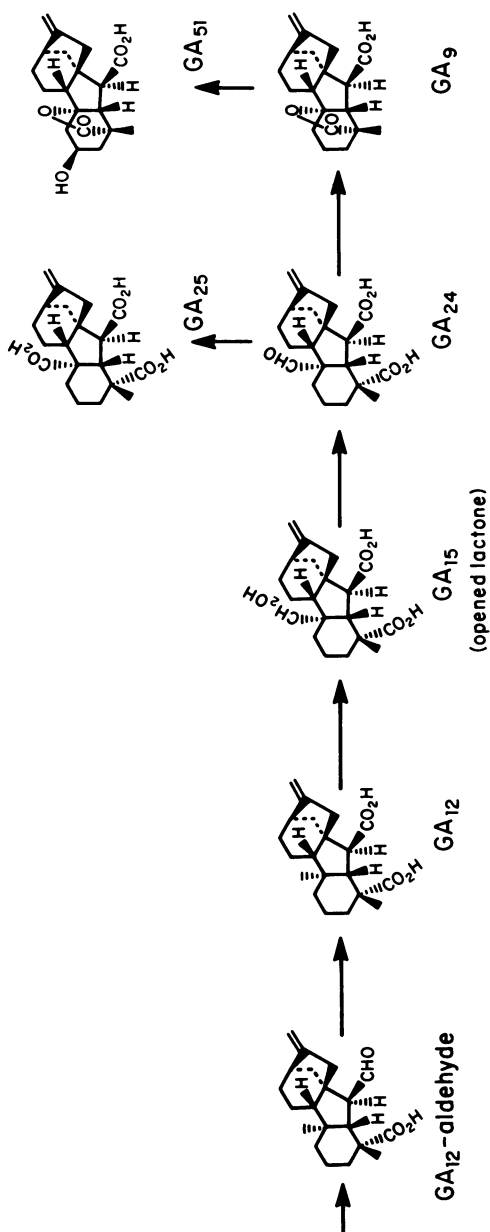


Figure 4. The non-hydroxylation pathway for pea (*Pisum sativum*).

### The Early-3-hydroxylation Pathway (Figure 5)

This pathway is present in the fungus (*G. fujikuroi*). All of the members shown in figure 5 are endogenous to the fungus; also at least eighteen of the actual or potential steps in this pathway have been analyzed by substrate/product feeding studies using [<sup>14</sup>C]-labeled substrates. In this pathway GA<sub>12</sub>-aldehyde is 3β-hydroxylated to GA<sub>14</sub>-aldehyde which, in turn, is oxidized at carbon-7, giving GA<sub>14</sub>. Gibberellin A<sub>14</sub> is then metabolized via an undefined series of intermediates to give the C<sub>19</sub> gibberellin, GA<sub>4</sub>. Gibberellin A<sub>4</sub> may be further metabolized, either by 13-hydroxylation to GA<sub>1</sub>, or by 1,2-dehydrogenation to GA<sub>7</sub>. Gibberellin A<sub>7</sub> is 13-hydroxylated to GA<sub>3</sub>. (This fungus is used in the commercial production of the agriculturally important C<sub>19</sub> gibberellins, GA<sub>3</sub>, GA<sub>4</sub>, GA<sub>7</sub> and GA<sub>9</sub>). There are several branch steps from the main early-3-hydroxylation pathway; e.g. GA<sub>14</sub> may be 16-hydroxylated (GA<sub>42</sub>), or oxidized at carbon-20 (GA<sub>36</sub> and GA<sub>13</sub>); in addition, GA<sub>4</sub> may be α-hydroxylated at carbon-1 (GA<sub>16</sub>) or carbon-2 (GA<sub>47</sub>). Gibberellin A<sub>13</sub> and GA<sub>36</sub> are shown as branches from the main pathway since they are apparently not metabolized when fed to the fungus (31). This pathway has been presented and discussed in greater detail in several reviews (34-36).

The first two members of the early-3-hydroxylation pathway are the C<sub>20</sub> gibberellins, GA<sub>14</sub>-aldehyde and GA<sub>14</sub>. Since these two gibberellins have yet to be identified from higher plants, there is no direct evidence for the presence of the pathway in higher plants; the natural occurrence of GAs that would be found late in the pathway (i.e. C<sub>19</sub>-GAs) is not critical evidence for the presence of the pathway, since C<sub>19</sub>-GAs can have more than one biosynthetic origin. Likewise, presumptive pathways based on feeding studies with GA<sub>14</sub>-aldehyde or GA<sub>14</sub> as substrates do not provide direct evidence for the presence of the pathway, because of substrate non-specificity. (As mentioned earlier, the fungus will convert non-native gibberellins through a series of non-native intermediates to non-native gibberellin products [12]). Feeds and refeeds of radiolabeled GA<sub>14</sub>-aldehyde and its metabolites, to cell-free systems from pumpkin endosperm, clearly document metabolism through an early-3-hydroxylation pathway in this system (37). However, the biological significance of this pathway in the intact pumpkin plant has yet to be established.

### Other Pathways

Six 12α-hydroxylated gibberellins have been identified as native to pumpkin (38). Their structures show them to be 12α-hydroxy derivatives of the C<sub>20</sub> gibberellins, GA<sub>12</sub>, GA<sub>14</sub>, GA<sub>37</sub>, GA<sub>13</sub> (named GA<sub>39</sub>), and the C<sub>19</sub> gibberellins, GA<sub>4</sub> (named GA<sub>58</sub>), and GA<sub>34</sub> (named GA<sub>49</sub>). Thus they are potential members of an early-12α-hydroxylation pathway analogous in oxidation and hydroxylation pattern to the early-3-hydroxylation pathway; alternatively, they may be a series of single step metabolites that branch from an early-3-hydroxylation pathway. Recent and extensive radiolabeled feeds to cell-free systems from pumpkin suggest both possibilities (37,38). In this system the conversions are highly

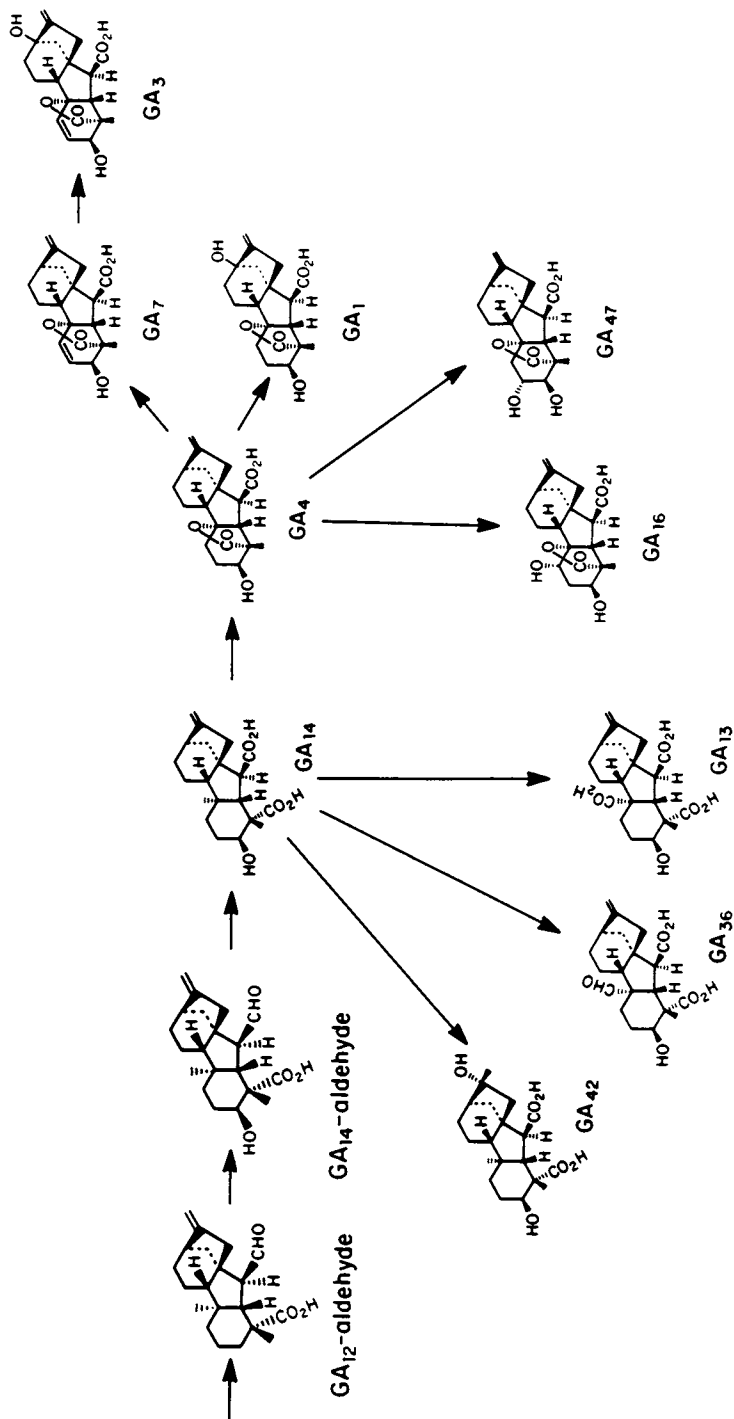


Figure 5. The early-3-hydroxylation pathway for the fungus, *Gibberella fujikuroi*.

pH dependent. The relationship of the 12 $\alpha$ -hydroxylated gibberellins to a specific pathway is, as yet, unresolved.

There are a number of additional gibberellins native to higher plants with unique hydroxylation patterns (for example, hydroxylated at positions 1, 11, 12 $\beta$ , or 15) that fit into no known pathways (2,3,39,40). These GAs cannot be assigned to a biosynthetic pathway until radiolabeled feeding studies have been made using the plants from which the gibberellins were isolated.

#### The Early-13-hydroxylation Pathway (Figure 6)

The gibberellins that comprise this pathway are both unique to higher plants (i.e. not present in the fungus) and widespread among higher plants. The pathway itself has been shown to be present in maize (Zea mays) (7,17,41), and pea (Pisum sativum) (27,42). This report will consider only the early-13-hydroxylation pathway for maize.

The first clue for the presence of the pathway in maize came from the identification of eight 13-hydroxylated GAs from very young tassels and vegetative shoots (17,41,43). No other GAs were found in these extracts. A comparison of the structures of these eight gibberellins clearly placed them in a single hypothetical pathway originating from the common GA-precursor, GA<sub>12</sub>-aldehyde (i.e. GA<sub>53</sub>, GA<sub>44</sub>, GA<sub>19</sub>, GA<sub>17</sub> [branch?], GA<sub>20</sub>, GA<sub>29</sub> [branch?], GA<sub>1</sub>, and GA<sub>8</sub> [branch?]) (5). The order of four specific steps has now been established from radiolabeled feeds to maize seedlings, where the fate of the label was followed after predetermined periods of incubation. The specific steps between GA<sub>12</sub>-aldehyde and GA<sub>53</sub> are still unresolved; in addition, the steps GA<sub>44</sub> to GA<sub>19</sub>; GA<sub>19</sub> to GA<sub>17</sub>; and GA<sub>19</sub> to GA<sub>20</sub> have yet to be demonstrated. This classical approach (feed of labeled substrate followed by identification of labeled immediate product, etc.) was greatly facilitated by the use of a series of GA-mutants that lack either all or specific combinations of endogenous GAs.

These five mutants, dwarf-1, dwarf-2, dwarf-3, dwarf-5 and anther ear-1 (Figure 7), are simple recessives, non-allelic to each other and expressed as a dwarf phenotype from the early seedling stage to maturity; in the dark as well as in the light (44). They are GA mutants in that each dwarf mutant resumes normal growth in response to exogenous GA; a constant supply of gibberellin is necessary for this normal growth (5,45). No other plant or animal hormone elicits such a response. The pattern of response to each member of the pathway varies depending on the mutant being tested (5) (see Figure 8). Such differential growth responses are expected only when the terminal gibberellin in the main pathway is bioactive per se. Other members (gibberellins and their precursors) are bioactive by their metabolism to the terminal bioactive gibberellin. Thus all members of the pathway subsequent to a genetic block would be bioactive, and those before the block inactive. It is the pattern of response for each mutant that suggests the position in the pathway blocked by each mutant. Initially, growth response data were used to suggest the specific position blocked by each of the five dwarf mutants. Subsequently, radiolabeled feeds, using either [<sup>14</sup>C]- or [<sup>13</sup>C,<sup>3</sup>H]-precursors, have provided definitive evidence for the positions



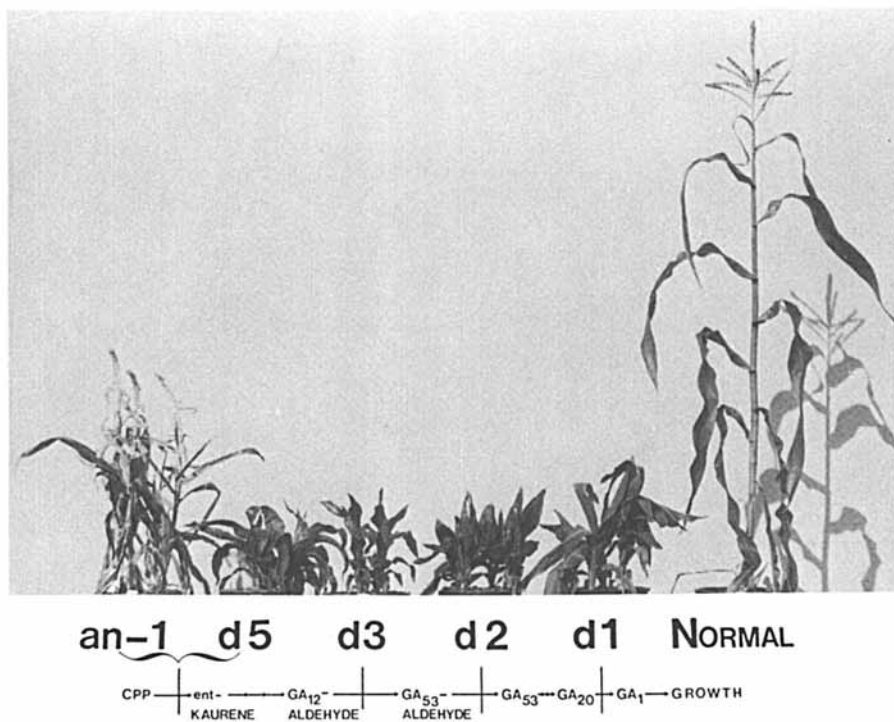


Figure 7. The five mature dwarf mutants and normal maize (*Zea mays*). The height of the mutants varies from 1/4 to 1/5 that of the normals.



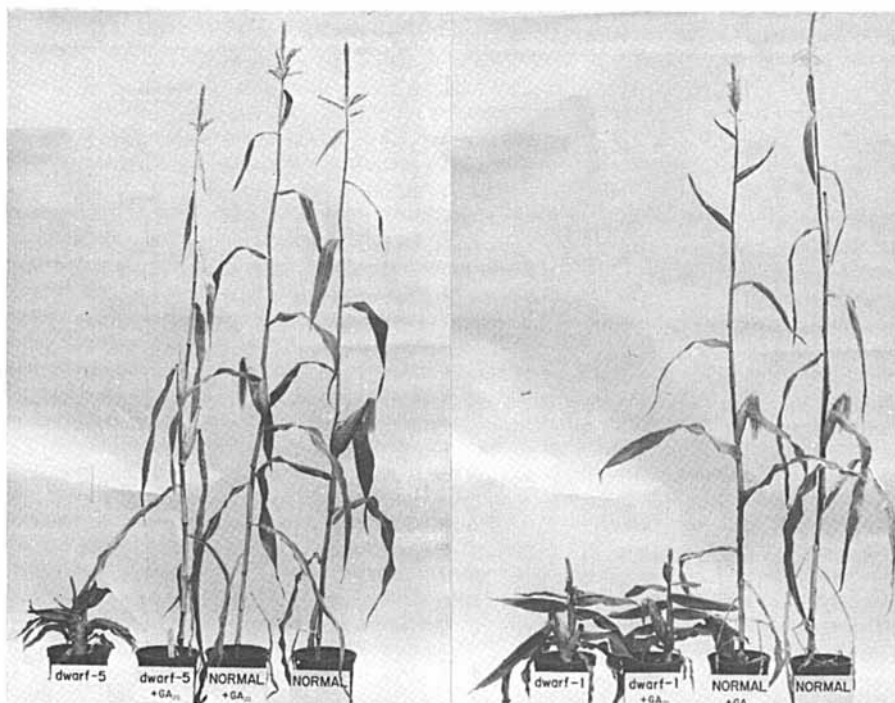


Figure 8. The differential growth response of the dwarf-5 and dwarf-1 mutants to  $GA_{20}$ . The dwarf-5 mutant (left set: dwarf-5; dwarf-5,  $+GA_{20}$ ; Normal,  $+GA_{20}$ ; Normal) responds by normal growth to treatment with  $GA_{20}$ , the dwarf-1 mutant (right set: dwarf-1; dwarf-1,  $+GA_{20}$ ; Normal,  $+GA_{20}$ ; Normal) does not respond when treated with the same level of  $GA_{20}$ .

blocked by the dwarf-5 and dwarf-1 mutants. This report will analyze the dwarf-5 and dwarf-1 systems only.

The dwarf-5 mutant. The dwarf-5 mutant controls the cyclization step, CPP to ent-kaurene (22). This conclusion is drawn from three lines of evidence: First, seedlings respond by normal growth to members of the main pathway, including ent-kaurene (5,46). These data suggest that the mutant blocks an early step in the pathway, before ent-kaurene.

Second, cell-free systems from normal seedlings synthesize ent-kaurene from radiolabeled GGPP, CPP and MVA, whereas cell-free systems from dwarf-5 seedlings synthesize ent-kaurene from these precursors at levels one-fifth that of the normal (22). It is interesting that the mutant system accumulates the isomer, ent-isokaurene, an isomer present in only trace amounts in the normal system; (ent-isokaurene is biologically inactive [47]). Presumably, the formation of the biologically inactive ent-isokaurene is favored in the mutant as a consequence of the genetically altered ent-kaurene synthetase B (Figure 9)(22).

The third line of evidence comes from the analysis of the endogenous GAs present in normal and dwarf-5 seedlings (43). Gibberellin A<sub>1</sub>, GA<sub>20</sub>, GA<sub>19</sub>, GA<sub>17</sub> and GA<sub>44</sub> have been identified from normal seedlings, whereas dwarf-5 seedlings give no evidence for the presence of four of these GAs (trace amounts of GA<sub>20</sub> were found in the dwarf-5 extract). These results would be expected if the dwarf-5 mutant controls the cyclization of CPP to ent-kaurene.

The dwarf-5 mutant has been used extensively to define the single steps, CPP to ent-kaurene (22); ent-kaurenol to ent-kaurenal, ent-kaurenal to ent-kaurenoic acid (48); GA<sub>53</sub> to GA<sub>44</sub> and GA<sub>20</sub> to GA<sub>29</sub> (17); GA<sub>20</sub> to GA<sub>1</sub> (7); and GA<sub>1</sub> to GA<sub>8</sub> (43,49,50). For each step a radiolabeled substrate was fed to the dwarf-5 mutant and the label recovered in the immediate product.

The dwarf-1 mutant. The dwarf-1 gene controls the 3 $\beta$ -hydroxylation of GA<sub>20</sub> to GA<sub>1</sub>. The original evidence comes from growth response data; i.e. GA<sub>1</sub> is active, and GA<sub>20</sub> and its precursors inactive (i.e. less than 1% the activity of GA<sub>1</sub>). By contrast, these same GAs and their precursors are all active in stimulating elongation in dwarf-5 seedlings (see Figure 8).

Conclusive evidence for the position of the genetic block comes from feeds of double labeled GA<sub>20</sub> to dwarf-1 seedlings. These seedlings do not metabolize GA<sub>20</sub> to GA<sub>1</sub>, whereas dwarf-5 seedlings do metabolize GA<sub>20</sub> to GA<sub>1</sub> (and GA<sub>29</sub>) (7).

Analysis of the endogenous GAs in dwarf-1 seedlings also supports the position that the dwarf-1 mutant controls the 3 $\beta$ -hydroxylation of GA<sub>20</sub> to GA<sub>1</sub>. Extracts of dwarf-1 seedlings contain GA<sub>20</sub>, GA<sub>19</sub>, GA<sub>17</sub> and GA<sub>44</sub> - and no GA<sub>1</sub>, whereas all five gibberellins are present in normal seedlings (43). The absence of endogenous GA<sub>1</sub> in dwarf-1 plants would be expected if the mutant blocks the conversion of GA<sub>20</sub> to GA<sub>1</sub>.

This mutant has been used to obtain data from radiolabeled feeds that document the steps GA<sub>20</sub> to GA<sub>29</sub> (7) and GA<sub>1</sub> to GA<sub>8</sub> (43).

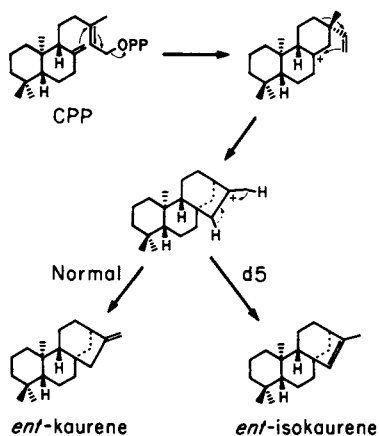


Figure 9. Proposed scheme for the conversion of CPP to *ent*-kaurene and *ent*-isokaurene (22). In the *dwarf*-5 mutant the pathway to *ent*-isokaurene is favored. *ent*-Isokaurene is biologically inactive (47).

### General Comments

Probably the most significant conclusion that comes from our genetic and biochemical studies is that GA<sub>1</sub> must be the only gibberellin in the early-13-hydroxylation pathway that is active per se in the control of shoot elongation in maize. Other members of the pathway are active through their metabolism to GA<sub>1</sub>. This conclusion is supported by data from three kinds of approaches:- bioactivity studies, radiolabeled feeds, and analysis of endogenous GAs in mutant and normal seedlings.

Unfortunately, the enzymological approach is still complicated by low levels of activity and instability of the enzymes (hydroxylases and oxidases) that catalyze the specific steps in the pathway(s). This is especially true for cell-free systems originating from young green shoots. Slow but steady progress is now being made on purification steps by Graebe's group in Gottingen (51), West's group at U.C.L.A. (52), Coolbaugh's group at Iowa (53) and MacMillan's group at the University of Bristol (54,55), which is also approaching the problem through the use of monoclonal antibodies (56). Our group at U.C.L.A. is using Robertson's mutator as a probe for cloning the dwarfing genes in maize (6,8).

### Acknowledgments

Portions of this work were supported by a grant to BOP from the National Science Foundation No. PCM 83-14338.

### Literature Cited

1. Phinney, B. O. In "The Biochemistry and Physiology of Gibberellins"; Crozier, A., Ed.; Praeger: New York, 1983; Vol. I, pp. 19-51.
2. Yamane, H.; Yamaguchi, I.; Kobayashi, M.; Takahashi, M.; Sato, Y.; Takahashi, N.; Iwatsuki, K.; Phinney, B. O.; Spray, C. R.; Gaskin, P.; MacMillan, J. Plant Physiol. 1985, 78, 899-903.
3. Takahashi, M.; Yamane, H.; Satoh, Y.; Takahashi, N.; Iwatsuki, K. Phytochemistry 1984, 23, 681.
4. MacMillan, J.; Takahashi, N. Nature 1968, 217, 170-1.
5. Phinney, B. O.; Spray, C. In "Plant Growth Substances 1982"; Wareing, P. F., Ed.; Academic: London, 1982; pp. 101-10.
6. Phinney, B. O.; Spray, C. R. Proc. 4th Ann. Plant Biochem. and Physiol. Symp. Univ. of Missouri-Columbia, 1985, pp. 67-74.
7. Spray, C. R.; Phinney, B. O.; Gaskin, P.; Gilmour, S. J.; MacMillan, J. Planta 1984, 160, 464-8.
8. Phinney, B. O.; Freeling, M.; Robertson, D. S.; Spray, C. R.; Silverthorne, J. In "Plant Growth Substances 1985"; Bopp, M., Ed.; Springer-Verlag: Berlin Heidelberg, 1985; pp. 55-64.
9. Ingram, T. J.; Reid, J. B.; Murfet, I. C.; Gaskin, P.; Willis, C. L.; MacMillan, J. Planta 1984, 160, 455-63.
10. Murakami, Y. In "Plant Growth Substances 1970"; Carr, D. J., Ed.; Springer-Verlag: Berlin, 1972; pp. 166-74.
11. Zeevaart, J. A. D., unpublished data.
12. Bearder, J. R.; MacMillan, J.; Wels, C. M.; Phinney, B. O. Phytochemistry 1975, 14, 1741-8.
13. Graebe, J. E.; Ropers, H. J. In "Phytohormones and Related Compounds - A Comprehensive Treatise"; Letham, D. S.; Goodwin, P. B.; Higgins, T. J. V., Eds.; Elsevier North-Holland: Amsterdam, 1978; Vol. I, pp. 107-204.

14. MacMillan, J. Biologia Plantarum (Prague) 1985, 27, 164-71.
15. Davies, J. K.; Jensen, E.; Junttila, O.; Rivier, L.; Crozier, A. Plant Physiol. 1985, 78, 473-6.
16. Gaskin, P.; Gilmour, S. J.; MacMillan, J.; Sponsel, V. M. Planta 1985, 163, 283-9.
17. Heupel, R. C.; Phinney, B. O.; Spray, C. R.; Gaskin, P.; MacMillan, J.; Hedden, P.; Graebe, J. E. Phytochemistry 1985, 24, 47-53.
18. Metzger, J. D.; Mardaus, M. C. Plant Physiol. 1986, 80, 396-402.
19. MacMillan, J. In "The Biosynthesis and Metabolism of Plant Hormones"; Crozier, A.; Hillman, J., Eds.; SEB Seminar Series No. 23, Cambridge University Press, Cambridge, 1984; pp. 1-16.
20. Reeve, D. R.; Crozier, A. Plant, Cell and Environment 1983, 6, 365-8.
21. Robinson, D. R.; West, C. A. Biochemistry 1970, 9, 70-9.
22. Hedden, P.; Phinney, B. O. Phytochemistry 1979, 18, 1475-9.
23. Coolbaugh, R. C. In "The Biochemistry and Physiology of Gibberellins"; Crozier, A., Ed.; Praeger: New York, 1983; Vol. I, pp. 53-98.
24. Alexander, K.; Akhtar, M.; Boar, R. B.; McGhie, J. F.; Barton, D. H. R. J. Chem. Soc. Chem. Commun. 1972, 383-5.
25. Arigoni, D.; Battaglia, R.; Akhtar, M.; Smith, T. J. Chem. Soc. Chem. Commun. 1975, 185-6.
26. Graebe, J. E.; Hedden, P.; Rademacher, W. In "Gibberellins - Chemistry, Physiology and Use"; Lenton, J. R., Ed.; British Plant Growth Regulator Group: Wantage, 1980; Monograph 5, pp.31-47.
27. Kamiya, Y.; Graebe, J. E. Phytochemistry 1983, 22, 681-9.
28. Crozier, A.; Kuo, C. C.; Durley, R. C.; Pharis, R. P. Can. J. Bot. 1970, 48, 867-77.
29. Reeve, D. R.; Crozier, A. In "Gibberellins and Plant Growth"; Krishnamoorthy, H. N., Ed.; Wiley Eastern: New Delhi, 1975; pp. 35-64.
30. Sponsel, V. M.(née Frydman); Hoad, G. V.; Beeley, L. J. Planta 1977, 135, 143-7.
31. Bearder, J. R.; MacMillan, J.; Phinney, B. O. J. Chem. Soc. Perkin Trans. I 1975, 721-6.
32. Bearder, J. R.; Frydman, V. M.; Gaskin, P.; Hatton, I. K.; Harvey, W. E.; MacMillan, J.; Phinney, B. O. J. Chem. Soc. Perkin Trans. I 1976, 178-83.
33. Frydman, V. M.; Gaskin, P.; MacMillan, J. Planta 1974, 118, 123-132.
34. Bearder, J. R. In "The Biochemistry and Physiology of Gibberellins"; Crozier, A., Ed.; Praeger: New York, 1983; Vol. I, pp. 251-387.
35. Hedden, P.; MacMillan, J.; Phinney, B. O. Ann. Rev. Plant Physiol. 1978, 29, 149-92.
36. Phinney, B. O. In "Plant Growth Substances"; Mandava, N. B., Ed.; ACS SYMPOSIUM SERIES No. 111, American Chemical Society: Washington, D.C., 1979; pp. 57-8.
37. Hedden, P.; Graebe, J. E.; Beale, M. H.; Gaskin, P.; MacMillan, J. Phytochemistry 1984, 23, 569-74.

38. Blechsmidt, S.; Castel, U.; Gaskin, P.; Hedden, P.; Graebe, J. E.; MacMillan, J. Phytochemistry 1984, 23, 553-8.
39. MacMillan, J. Proc. 4th Ann. Plant Biochem. and Physiol. Symp. Univ. of Missouri-Columbia, 1985, pp. 53-66.
40. Gaskin, P.; Gilmour, S. J.; Lenton, J. R.; MacMillan, J.; Sponsel, V. M. J. Plant Growth Regul. 1984, 2, 229-42.
41. Hedden, P.; Phinney, B. O.; Heupel, R.; Fujii, D.; Cohen, H.; Gaskin, P.; MacMillan, J.; Graebe, J. E. Phytochemistry 1982, 21, 391-3.
42. Sponsel, V. M. Physiol. Plant. 1985, 65, 533-8.
43. Spray, C. R.; Yamane, H.; Phinney, B. O.; Gaskin, P.; MacMillan, J., unpublished data.
44. Sembdner, G.; Schreiber, K. Flora, oder Allemeine Botanische Zeitung Abt. A 1965, 156, 359-63.
45. Phinney, B. O. Proc. Natl. Acad. Sci. U.S.A. 1956, 42, 185-9.
46. Katsumi, M.; Phinney, B. O.; Jeffries, P. R.; Henrick, C. A. Science 1964, 144, 849-50.
47. Phinney, B. O., unpublished data.
48. Wurtele, E. S.; Hedden, P.; Phinney, B. O. J. Plant Growth Regul. 1982, 1, 15-24.
49. Davies, L. J.; Rappaport, L. Plant Physiol. 1975, 55, 620-5.
50. Davies, L. J.; Rappaport, L. Plant Physiol. 1975, 56, 60-6.
51. Graebe, J. E., personal communication.
52. West, C. A., personal communication.
53. Coolbaugh, R. C., personal communication.
54. Smith, V. A.; MacMillan, J. J. Plant Growth Regul. 1984, 2, 251-64.
55. Smith, V. A.; MacMillan, J. Planta 1986, 167, 9-18.
56. MacMillan, J., personal communication.
57. Crozier, A. In "The Biochemistry and Physiology of Gibberellins"; Crozier, A., Ed; Praeger: New York, 1983; Vol. I, pp. 1-18.

RECEIVED June 2, 1986

## Chapter 4

# Fatty Acids in Plants: A Model System

Glenn Fuller<sup>1</sup> and P. K. Stumpf<sup>2</sup>

<sup>1</sup>Western Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Albany, CA 94710

<sup>2</sup>Department of Biochemistry and Biophysics, University of California, Davis, CA 95616

Fatty acids in plants are synthesized from acetate in the chloroplasts of leaf cells and the plastids of seeds. In leaf cells there are active desaturase enzymes which convert the fatty acids to linoleic and linolenic acids in large amounts. The predominant acyl lipids are triglycerides in seeds, phospholipids and glycolipids in leaves. Lemna minor was used to examine lipid synthesis in a plant which can grow under both light and dark conditions when provided proper nutrition.

In interactions of plants with their environment, changes in the membrane lipids of the host are often in evidence, especially chemical changes in the composition of fatty acids. The biosynthetic pathways to these fatty acids are modified and it is these changes which are the subject of much current study. De novo synthesis of saturated fatty acids in both procaryotes and eucaryotes is well understood. Subsequent desaturation reactions and pathways by which acyl glycerides are formed are not as well defined. In this chapter we will discuss fatty acid and triglyceride biosynthesis and will present data on using duckweed, a small aquatic weed, to study some of the reactions of lipids in plants.

### Fatty Acid Synthesis

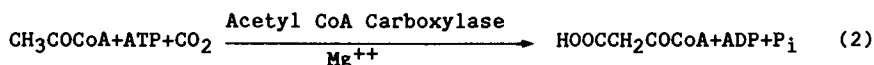
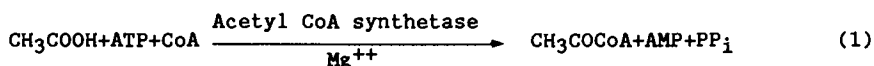
Synthesis of fatty acids in plants has been the principal subject of a number of recent reviews (1, 2, 3, 4). The properties of acyl lipids are determined by their fatty acid composition and distribution. For instance, Van der Waals bonding is stronger for saturated fatty acids than for cis-unsaturated acids having the same carbon number (5); thus, the saturated acids and their acyl lipids have higher melting points. There is some evidence that lower melting points or phase transition temperatures in membrane lipids correlate with chill resistance (6). The melting range, oxidation stability, and rheological properties of triglycerides

This chapter not subject to U.S. copyright.  
Published 1987, American Chemical Society

are all influenced by their composition and are critical to their commercial uses.

Each plant cell manufactures its own fatty acids, since there is no lipid transport in plants. The synthesis begins with acetate which is formed from pyruvate, formed from phosphoglycerate in the Calvin cycle in leaf tissue or via degradation of sugars in seeds or fruits. De novo synthesis of fatty acids takes place in the chloroplasts of vegetative tissues or in plastids of other plant tissues. Acetate is first esterified to the -SH function of coenzyme A (CoA) via an enzyme, acetyl CoA synthetase. Coenzyme A is an adenosine derivative attached to a 4'-phosphopantetheine moiety, a chemical subunit which is ubiquitous in the metabolism of fatty acids.

#### Formation of Acetyl and Malonyl CoA



The driving force for the reaction is the conversion of ATP to AMP. Acetyl CoA is then carboxylated by the enzyme acetyl-CoA carboxylase to form malonyl CoA. The carboxylase is actually a multienzyme complex in which one of the proteins is attached to biotin, the carrier of the carboxyl group. Again, the driving force for the reaction is hydrolysis of ATP. The carboxyl group added in this reaction is lost in the later condensation reactions. Although this seems inefficient, the condensation process is actually driven by the change in free energy resulting from decarboxylation.

The Fatty Acid Synthase (FAS) Sequence. The subsequent reactions of fatty acid synthesis are shown in Figure 1. The next sequence of reactions is the transfer of both the acetate and malonate moieties from CoA to acyl carrier protein (ACP). ACP is a protein on which the 4'-phosphopantetheine group already referred to is attached to the hydroxyl of a serine which is located approximately in the middle of the chain (amino acid number 36 of 77) in the ACP of *E. coli*. The ACP of both eucaryotic and procaryotic organisms has considerable homology. The ACP of *E. coli* can be used to carry acyl groups through the FAS reactions with both animal and plant FAS enzymes. Transfer of acyl groups to ACP is essential for the reactions to take place in plants. The enzymes responsible are acetyl-CoA:ACP transacylase and malonyl-CoA: ACP transacylase, also called acetyl and malonyl transferases.

There is evidence that in eucaryotic organisms other than plants the fatty acid synthase enzymes are associated in a complex in which several functions exist in a single protein. The synthase in animal liver has been shown to be a complex of two identical



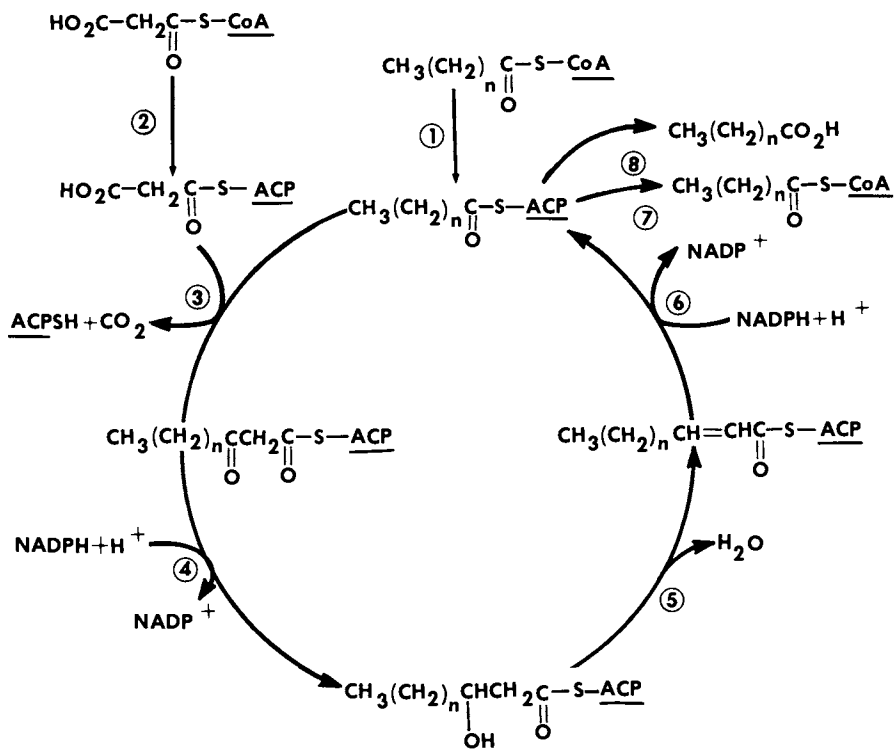


Figure 1. Fatty Acid Biosynthesis.

protein subunits oriented head to tail in which the various functions and the ACP are held together (7). Similarly in yeast the synthase is a complex of six  $\alpha$  and six  $\beta$  units. Each unit contains several of the specific functions of the system. In *E. coli* and other procaryotes the various enzymes of the FAS appear to be non-associated proteins in the cytosol. The FAS system in higher plants is similar to that of the procaryotes in that individual functions can be identified with specific proteins found in the tissue plastids (8). All of the ACP in plant leaf cells is found in the chloroplasts (9).

After conversion to acetyl-ACP and malonyl-ACP, two carbons of the malonyl-ACP are introduced via the condensing enzyme,  $\beta$ -ketoacyl-ACP synthetase. Loss of the malonyl carboxyl drives the reaction and in the first step of the sequence acetoacetyl-ACP is formed. The  $\beta$ -ketoacyl-ACP is then reduced to  $\beta$ -hydroxyacyl-ACP by NADPH and the enzyme  $\beta$ -ketoacyl-ACP reductase. The hydroxy acid is dehydrated to form a trans-2,3-enoyl-ACP which can be reduced by NADH or NADPH to the saturated ACP derivative (butyrate in the first series of steps). Condensation with malonyl-ACP is then repeated and the cycle continues to produce acyl-ACP derivatives with two additional carbon atoms until palmitoyl-ACP results. A second  $\beta$ -ketoacyl-ACP synthetase accomplishes addition of two more malonyl carbon atoms to allow the formation of stearoyl-ACP. The  $C_{16} \rightarrow C_{18}$   $\beta$ -ketoacyl-ACP synthetase has been shown to be a separate enzyme since it is more easily inhibited by arsenite and is less sensitive to the antibiotic, cerulenin, than the  $\beta$ -ketoacyl-ACP synthetase forming  $C_4$  to  $C_{16}$  keto acids.

Desaturation. Although the sequence produces stearate, there is very little stearic acid found in plant lipids. An active desaturase, the  $\Delta^9$  stearoyl ACP desaturase, also found in the chloroplast or plastids, forms a cis double bond between  $C_9$  and  $C_{10}$  of the carbon chain. This system requires both NADPH and ferredoxin. Stearoyl ACP is the physiological substrate. The major product is oleoyl-ACP, which may then be hydrolyzed or converted to oleoyl-CoA. Animals also produce oleic acid as a major product.

The animal and plant desaturase systems diverge at this point. Animal systems desaturate oleate only between the  $C_9$  double bond and the carboxyl group, while plants desaturate between the  $C_{10}$  carbon and the  $\omega$ -methyl. The plant desaturase systems giving rise to linoleic acid and  $\alpha$ -linolenic acid are not yet well characterized. This is because the desaturases are membrane-bound and have not been isolated. Current indications are that phosphatidyl choline with oleic acid at the 2-position is the substrate for the 18:1 $\rightarrow$ 18:2 reaction (2). There is evidence (10) that the 18:2 $\rightarrow$ 18:3 reaction takes place in the chloroplast and that the substrate is monogalactosyldiacylglyceride (MGDG).

#### Formation of Acyl Lipids

Synthesis of polar and non-polar lipids in plants as well as animals is via the glycerol phosphate or Kennedy pathway (5). In this pathway 3-sn-glycerol phosphate, formed by reduction of dihydroxyacetone phosphate, is acylated by acyl CoA in the 1- and

American Chemical Society

Library

1155 16th St., N.W.

Washington, D.C. 20036

2-positions. The transferases accomplishing this reaction appear to be different since there is a preference for saturated acids in the 1-position and shorter chain or less saturated acids in the 2-position. The diacyl phosphatidic acid produced may then be hydrolyzed to diacyl glycerol and further acylated to triglyceride or it may undergo reactions to form phospholipids or glycolipids. Formation of phospholipids is accomplished by reaction of diacylglycerol or phosphatidic acid with cytidine diphosphate (CDP) or CDP derivatives of ethanolamine or choline. Galactosyl glycerides are formed by reaction of diacyl glycerol with uridine diphosphate (UDP) derivatives of galactose.

### A plant model system

In our work we were seeking a plant system with which some of the specific reactions, e.g., desaturation and acyl lipid formation, could be studied. Lemnaceae (duckweeds) are small aquatic plants which grow rapidly, reproduce vegetatively and have a relatively simple morphology (11). They grow autotrophically in inorganic medium, heterotrophically in the dark with a carbon source, or photoheterotrophically. Because of their small size and simple growth requirements they can readily be maintained in axenic cultures, and hence, are an excellent model system for study of plant metabolism. Although Lemna minor and other duckweed species have been used extensively for biochemical studies, relatively little has been reported concerning fatty acid metabolism in Lemna. We proposed to determine differences, if any, in lipid metabolism under photoautotrophic, photoheterotrophic and totally heterotrophic growth conditions.

### Methods and procedures

Lemna minor in non-sterile culture was treated with Clorox solution diluted 9:1 with water. After four minutes in this solution the fronds were washed twice with sterile water and placed in a sterile solution of modified Hillman M medium (11) to which 1% sucrose had been added. After growth in this sterile culture medium, plants were repeatedly transferred throughout the experiments to 1) inorganic Hillman M medium; 2) Hillman M medium supplemented with 1% sucrose; and 3) Hillman M medium with 1% sucrose supplemented with 600 mg/liter tryptone and 100 mg/liter yeast extract. Lemna was grown in sterile flasks (2800-ml Fernbach flasks and 125 ml Erlenmeyer flasks) under continuous light at ca.  $180 \mu\text{mol m}^{-2} \text{sec}^{-1}$ . The flasks were stationary and maintained at ambient temperature of the laboratory. Cultures in sucrose and sucrose-tryptone medium were also grown in the dark. Light-grown plants were harvested after two weeks in the culture flask, dark-grown cultures after 6 weeks. Fronds were ground and extracted with 3:2 hexane-isopropanol and a portion of the lipids converted by methanol- $\text{H}_2\text{SO}_4$  esterification to esters for gas chromatography (12). Other portions of the extracted lipids were separated by 3-directional TLC (13) to determine distribution of lipids. Some 0.4g samples (wet wt.) of Lemna minor fronds were incubated in 1.5 ml of medium under the same conditions as they were grown (light, dark, different

media) with one  $\mu\text{Ci}$  of  $1\text{-}^{14}\text{C}$  acetate (sodium acetate- $1\text{-}^{14}\text{C}$ ,  $0.133 \mu\text{Ci}/\mu\text{l}$ ). Fatty acid distribution was measured using a radioactive detector and a mass detector on a gas chromatograph (DEGS column) or by collection of fractions from an HPLC column and lipid distribution by autoradiography of the TLC plates.

### Results

Table I reports the distribution of fatty acids in Lemna minor grown

Table I. Fatty Acids in Lemna Minor<sup>a</sup>

Medium	Fatty Acid (%)						
	Emergence before		Unknowns emerging before				
	16:0	16:0	18:0	18:0	18:1	18:2	18:3
Inorganic (light)	12	47	7	3	tr	7	24
Sucrose (light)	2	27	tr	3	6	21	38
Tryptone (light)	9	25	tr	tr	2	27	37
Sucrose (dark)	4	37	2	3	14	23	17
Tryptone (dark)	1	27	tr	2	16	28	25

<sup>a</sup> Determined by GC of fatty acid methyl esters.

in different media in light and dark. Growth in inorganic medium was less than half the rate of photoheterotrophic growth in sucrose and sucrose-tryptone. (Doubling time ca. 5 days vs. 36-48 hrs.) This is reflected in a slower de novo synthesis of fatty acid and a high accumulation of 16:0 and lower fatty acids. Among the  $\text{C}_{18}$  acids there were high levels of 18:3 acid in the autotrophic culture, perhaps indicating that the chloroplasts were very active. Sucrose and sucrose-tryptone in the light gave somewhat more normal ratios of 18-carbon acids with linolenic acid still predominating. The plants grown in the dark had lower than normal 18:3 acids, probably reflecting the absence of normal chloroplast organelles.

Labelled acetate incorporation. Lemna fronds grown in light and dark in sucrose medium were incubated with  $1 \mu\text{Ci}$  of  $1\text{-}^{14}\text{C}$  acetate for two, four and six hours. The most pronounced differences were at six hours; results are shown in Table II. With light-grown plants, incorporation of acetate was rapid and relative  $^{14}\text{C}$  activity of the various acids closely approached the composition ratios of these acids. In the dark, however, labelled 18:2 acid was low and 18:3 was practically not formed in six hours, indicating low 18:2 desaturase activity and extremely low 18:3

Table II.  $^{14}\text{C}$ -Acetate Incorporation into Fatty Acids of  
Lemna Grown in Sucrose Medium

6-Hour Incubation

	Light-grown <sup>a</sup>			Dark-grown <sup>a</sup>		
	16:0 &			16:0 &		
	18:1	18:2	18:3	18:1	18:2	18:3
% Total Fatty Acids <sup>b</sup>	33	21	38	33	24	33
% of $^{14}\text{C}$ Activity <sup>c</sup>	20	23	28	43	15	3

<sup>a</sup> Plants were incubated under the same light and dark conditions under which they were grown.

<sup>b</sup> By GC of methyl esters from Lemna grown on sucrose medium.

<sup>c</sup> By collection and counting of fractions from HPLC separations of fatty acid isopropylidenehydrazides.

activity. Thus, for rapid incorporation of acetate an active photosynthesizing system appears necessary. Lipids were separated into classes by three-directional TLC (see Table III). The incorporation of acetate into the lipids in light was rapid. In the dark,  $^{14}\text{C}$ -acetate was not quickly incorporated into the galactolipids. This fact correlates with the slow desaturation to  $\alpha$ -linolenate and is not surprising since up to 90% of the fatty acid in galactolipids is linolenate.

### Conclusions

Lemna minor was shown to be a representative plant when grown autotrophically and photoheterotrophically. Lipid and fatty acid patterns were in the range expected for plants. Growing the plants in the dark did not stop fatty acid biosynthesis but changed the pattern in that the amount of 18:3 acid was decreased. These results suggest a number of experiments to modify action of critical enzymes, especially the desaturases and makes Lemna a useful system for study of the reactions catalyzed by such enzymes. In addition to its ability to grow heterotrophically in the dark, Lemna minor is easily grown in sterile culture, a useful aspect when studies could be influenced by microbial-plant interactions. Moreover, large quantities of an active growing tissue under completely controlled conditions can be made available to the investigator.

Table III.  $^{14}\text{C}$ -Acetate Incorporation into Lipids of *Lemna*<sup>a</sup>  
Grown in Sucrose Medium

Lipid Class <sup>b</sup>	<u>Light-grown</u>		<u>Dark-grown</u>	
	Total Lipids	$^{14}\text{C}$ Lipids	Total Lipids	$^{14}\text{C}$ Lipids <sup>c</sup>
<u>Phospholipids</u>				
PC	++++	++++	++++	++++
PE	++	+++	++	+++
PG	++	+++	+	+++
PA	+	+	+	+
PI	+	+	+	+
<u>Glycolipids</u>				
MGDG	++++	+++	++	?
DGDG	++	+	++	-
<u>Sulfolipid</u>				
SQDG	+	+	-	-
<u>Netural Lipids</u>				
FFA	+	++	+	++
TG	+	++	+	+
MG & DG	+	+	+	+
SE	+	+	++	+

<sup>a</sup> After 6-hour incubation in dark or light. From autoradiogram of TLC plate and charring of total lipid plate.

<sup>b</sup> Abbreviations PC = Phosphatidylcholine; PE = Phosphatidylethanolamine, PG = Phosphatidylglycerol; PA = Phosphatidic acid; PI = Phosphatidylinositol, MGDG = Monogalatosyldiglyceride; DGDG = Digalactosyldiglyceride; SQDG = Sulfoquinovosyldiglyceride; TG = Triglyceride; MG = Monoglyceride; DG = Diglyceride; SE = Sterol Ester; FFA = Free fatty acid.

<sup>c</sup> Qualitative inspection of charred spots on TLC plate and of autoradiogram, + = very light, ++++ very heavy.

Acknowledgments

The authors wish to thank Elaine Tobin for suggesting modifications to Lemna media and Raymond Pacovsky for preparation of Figure 1. Thanks are also due to Gisela Travis and Emily Grey for typing this and several other manuscripts in the book.

Literature Cited

1. Stumpf, P. K. in "Fatty Acid Metabolism and Its Regulation", Numa, S., Ed., Elsevier, New York, 1984, Chap. 6.
2. Roughan, P. G. and Slack, C. R. 1982, Ann. Rev. of Plant Physiol. 33:97-132.
3. Jeffcoat, R., 1977, Biochem. Soc. Transactions 5:811-818.
4. Hitchcock, C. and Nichols, B. W., "Plant Lipid Biochemistry", Academic Press, London, 1971.
5. Gurr, M. I. and James, A. T., "Lipid Biochemistry. An Introduction", 3d edition, Chapman and Hall, London, 1980.
6. Bishop, D. G. in "Biosynthesis and Function of Plant Lipids", Thomson, W. W.; Mudd, J. B. and Gibbs, M. Eds., Amer. Soc. of Plant Physiologists, Rockville, MD, 1983, pp. 86-89.
7. Wakil, S. J., Stoops, J. K. and Joshi, V. C., 1983, Ann. Rev. Biochem. 52: 537-579.
8. Stumpf, P. K., Shimata, T., Eastwell, K., Murphy, D. J., Liedvogel, G., Ohlrogge, J. B. and Kuhn, D. B. in "Biochemistry and Metabolism of Plant Lipids", Wintermans, J. F. G. M. and Kuiper, P. J. C. Eds., Elsevier Biomedical Press i,B.V. (1982), pp. 3-11.
9. Ohlrogge, J. B., Kuhn, D. N. and Stumpf, P. K. (1979) Proc. Nat. Acad. Sci. U.S.A. 76: 1194-1198.
10. Murphy, D. J., Harwood, J. L., Lee, K. A., Roberto, F., Stumpf, P. K. and St. John, J. B. (1985), Phytochemistry 24: 1923-1929.
11. Posner, H. in Methods in Developmental Biology, Witt, F. H. and Wessels, N. K., Eds., Crowell, New York, 1967, pp. 301-317.
12. American Oil Chemists Society. Official and Tentative Methods. 3d ed. 1984. Method No. Ce2-66.
13. Kramer, J. K. G., Fouchard, R. C. and Farnworth, E. R., 1983, Lipids, 18: 896-899.

RECEIVED September 26, 1986

## Chapter 5

# Chemical and Biological Aspects of Brassinolide

Werner J. Meudt

Plant Hormone Laboratory, Agricultural Research Service, U.S. Department of  
Agriculture, Beltsville, MD 20705

Brassinolide (BR), (2 $\alpha$ ,3 $\alpha$ ,22 $\alpha$ ,23 $\alpha$ -tetrahydroxy-24 $\alpha$ -methyl-B-homo-7-oxa-5 $\alpha$ -cholestan-6-one), a biologically active steroidal lactone first isolated from rape Brassica napus L.), pollen, stimulates growth of green plant tissues. Although the mechanism responsible for observed BR effects remains to be determined, the action of BR on growth is oligodynamic, and rapid. BR affects specific target tissues that are sensitive to the plant hormone indole-3-acetic acid (IAA)-induced growth (apparently without affecting IAA uptake and/or transport) and tissues that are gravi-perceptive. Structural analogues of BR were synthesized and the stereospecificity for its biological activity determined. Brassinosteroids thus provide plant scientists with a plant sterol for which physiological significance is demonstrated.

The isolation of a growth stimulating chemical agent from pollen is not new. Hans Fitting, a renowned plant physiologist (1877-1970), isolated the first phytohormone, from orchid pollen (1) and recognized its importance in the development of plants. Fitting adopted the term "hormone" (first proposed by E.H. Starling in 1905 (2)) from the medical field and introduced it to the field of developmental physiology of plants.

Fitting proclaimed that "Alle derartigen Stoffe, die im eigenen Stoffwechsel des Organismus erzeugt, ohne Nahrungsstoffe zu sein... welche die Entwicklungsvorgänge beeinflussen, also entwicklungsphysiologisch von Bedeutung sind, "Hormone" zu nennen."

Fitting's attempt to identify his "Pollen Hormone" chemically was unsuccessful. Today, however, we know that pollen tissues are the source of the known plant hormones (auxin, gibberellins, cytokinin), as well as "brassinolide."

The year of Fitting's death, 1970, was also the year that Mitchell et al (3) reported on the presence of a biologically active agent in rape (Brassica napus L) pollen that caused growth aberrations when applied to second internodes of 7-day old bean

This chapter not subject to U.S. copyright.  
Published 1987, American Chemical Society



seedlings. The growth effect observed was sufficiently different from growth effects induced by gibberellins or auxins for them to suspect the presence of a new class of plant growth stimulator, which subsequently was named "brassinolide" (BR). Brassinolide and structurally related brassinosteroids have since been isolated from various plant sources (See below).

Typically, picomole quantities of brassinolide applied to young bean seedlings will cause cell elongation, cell division, and splitting of the treated internode. The symptoms are confined to the treated area which suggests little or no movement of the material up or down the plant axis (4). Although first incorrectly identified as a fatty acid glucoside (5) the growth response observed was primarily attributed to the presence of the steroidal lactone, brassinolide (6).

Subsequent studies in our laboratory showed that biologically active brassinosteroids characteristically increases the sensitivity of internodal tissues of light grown bean seedlings to auxin treatments and to geotropic stimulations. Acknowledging that other auxin induced responses, such as pH changes of the incubation media (7) and ethylene production can be increased by brassinosteroids (8,9), the effect of BR on growth is not explicable on the basis of ethylene production or proton excretion because these effects are also brought about by steroids other than brassinolide (10) and may also occur in mature and aged tissues that otherwise do not grow in response to auxin, BR or geotropic stimulations. The brassinolide effect is rapid, oligodynamic and is dictated by stringent structural requirements of the molecule (11) as well as by target tissue specificity.

The review deals with the chemistry and physiology of brassinosteroids from which it may be deduced that BR accelerates certain cellular processes that regulate the sensitivity of tissues to auxin and geotropic stimulations and thus may fulfill a regulatory function that permits plant tissue to react to environmental (geotropism) as well as chemical (auxin) perturbations.

### Chemistry of Brassinolide

The discovery of brassinolide by Mitchell et al. about 15 years ago (3), and its subsequent chemical identification that showed it to be a steroidal B-ring lactone (6), introduced chemists to a novel steroidal structure. Characteristically, plant sterols contain 23 carbons comprising an unsaturated 1,2-cyclopenten-anthrene system. Joined to this nucleus are angular methyl groups at carbons 10 and 13 and a nine-carbon side chain at carbon 17. The structure of brassinolid is different from other phytosteroids, in that its molecule is a steroidal lactone with the oxygen function in the enlarged B-ring, and the structural skelton is completely saturated. The enlarged B-ring lactone, composed of carbons 6,7,8,9, and 10, is unprecedented in a natural sterol. In addition to the structural characteristics indicated above, biologically active brassinosteroids must also contain four hydroxyl groups, each positioned at carbons 2 and 3 of the A-ring and at carbons 22 and 23 in the side chain. The 2,3-hydroxyl groups project to the rear (alpha) of the basic structure. The junction between rings A and B is trans, yielding the

5 alpha series of compounds representing almost completely planar molecules. Biologically active brassinosteroid molecules also possess a methyl group at carbon 24, which is also alpha-orientation.

The chemical identity and biological activity has been verified through the use of synthetic brassinolide. A number of biologically active epimers and biologically inactive isomers were used in the study of structure activity relationships (11,12).

The structure of brassinolide, along with its three biologically active 22,23-cis glycolic isomers, is shown in Figure 1 (structures I, II, and III). The biological activity of the isomers is about 50% that of brassinolide (12). Activity is lost whenever the orientation of the 2,3-cis glycolic groups are beta, as in structure IV in Figure 1.

In summary the structural requirements are: a trans A/B ring system (alpha-hydrogen), a 6-ketone or a 7-oxa-6-ketone system in ring B, cis alpha-oriented hydroxyl groups at C-2 and C-3, cis hydroxy groups at C-22 and C-23 as well as an alkyl substituent at C-24.

It is of interest that the structure of brassinolide resembles that of the insect molting hormone, ecdysone, which also is of plant origin. The structure of ecdysone is shown in Figure 1 and differs from the structure of brassinolide in that the orientation of the vicinal hydroxyl group at C-2 and C-3 is beta, the A/B junction is cis rather than trans as in the brassinolide structure and that ecdysone lacks the lactone oxygen in the B-ring.

Ecdysones, that control all of the processes that are connected with the ecdyses of an insect are growth and differentiation hormones in insects that are similar in function as estrogens and androgens of mammals. It is highly conjectural, but thought provoking, that similarity of structure should reflect similarity in function. It might be suggested that brassinolides have similar "signal" functions in plants as steroidal hormones have in animals by functioning as a primary chemical messenger during early events of embryogenesis and growth.

#### Naturally Occurring Brassinosteroids in Plants.

Since the discovery of brassinolide in rape pollen, 11 additional biologically active isomers have been isolated from higher plants. With the exception of pollen tissues, all of the brassinosteroids that were isolated were found only in young, immature, actively growing and differentiating plant tissues, including immature seeds and shoots of a variety of plants.

The trivial and IUPAC equivalent names of brassinosteroids and related compounds are given in Table 1. It should be noted, with the exception of typhasterol and teasterone, that the stereochemistry of the 2,3- and 22,23- diol groupings are alpha oriented, as in the brassinolide structure, and that they differ from the brassinolide structure only by the alkyl substituent at C-24 and the degree of oxidation of the B-ring (Figure 2). All of the brassinosteroids listed are biologically less active than brassinolide.

It is suggested that the biological activity of some of these brassinosteroids might be due to their conversion to brassinolide by the plant tissue during the bioassay (15). This will require further investigation.

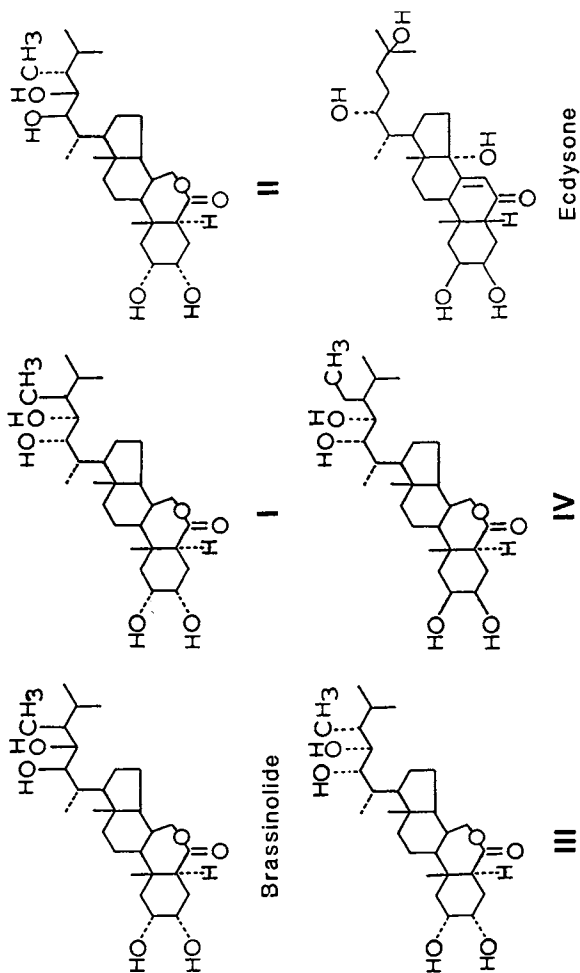


Figure 1. Structures of BR and its 3 biologically active 22, 23-cis glycolic isomers (I, II, and III). Structure IV is the 2,3-beta-dihydroxy isomer of III and is biologically inactive. Ecdysone, (lower right) is the structure of the insect molting hormone shown here for purpose of comparison with structure of brassinosteroids.

Table I. Trivial and IUPAC Equivalent Names

---

Brassinolide	= (22R,23R)-2 $\alpha$ ,3 $\alpha$ ,22,23-Tetrahydroxy-24S-methyl-B-homo-7-oxa-5 $\alpha$ -cholestan-6-one
I	= (22S,23S)-2 $\alpha$ ,3 $\alpha$ ,22,23-Tetrahydroxy-24S-methyl-B-homo-7-oxa-5 $\alpha$ -cholestan-6-one
II	= (22R,23R)-2 $\alpha$ ,3 $\alpha$ ,22,23-Tetrahydroxy-24R-methyl-B-homo-7-oxa-5 $\alpha$ -cholestan-6-one
III	= (22S,23S)-2 $\alpha$ ,3 $\alpha$ ,22,23-Tetrahydroxy-24R-methyl-B-homo-7-oxa-5 $\alpha$ -cholestan-6-one
IV	= (22S,23S)-2 $\alpha$ ,3 $\alpha$ ,22,23-Tetrahydroxy-24S-ethyl-B-homo-7oxa-5 $\alpha$ -cholestan-6-one
Ecdysone	= (22R)-2 $\alpha$ ,3 $\alpha$ ,14 $\alpha$ ,22,25-Pentahydroxy-5 $\beta$ -cholest-7-en-6-one
6-Deoxocastasterone	= (22R,23R)-2 $\alpha$ ,3 $\alpha$ ,22,23-Tetrahydroxy-24S-methyl-5 $\alpha$ -cholestane
Deoxodolichosterone	= (22R,23R)-2 $\alpha$ ,3 $\alpha$ ,22,23-Tetrahydroxy-5 $\alpha$ -ergost-24(28)-ene
28-Norbrassinone	= (22R,23R)-2 $\alpha$ ,3 $\alpha$ ,22,23-Tetrahydroxy-5 $\alpha$ -cholestan-6-one
28-Norbrassinolide	= (22R,23R)-2 $\alpha$ ,3 $\alpha$ ,22,23-Tetrahydroxy-B-homo-7-oxa-5 $\alpha$ -cholestan-6-one
Dolichosterone	= (22R,23R)-2 $\alpha$ ,3 $\alpha$ ,22,23-Tetrahydroxy-5 $\alpha$ -ergost-24(28)-en-6-one
Dolicholide	= (22R,23R)-2 $\alpha$ ,3 $\alpha$ ,22,23-Tetrahydroxy-B-homo-7-5 $\alpha$ -ergost-24(28)-en-6-one
Castasterone	= (22R,23R)-2 $\alpha$ ,3 $\alpha$ ,22,23-Tetrahydroxy-24S-methyl-5 $\alpha$ -cholestan-6-one
Homodolichosterone	= (22R,23R)-2 $\alpha$ ,3 $\alpha$ ,22,23-Tetrahydroxy-5 $\alpha$ -stigmast-24(28)-en-6-one
28-Homodolichonlide	= (22R,23R)-2 $\alpha$ ,3 $\alpha$ ,22,23-Tetrahydroxy-B-homo-7-oxa-5 $\alpha$ -stigmast-24(28)-ene-6-one
28-Homobrassinone	= (22R,23R)-2 $\alpha$ ,3 $\alpha$ ,22,23-Tetrahydroxy-24S-ethyl-5 $\alpha$ -cholestan-6-one
28 Homobrassinolide	= (22R,23R)-2 $\alpha$ ,3 $\alpha$ ,22,23-Tetrahydroxy-24S-ethyl-B-homo-7-oxa-5 $\alpha$ -cholestan-6-one
Teasterone	= (22R,23R)-3 $\beta$ ,22,23-Trihydroxy-24S-methyl-5 $\alpha$ -cholestan-6-one
Typhasterol	= (22R,23R)-3 $\alpha$ ,22,23-Trihydroxy-24S-methyl-5 $\alpha$ -cholestan-6-one

---

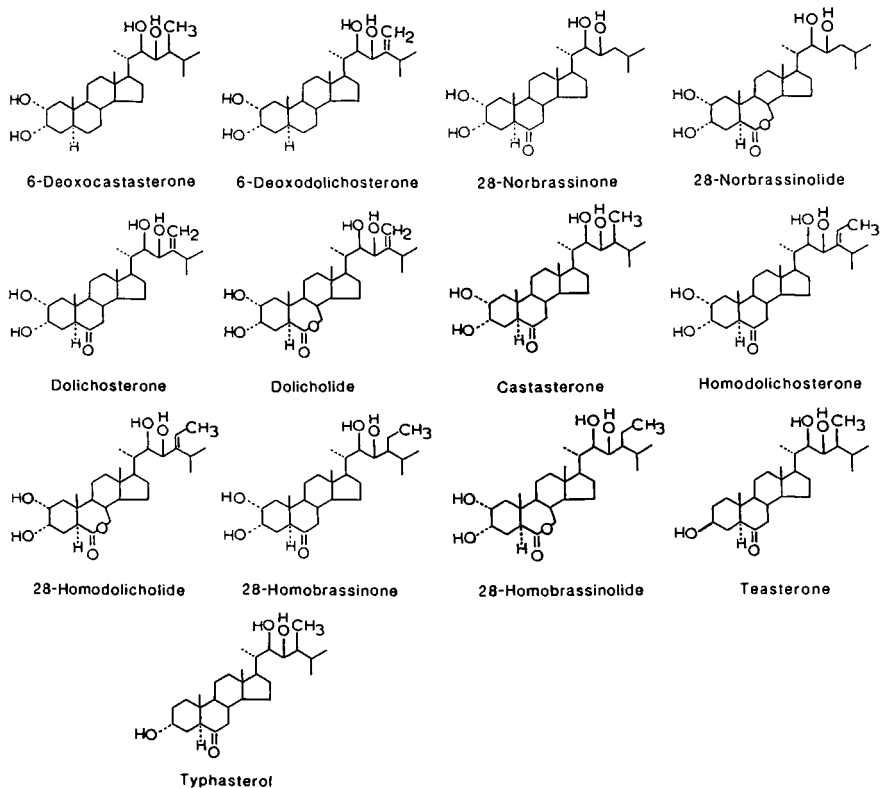


Figure 2. Structure of naturally occurring brassinosteroids. Trivial and IUPAC equivalent names are given in Table I.

Brassinolide. Brassinolide, the most biologically active brassinosteroid, was first isolated from rape pollen (3) affording about 40 micrograms of brassinolide per kg of fresh pollen (52). Brassinolide represents only about 0.01% of the total steroid content in rape pollen. Rape pollen is a rich source of steroid in general, constituting about 0.1% of the fresh weight. The major steroids being 24-methylene cholesterol (13), avenasterol, cholesterol, and  $\beta$ -sitosterol (14). Brassinolide has since been isolated from a variety of plants including chestnut gall (17,27), immature seeds of chinese cabbage (15,23) and in tea leaves (26). The concentrations are usually very low ranging from 0.25  $\mu\text{g}/\text{kg}$  (17) to 0.57  $\mu\text{g}/\text{kg}$  (27) in chestnut gall tissues. In comparison, the content of castasterone in these tissues is about five times as high and that of 6-deoxocastasterone was 20 to 100-fold higher than brassinolide. Small amounts of brassinolide were also detected in immature seeds of Chinese cabbage (15,23) and in tea leaves (26). The amounts in each instance were less than 10 ng/kg Fr. wt.

6-Deoxocastasterone. 6-Deoxocastasterone lacks both oxygen functions in the B-ring of brassinolide. It is present in extracts of immature seeds of *Phaseolus vulgaris* (15), in young insects galls of chestnut trees (*Castanea crenata* Sieb Et Zucc.) (16) and in healthy tissues including shoot, leaf and flower bud of the chestnut. The larvae collected from the gall contained at best a trace amount of activity which, most likely was derived from the host tissues and thus the brassinosteroid is exclusively of plant origin. The amounts present in the various tissues range between 9 and 25  $\mu\text{g}$  of the 6-Deoxo analogue per kg fresh weight. The biological activity is about one-fiftieth of that of brassinolide (15).

6-Deoxodolichosterone. 6-Deoxodolichosterone which is the dehydro derivative of 6-deoxocastasterone, is found in immature *P. vulgaris* seeds (15). The amounts isolated and the biological activity were not indicated by the authors.

28-Norbrassinone. 28-Norbrassinone or brassinone or 28-nor-castasterone is the 6-ketone analogue of 28-norbrassinolide, lacking the lactone function in the B-ring and an alkyl group at C-24. It is present in immature seeds and sheaths of Chinese cabbage (*Brassica campestris* cv. Pekinensis), leaves of green tea (*Thea sinensis*), and insect galls of chestnut trees at quantities of 0.78, 2.0 and 11 ng per kg fresh weight, respectively (16). The presence of brassinone in insect galls could, however, not be confirmed. Arima et al. (17) claim that the SIM peak of brassinone reported by Abe et al. (16) had a shorter retention time than authentic brassinone and that the peak in question might be due to 6-deoxocastasterone. The biological activity of brassinone is, at best, 1% of brassinolide (12,18,19).

Dolichosterone. Dolichosterone is the methane derivative of castasterone and differs from the brassinolide structure by the absence of the lactone function in the B-ring and by having a methylene rather than a methyl function at C-24. The brassinosteroid was isolated from immature seeds of *Dolichos lablab* (hyacinth-bean)

(20,21) and from fresh rice (*Oryza sativa* cv. Aborio J. ) shoots (22). The hyacinth bean seeds yielded about 1.5  $\mu\text{g}$  of this ketone per kg of fresh tissue, and the rice shoots yielded only about 8 ng per kg fresh weight. This ketone is biologically less active than brassinolide, but no quantitative activity data were presented.

Castasterone. Castasterone is the 6-ketone analogue of brassinolide and is the most biologically active analogue of brassinolide. It is about 50% as active as brassinolide (12,23). Castasterone was first isolated from chestnut insect galls (23) and subsequently has been found to be widely distributed in plants, including immature seeds and sheaths of Chinese cabbage (24), rice shoots (22) green tea leaves (16,25,26), immature seeds of hyacinth-bean (21), gall, leaf, shoot, and flower buds of *C. crenata* (17,27), and immature seeds of *P. vulgaris* (15).

Chestnut gall, non-gall tissues as well as green tea leaves are a rich source of castasterone, yielding about 2 to 7  $\mu\text{g}$  per kg tissues (16); all of the other tissues examined contain 100 ng/kg or less.

Homodolichosterone. Homodolichosterone thus far has been isolated from immature seed of *D. lablab* (20) (.6  $\mu\text{g}/\text{kg}$  immature seeds). It is the ethylidene analogue of castasterone and is only slightly biologically active (data not given) (20).

28-Homobrassinone. 28-Homobrassinone or 24-ethylbrassinone was isolated from Chinese cabbage (0.1 ng/kg) and from green tea leaves (0.5 ng/kg) (16). The biological activity of this ketone was tested on radish and tomato seedlings and found to be only 0.1% as active as brassinolide and was inactive on tomatoes (18).

28-Norbrassinolide. 28-Norbrassinolide was isolated from Chinese cabbage (about 1 ng/kg) (16). It differs in structure from brassinolide by the absence of an alkyl function at C-24 and is biologically as active as its 6-ketone analogue (brassinone). It has 1% of the activity of brassinolide as assayed in the bean test (12) but was as active as brassinolide in the radish test and had 10% of the activity of brassinolide in the tomato test (18).

Dolicholide. Dolicholide was first isolated from immature hyacinth-bean (*Dolico lablab* L.) (about 5 ng/kg fresh weight) (28) and subsequently from immature bean seeds (*Phaseolus vulgaris* cv. Kentucky Wonder) (15). It resembles brassinolide in structure except that it is unsaturated, carrying a methylene at C 24. The biological activity is about one tenth of that of brassinolide in the rice-lamina inclination assay (28).

28-Homodolicholide. 28-Homodolicholide is the ethylidene analogue of brassinolide which, with brassinolide and castasterone, was isolated from immature seed of *D. lablab* (21). The seeds contained about 300 ng/kg fr. wt. The biological activity has not yet been determined.

28-Homobrassinolide. Since sitosterol and cholesterol are widely distributed in the plant kingdom, the presence of 28-norbrassinolide and 28-homobrassinolide might be expected. This prompted Abe et al. (16) to focus their attention on isolation and identification of these two brassinosteroids from Chinese cabbage, green tea, and insect galls of the chestnut tree. Thus far only the 28-norbrassinolide has been found. They were not able to detect 28-ethylbrassinolide, even though the corresponding ketone analogue (24-ethyl-brassinone) was present in Chinese cabbage and in green tea leaves.

Typhasterol. Typhasterol and Teasterone are the first tri-hydroxy 6-ketone sterols (2-deoxy-castasterones and its 3-epimer) isolated that show biological activity in the rice lamina joint bending test despite the lack of a hydroxyl group at C-2. Typhasterol was isolated from cattail (Typha latifolia L) pollen (29), and both teasterone and typhasterol were present at 60 and 15 ng respectively per kg fr. wt. of leaves and were isolated from the less polar active fraction obtained from tea leaves (30). The biological activity of these two ketones as tested in the rice test, is about one tenth that of brassinolide (30). Typhasterol has recently been synthesized (31), but its biological activity was not tested.

#### Quantitative Bioassays for Brassinosteroids.

Brassinolide was tested on 17 bioassays for growth substance. The results led to claims that brassinolide possesses a broad spectrum of biological activity, including gibberellin-, auxin- and cytokinin-like activity (32,34). These claims must be treated with some caution however, since the claimed "specificity" of some of the bioassays selected is questionable. At present three bioassay techniques (35,36,37) are used routinely for the detection of brassinolide activity. All three assays are sensitive to auxin, which is a prerequisite for the detection of brassinolide-like compounds. This is not to say that brassinolide has auxin-like activity, but rather there seems to be an interaction of cooperative action between auxin and BR.

The three main bioassays used in studies for the isolation and characterization of brassinolide and other brassino-steroids are: the bean second internode bioassay (35); rice-lamina inclination test (36,38,40) as modified by Arima et al. (40); and bean first internode bioassay (37).

Bean Second Internode Bioassay. This assay was developed by Mitchell and Livingston (35) for the detection of growth stimulants and inhibitors in general. Test materials to be assayed are first dispersed in 250 g of fractionated lanoline, and then applied to the second internode of a 7-day old bean plant (Phaseolus vulgaris L. cv Pinto). The second internode should not be more than 2 mm long for maximum effect. The response usually is measured 4 days after treatment and is semi-quantitative. One ng of brassinolide causes elongation, but 100 ng or more causes the treated internode to thicken and split (characteristic of a brassinolide response). The growth response to brassinolide is restricted to the division and elongation of undifferentiated parenchyma cells in the treated



internode. Parenchyma cells that are slightly more mature in their anatomical development fail to grow in response to brassinolide treatments. Tissues subtending the treated portion of the internode also do not respond (4).

Rice - Lamina Inclination Test. Rice seeds (*Oryza sativa* L. cv. Koshihikari) are germinated in water for 2 days in the light and then planted on 1% agar and grown for 7 days in the dark, at which time the plantlets are exposed to red light for 1 to 2 hours per day. Leaf sections, excised from the second leaf of the etiolated rice seedlings are used for the bioassay of BR. The excised leaf sections consist of laminae, lamina joints, and 2 cm of sheaths. The isolated segments are floated on water for 1 day in the dark. Explants, which are bent 15 degrees, are selected and placed in a petri dish containing 20 ml of aqueous solution of test sample. As in an IAA response, biologically active BR induce the laminae to bend. The angle between the laminae and sheaths, is measured 2 days after treatment. This test is faster than the bean second internode test and has the capability of detecting nanogram quantities of brassinolide.

Bean First Internode Bioassay. This assay was originally designed for the detection of auxin activity (37). It takes advantage of the fact that unilaterally applied auxin causes bending of the treated bean internode, which reaches a maximum rate about 20 minutes after auxin application. The sections will respond to 10 pmol of IAA and BR increase the sensitivity of the sections to auxin by one order of magnitude.

The bioassay is performed on isolated first internode section of 7-day-old bean plants (*P. vulgaris* L. cv. Bush Burpee Stringless Bush Bean). The seedlings are grown in soil or perlite in a temperature controlled light room (25C) equipped with cool white (SHO 120 V-200 W) fluorescent lights at a photoperiod of 4 hours and an irradiance of 4-8 Wm<sup>-2</sup> (400-700nm). Under these conditions, the seedlings are partially etiolated, the first internodes are 4-5 cm long, and the second internode has not started to develop. The first internode section is isolated and cut to a uniform length of 4 cm. The sections are placed into scintillation vials equipped with a sponge plug saturated with 0.01M phosphate buffer pH 6.1. Three cm of the upper portion of the section protrudes from the neck of the vial.

The test material is first dissolved in ethanol and 10 $\mu$ l are transferred to a small (5mm diameter) filter paper disk. The disks are dried and sandwiched between the lower part of the internode and the moistened sponge close to the vial. The position of the apical portion of the section is recorded and the vial is placed in a high humidity chamber. Subsequent measurements are taken at hourly intervals, over a period of 4 hours.

Biologically active brassinosteroids by themselves cause little curvature; however, if a brassinolide treatment is co-applied with auxin the ensuing curvature is greater than the sum total of IAA and brassinolide applied separately (Figure 3).

This bioassay has the advantage that test materials may be applied in sequence by removing one treated disk and replacing it with another. Brassinolide is most effective when it precedes or

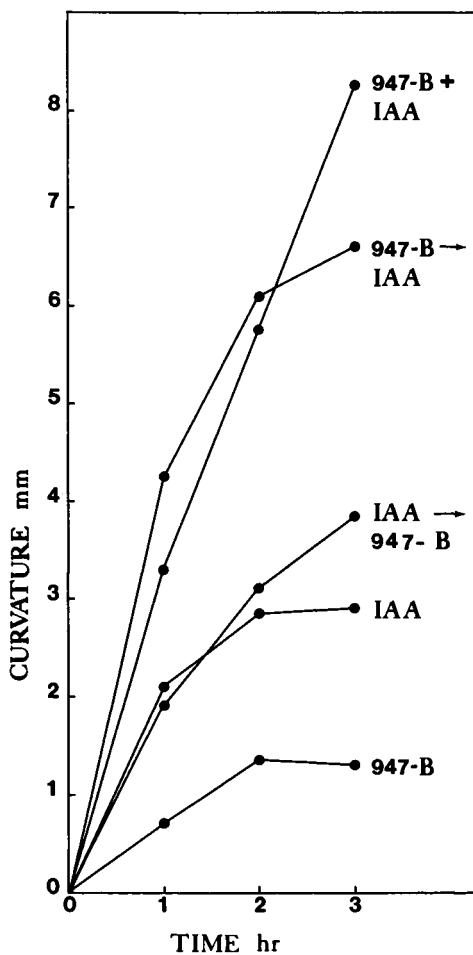


Figure 3. Effect of sequential treatments on bending response of bean first internode sections. Sections were treated with disks containing BR (947-B) ( $1.0 \mu\text{g}/\text{segment}$ ), IAA ( $0.1 \text{ nmol}/\text{segment}$ ). Order of treatment is as indicated: (947-B $\rightarrow$  IAA) indicates that sections were first treated for 10 minutes with BR followed by IAA, (IAA $\rightarrow$  947-B) indicates the reverse. Sections treated with disks containing only solvent did not bend.

coincides with IAA treatments. No BR response is observed when the sequence of application is reversed. This assay is sensitive to 1.0 ng quantities of BR.

#### Auxin-Brassinolide Interaction

The rate of change of horizontal displacement of the apical portion of bean first internode sections treated unilaterally in the manner described above (bioassay) with brassinolide and/or IAA was monitored by using an angular transducer (in accordance with the technique described by Meudt and Bennett (37)).

The results, presented in Figure 4, show that 0.1 nmol of IAA caused a transient growth, with a maximum rate reached about 20 minutes after auxin application. The start of the bending was preceded by a 10-minute lag. Growth rates diminished gradually only to be interrupted by periodic oscillation of the rates (curve A). Brassinolide lacks significant biological activity of its own, particularly during the first 40 minutes, after which bending does occur and reaches a maximum rate about 75 minutes after initial start of the treatment (trace B). Since the growth pattern in response to increasing amounts of BR does not change, we assume that the bending observed is due to the interaction of BR and endogenous auxin rather than to brassinolide itself. When brassinolide is applied in combination with auxin, the auxin-induced growth is greatly enhanced. Brassinolide apparently does not affect the first part of the growth kinetics induced by IAA. This suggests that the rate of uptake of IAA is not affected and that brassinolide has little effect on the metabolic events that bring about the first spurt of growth induced by IAA. The change in BR-induced growth kinetics occurs primarily during the second growth phase (C), i.e., after 30 minutes. This suggests that brassinolide regulates some metabolic event that is responsible for sustained auxin action on growth rather than initial processes involving uptake and (or) transport. The data indicate the existence of a strong synergistic interaction between brassinolide and IAA; however, follow-up experiments showed this apparent synergistic relationship does not hold true when the two treatments are applied in certain sequence. Results shown in Figure 5 demonstrate that brassinolide stimulates auxin-induced growth when brassinolide treatment precedes the auxin treatment (solid line). This apparent mutual relationship does not hold true when the sequence of application is reversed (dotted line). The growth rate of IAA pretreated sections is not increased by a subsequent treatment with brassinolide. These sections are, however, sensitized to auxin, as shown when the brassinolide treatment is again replaced by IAA (dotted line - 3rd hr).

Figure 6 shows that by pretreating IAA-sensitive tissues with as little as 10 pmol of IAA reduces the sensitivity of the tissues to subsequent auxin applications and that an application of 1 nmol of IAA desensitizes the tissues completely. This attenuation of IAA-induced growth after a chronic stimulation is prevented by pretreating the tissues with as little as 100 pmol of brassinolide. One possible explanation of these results is that the initial application of IAA reduced the ability of tissues to take up additional IAA by blocking putative transport channels.

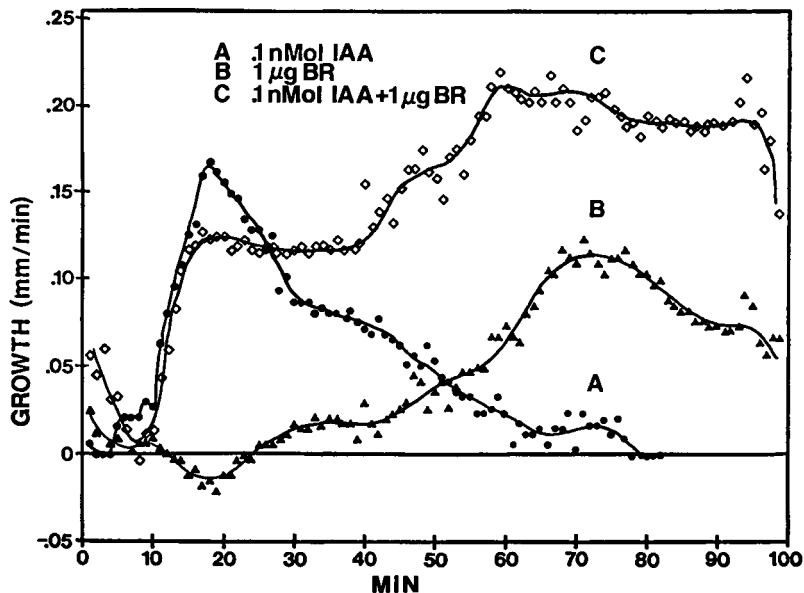


Figure 4. Rate of horizontal displacement of apical portion of bean internode sections treated with IAA (A) and BR (B) and IAA plus BR (C).

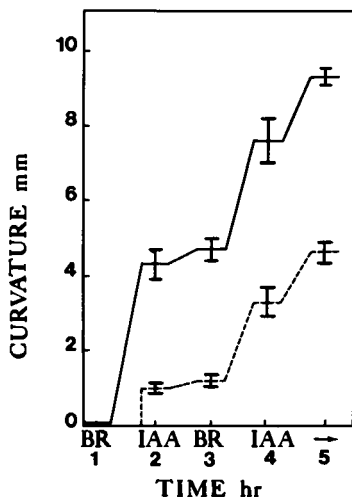


Figure 5. Effect of alternating BR and IAA treatments on curvature of bean first internode sections. Sections were treated alternately either with 100 pmol of BR followed by 100 pmol of IAA (solid line) or first with IAA followed by BR (dotted line). Treatments were exchanged at hourly intervals and measurements were taken at the end of each treatment.

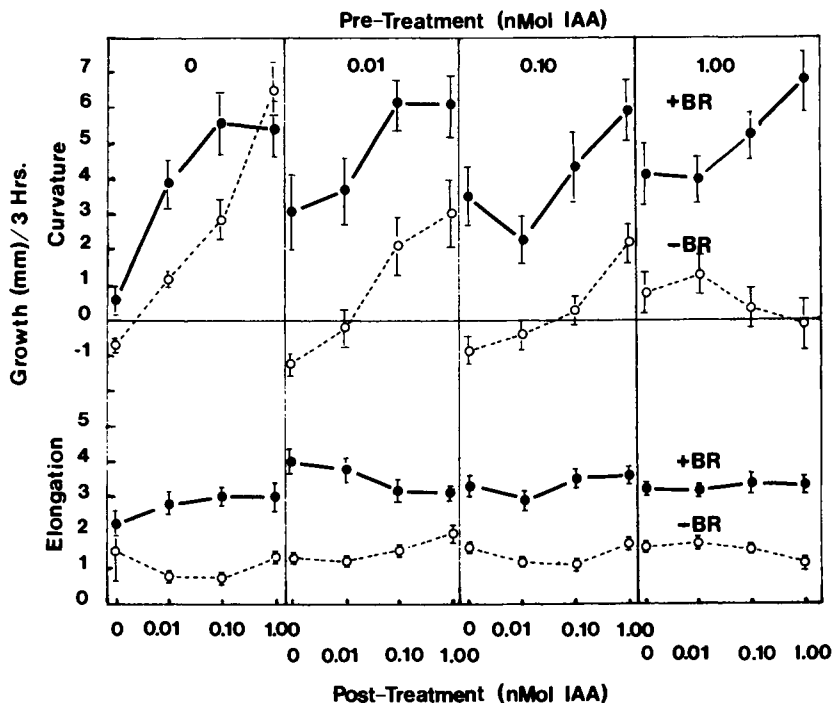


Figure 6. Prevention of IAA induced autogenous growth inhibition (bending of bean internode sections) (upper data) by BR. Bean internode section were pretreated for 1 hour with either IAA (dotted line) or IAA plus 200 pmol of BR (solid line). Data in lower graph show longitudinal growth of the internodes.

Brassinolide somehow keeps these channels open, thus increasing the uptake or transport capacity of a brassinolide-treated tissue for IAA.

Analysis of tissues for IAA as determined by reverse isotope dilution assays reveals however, that brassinolide does not affect auxin uptake or auxin movement within the tissues (41). In this same study we also observed that bean internode sections treated with brassinolide contained significantly less IAA after 2 hours than tissues treated with IAA alone, even though BR potentiated IAA-induced growth by more than 400%.

#### Brassinolide Effect on Proton Secretion

The rapidity with which BR acts and its chemical nature suggests that membrane sites associated with IAA action should be affected. Indeed, one can observe IAA-induced rapid changes of electropotentials across cytoplasmic membranes that precede cell enlargement, and brassinolide stimulates this process (7).

Unfortunately, changes in electropotentials across cytoplasmic membranes may also be induced by steroids that are structurally unrelated to BR (10) and do not stimulate auxin induced growth.

#### Brassinolide-Gibberellic Acid (GA) Interaction

The physiological effect of biologically active brassinosteroid on the growth of dwarf rice seedlings was studied by Takenò and Pharis (42), who found that nanogram quantities of brassinolide increased seedling vigor and characteristically caused bending of the second rice leaf lamina. They also pointed out that the physiological effect of BR is an auxin-mediated response and that the effect is quite different from that of GA<sub>3</sub>, which does not cause bending of the second leaf lamina. GA<sub>3</sub> causes marked elongation of the second rice leaf sheath and inhibits BR-induced bending of the leaf lamina. In our bioassay using the bean first internode assay (37) we observed that GA<sub>3</sub> caused no bending of the internode but stimulated elongation of the stem section. Whereas brassinolide stimulated auxin-induced growth in these tests, GA<sub>3</sub>-induced growth was inhibited (Figure 7). These results were confirmed by using the dwarf pea bioassay (Figure 8). In both systems, the GA response is inhibited by BR.

#### Brassinolide and Ethylene Interaction

Yopp et al. (43) reported that brassinolide enhances hook closure of the bean seedling in the dark and also enhances production of ethylene. They suggest that brassinolide acts on hook closure through an effect on ethylene synthesis in the tissues. Data from Arteca et al. (8, 9) seem to support that brassinolide stimulated auxin-induced ethylene synthesis, particularly in the presence of calcium. A reinvestigation of the possible role of ethylene in the brassinolide action on growth using bean internode sections reveal that auxin-induced ethylene production is stimulated by brassinolide but as seen in Figure 9, the apparent stimulation of ethylene production can be induced in young as well as in mature tissues even though mature

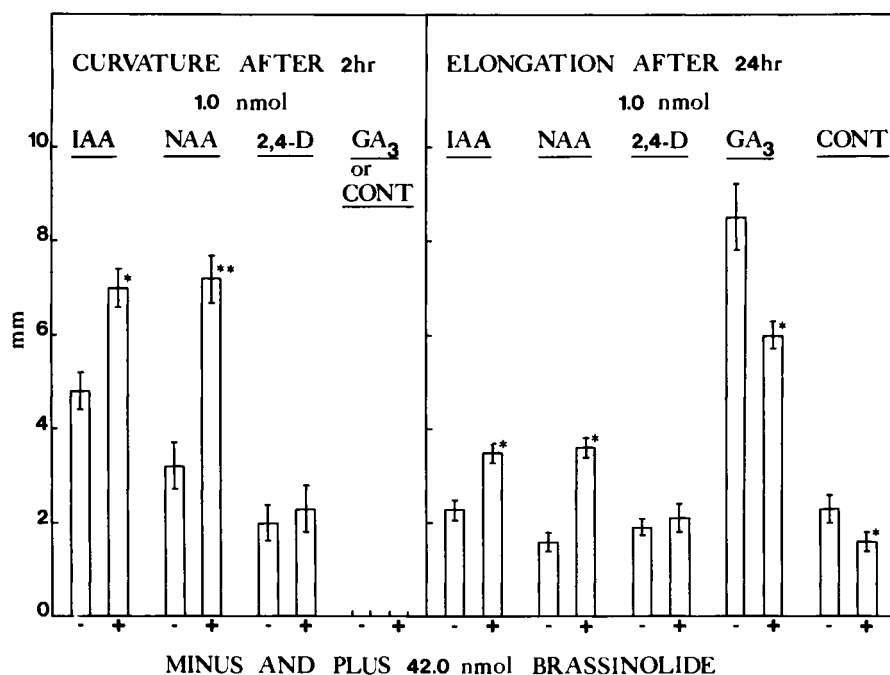


Figure 7. Effect of BR treatment of curvature (left) and elongation (right) of bean first internode sections treated with disks containing either 1 nmol of IAA or NAA (naphthalene-acetic-acid) or 2,4,-D (2,4-dichloro-phenoxy acetic acid) or GA<sub>3</sub> (gibberellic acid) plus and minus 42 nmoles of BR.

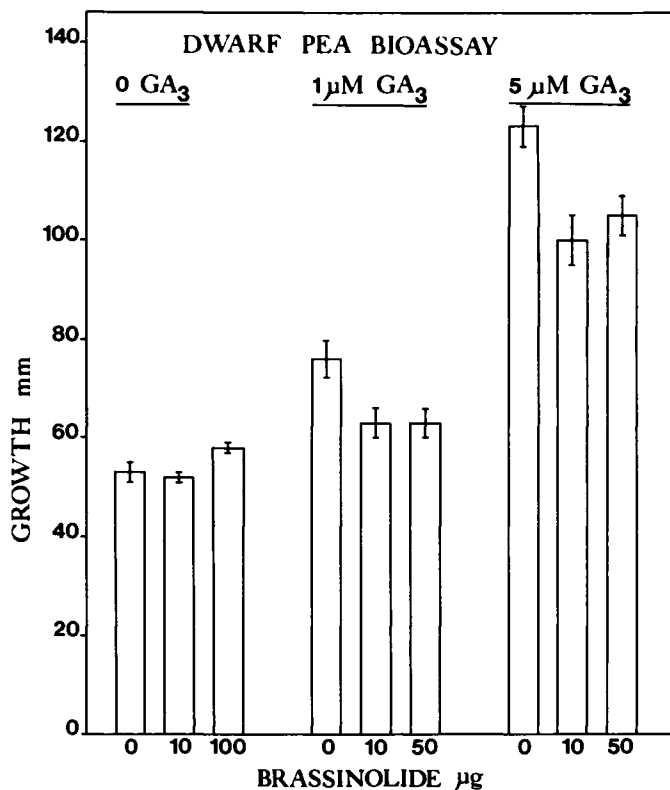


Figure 8. Effect of BR on GA induced growth of dwarf pea seedlings. Plants were treated with 5 µl of 0.1, or 5 µM GA<sub>3</sub> plus and minus 10, 50, or 100 µg of BR. Treatments were applied to the plumule of isolated seedlings. Length measurements were taken 6 days after treatment.



tissues are not able to grow in response to auxin or brassinolide treatments. Furthermore, the increase of ethylene production is a slow process, taking at least 4 hours, while the growth response is rapid (observable within 60 minutes after auxin and or brassinolide treatment). I interpret these results to mean that brassinolide and auxin-induced growth involve metabolic events that are independent from auxin and BR-enhance ethylene biosynthesis.

#### Effect of Brassinolide or Geotropism

Asymmetric growth of isolated bean internode may be induced either by unilateral applications of auxin to an upright positioned section or through geostimulation by placing an isolated internode section in a horizontal position. The latter happens in the absence of exogenous supply of auxin. Both events are stimulated by brassinolide. An example of the effect of BR on geostimulated bending of isolated bean sections is shown in Figure 10. For this example, the internode section were dipped for a few seconds in aqueous solution of .01 ppm BR prior to securing them in a scintillation vial fitted with a water saturated sponge and placing them in a horizontal position. The results indicate that BR shortens the time of graviperception of the sections which reflect an accelerated growth response. The rate of bending of control section reaches maximum about 2 hours after the beginning of geostimulation - while BR treated sections attain their maximum rate of growth in less than one hour after geostimulation. Exogenous auxin in this system does not enhance either this intrinsic geotropic response or the BR effect.

Although auxin seems to have no stimulating effect on geotropic bending of isolated bean internode sections, it does influence the growth of pulvini. Figure 11 shows that the elongation of pulvini, which are the graviperceptive organs of grass shoots (44) are stimulated to grow in response to auxin and BR applications. In this experiment, 1.0  $\mu$ g of BR or IAA was applied as lanolin preparation around pieces of isolated nodal pulvini obtained either from normal or lazy corn. In both cases, IAA stimulated the growth of internodal pulvini tissue and BR enhanced the auxin effect (data kindly provided by Drs. P.B. Kaufman and P. Dayanandan).

#### Effect of Brassinolide on Crop Production

Brassinolide applied as a lanolin preparation, a spray, or a seed treatment enhances crop production by stimulating overall growth of plants when applied to young seedlings (45,46).

Typically, the slow growing plants in a population were affected more than rapidly growing ones (45,46) and that it is affected by light. Low light intensities favor BR stimulated cell division (47,49). Crop plants grown under field conditions also benefit from brassinosteroid treatments. Lettuce, radishes, and potatoes all mature at an accelerated rate (50).

Head lettuce seedlings, for instance, sprayed with 0.01 ppm headed 2 weeks earlier than control seedlings sprayed with H<sub>2</sub>O (50). Potato tubers developed about 3 weeks earlier when plants were treated with 0.1ppm BR than control plants treated with just water. Plants grown under fertilizer stress seemed to respond most favorably to BR treatment.

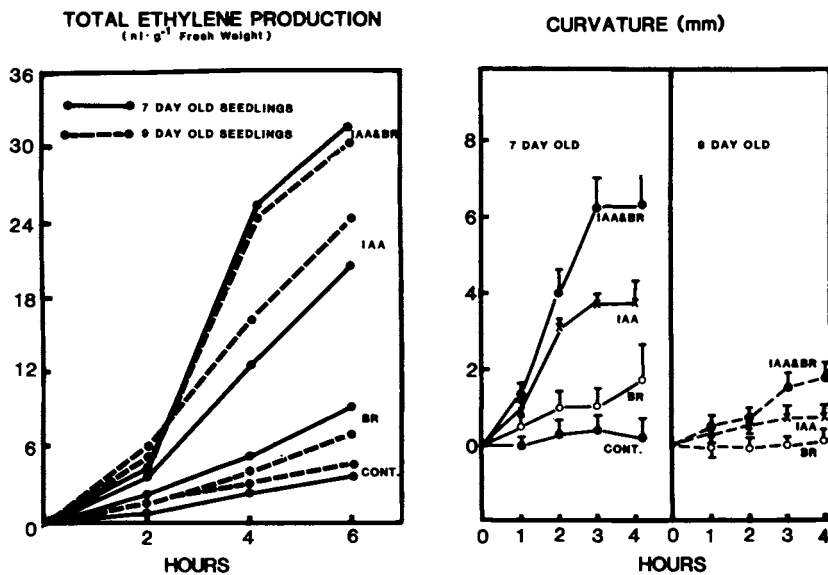


Figure 9. Correlation between ethylene production (left) and growth (right) of bean first internode sections from 7- and 9-day old seedlings. Treatments consisted of 1 nmol IAA and 1 g BR.

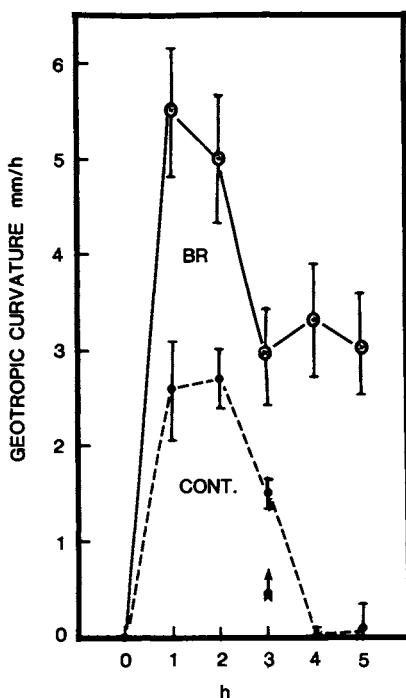


Figure 10. Effect of 0.01 ppm BR on geotropic induction of curvature of isolated bean internode sections.

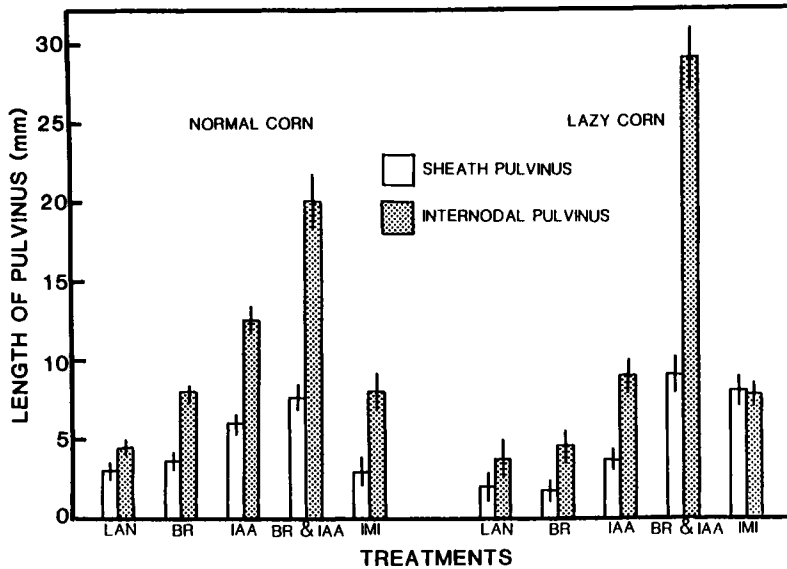


Figure 11. Effect of BR on growth of normal- and Lazy Corn pulvini. IAA and BR (1 g) was mixed with lanolin and applied to the pulvini (IMI=IAA-myo-inositol (Data of Kaufman and Dayanadan).

Encouraging results on the use of BR in agricultural practices are also being generated by Hamada et al. (53) who showed an increase in crop yield of rice, corn, cucumbers, sweet-potatoes etc. in response to repeated (3x) applications of 10ppm BR. BR also increased cold resistance in egg plants and cucumbers; enhanced disease resistance against soft rot in Chinese cabbage and against sheath blight in rice plants; decreased plant injury and enhanced recovery of plants treated with various herbicides.

### Conclusion

Classically, plant sterols are considered either as structural constituents of cytoplasmic membranes or as precursors to animal sex hormones and insect molting hormones. It is for that reason that in the past, the subject of "Steroid Hormones in Plants" was of more interest to phytoendocrinologists dealing with insect molting hormones of plant origin (51) than to plant physiologists. Steroids in general and animal sex-hormones in particular have, however, been tested on various plant systems with the idea that they act on sex expression of flowering of monoecious plants in a manner similar to their actions in animal systems. Such evidence is, however, very tenuous and circumstantial.

Brassinolide and biologically active brassinosteroids provide plant scientists, however, with phytosteroids to which a hormonal function in plants can be assigned. The cellular mechanism of BR action can at present only be reconciled with observed physiological effects, and based on this evidences, it is proposed that BR fulfills a regulatory function in plants. The function being that BR regulates the sensitivity of plant tissues to auxin and to geotropic stimulation. It is suggested that BR somehow influences the "responsiveness" of tissues to auxin without affecting the intercellular auxin concentrations. It is conceivable that the BR induced amplification of auxin action on target cells involves interactions of BR with some membrane associated receptors with high affinity for auxin and/or BR which brings auxin closer to the protein synthesis machinery of the cell. It may thus function as a modulator of auxin (54) and gravi-sensitive gene expression.

Alternative suggestions proposed are that ethylene production (8,9) or proton secretion (7) be the biochemical locus of action of BR. In these cases BR enhanced growth ought to be explicable on the basis of either one of the chemical changes observed. Unfortunately, these BR induced biochemical changes lack the required selectivity of target tissues and chemical structures specificity. Both phenomena may be induced by compounds other than brassinosteroids and also in aged tissues that are otherwise incapable of auxin induced cell enlargement.

### Literature Cited

1. Fitting, H. Biol. Ztb. 1909, 29,193.
2. Starling, E. H. Lancet 1905 ii, 423.
3. Mitchell, J. W.; Mandava, N. B.; Worley, J. R.; Plimmer, J. R.; Smith, M. V. Nature (London) 1970, 225,1065.
4. Worley, J. F.; Mitchell, J. W. J. Am. Soc. Hortic. Sci. 1971, 96, 270.

5. Mandava, N. B.; Mitchell, J. W. *Chem. Ind.* 1972, 930.
6. Grove, M. D.; Spencer, G. F.; Rohwedder, W. K.; Mandava, N. B.; Worley, J. F.; Warthen, J. D., Jr.; Steffens, G. L.; Flippen-Anderson, J. L.; Cook, J., Jr. *Nature* (London) 1979, 281,216.
7. Cerana, R.; Bonnetti, A.; Marre, M. T.; Lado, P.; Marre, E. *Physiol. Plant.* 1983, 59, 23.
8. Arteca, R. N.; Tsai, D. S.; Schlaghaufer, C.; Mandava N. B. *Physiol. Plant.* 1983, 59, 539.
9. Arteca, R. N. *Physiol. Plant.* 1984, 62, 102.
10. Cerana, R.; Lado, P.; Anastasia, M.; Ciuffreda, P.; Allevi, P. *Z. Pflanzenphysiol.* 1984, 114, 221.
11. Thompson, M. J.; Mandava, N. B.; Flippen-Anderson, J. L.; Worley, J. F.; Dutky, S. R.; Robbins, W. E.; Lusby, W. *J. Org. Chem.* 1979, 44 5002.
12. Thompson, M. J.; Meudt, W. J.; Mandava, N. B.; Dutky, S. R.; Lusby, W. R.; Spaulding D. W. *Steroids* 1982, 39, No. 89.
13. Standifer, L. N.; Devys, M.; Barbier, M. *Phytochemistry* 1968, 7,1369.
14. Knights, B. A., (1968) *Phytochemistry* 7, 1707.
15. Yokota, T.; Morita, M.; Takahashi, N. *Agric. Biol. Chem.* 47, 2149.
16. Abe, H.; Morishita, T.; Uchiyama, M.; Takatsuto, S.; Ikekawa, N.; Ikeda, M.; Sasse, T.; Kitsuya, T.; Marumo, S. *Experientia* 1983, 39, No. 351.
17. Arima, M.; Yokota, T.; Takahashi, N. *Phytochemistry* 1984, 23, 1587.
18. Takatsuto, S.; Yazawa, N.; Ikekawa, N.; Takematsu, T.; Takeuchi, Koguchi, M. *Phytochemistry* 1983, 22, 2437.
19. Okada, K.; Mori, K. *Agric. Biol. Chem.* 1983, 47, 89.
20. Baba, J.; Yokota, T.; Takahashi, N. *Agric. Biol. Chem.* 1983, 47,
21. Yokota, T.; Baba, J.; Takahashi, N. *Agric. Biol. Chem.* 1983, 47 1409.
22. Abe, H.; Nakamura, K.; Morishita, T.; Uchiyama, M.; Takatsuto, S Ikekawa, N. *Agric. Biol. Chem.* 1984, 48,1103.
23. Yokota, T.; Arima, M.; Yakahasi, N. *Tetrahedron Letters* 1982, 32 1275.
24. Abe, H.; Morishita, T.; Uchiyama, M.; Marumo, S.; Munakata, K.; Takatsuto, S.; Ikekawa, N. *Agric. Biol. Chem.* 1982, 46, 2609.
25. Abe, H.; Morishita, T.; Uchiyama, M.; Takatsuto, S.; Ikekaa, N. *Biol. Chem.* 1984, 48, 2171.
26. Morishita, T.; Abe. H.; Uchiyama, M.; Marumo S.; Takatsuto, S.; Ikekawa, N. *Phytochemistry* 1983, 22, 1051.
27. Ikeda, M.; Takatsuto, S.; Sassa, T.; Ikekawa, N.; Nukina, M: *Agr Biol. Chem.* 1983, 47655.
28. Yokota, T.; Baba, J.; Takahashi, N. *Tetrahedron Letters* 1982, 23,4965.
29. Schneider, J. A.; Yoshihara, K.; Nakanishi, K.; Kato, N. *Tetrahedron Letters* 1983, 24, 3859.
30. Abe, H.; Morishita, T.; Uchiyama, M.; Takatsuto, S.; Ikekawa, N. *Agric. Biol. Chem.* 1984, 48, 2171.
31. Takatsuto, S.; Yazawa, N.; Ishiguro, M.; Morisaki, M.; Idekawa, *Chem. Soc.* 1984, *Perkin Trans.*, 1, 139.
32. Yopp, J. H.; Ladd, D.; Jaques, D. In "Tenth International Confer on Plant Growth Substances," Madison, Wisconsin, 1979, 25.

33. Yopp, J. H.; Mandava, N. B.; Sasse, J. M. Physiol. Plant. 1981, 53, 445.
34. Mandava, N. B.; Sasse, J. M.; Yopp, J. H.; Physiol. Plant. 1981, 53, 453.
35. Mitchell, J. W.; Livingston, G. A.; "Methods of Studying Plant Hormones and Growth Regulating Substances;" Agricultural Handbook 336, Agri. Res. Ser. USDA, 1968.
36. Meada, E. Physiol. Plant. 1965, 18, 813.
37. Meudt, W. J.; Bennett, H. W. Physiol. Plant. 1979, 44, 422.
38. Wada, K.; Maurmo, S.; Ikekawa, N.; Morisaki, M.; Mori, K. Plant Physiol. 1981, 22, 323.
39. Ishiguro, M.; Takatsuto, S.; Morisaki, M.; Ikekawa, N. 1980, J. Soc., Comm. 1980, 962.
40. Arima, M.; Yokta, T.; Takahashi, N. Phytochemistry 1984, 23, 1587.
41. Cohen, J. D.; Meudt, W. J. Plant Physiol. 1983, 72, 691.
42. Takeno, K.; Pharis, R. P. Plant Cell Physiol., 23, 1275.
43. Yopp, J. H.; Colclasure, G. C.; Mandava, N. B. Physiol. Plant 1979, 46, 247.
44. Kaufman, P. B.; Dayanadan, P; Meudt, W. J. Plant Physiol. Suppl 69, 12.
45. Mitchell, J. W.; Gregory, L. E. Nature New Biol. 1972, 239, 253.
46. Gregory, L. E. Am. J. Bot. 1981, 68, 586.
47. Krizek, D. T.; Worley, J. F. Bot. Gaz. 1973, 134, 147.
48. Krizek, D. T.; Worley, J. F. Physiol. Plant. 1983, 51, 259.
49. Krizek, D. T.; Mandava, N. B. Physiol. Plant. 1983, 57, 317.
50. Meudt, W. J., Thompson, M. J., and Bennett, H. W. Proc. 10th Ann. Growth Regulator Soc. Am., 1983, p. 312.
51. Heftmann, D. 1985. Lloydia 38, 195.
52. Mandava, N. B.; Kozempel, M.; Worley, J. F.; Mathes, D.; Warthen, J. D. Jr.; Jacobson, M.; Steffens, G. L.; Kenney, H.; Grove, M.D. Ind. Eng. Chem. 1978, 17, 351.
53. Hamada, K.; Nishi, S.; Uezono, T.; Fujiwara, S.; Nakazawa, YU. 12th International Conference Plant Growth Substances, 1985, p. 43.
54. Meudt, W. J., Thompson, M. J. Proc. 10th Ann. Growth Regulator Soc. Am., 1983, p. 306.

RECEIVED January 10, 1986

## Chapter 6

# Biochemistry of Conifer Resistance to Bark Beetles and Their Fungal Symbionts

Mark A. Johnson and Rodney Croteau

Institute of Biological Chemistry, Washington State University, Pullman,  
WA 99164-6340

Conifers exhibit resistance to bark beetle attack and fungal invasion because of an induced synthesis and flow of secondary resin which contains high levels of volatile monoterpenes. Monoterpene olefin synthases (geranyl pyrophosphate cyclases) were found in higher levels in fungus-infected lodgepole pine seedlings than in uninfected controls. Cyclases responsible for the formation of (+)- $\alpha$ -pinene and (+)-limonene, monoterpenes which inhibit fungal growth and repel bark beetles, have been studied in herbaceous species. Two distinct types of enzymes synthesize antipodal monoterpene hydrocarbons. The differential expression of the cyclases responsible for co-production of enantiomeric monoterpenes may determine the highly selective resistance response conifers exhibit toward bark beetles and their fungal symbionts.

Resin secretion is part of a resistance mechanism conifers employ against bark beetles and their associated pathogenic fungi (1-3). Conifer resins are contained in a system of ducts or cortical blisters and consist of a mixture of terpenes and benzenoid compounds (4). The terpenoid fraction of wood resins of the Pinaceae (especially pines) generally contain 20 to 50% volatile mono- ( $C_{10}$ ) and sesquiterpenes ( $C_{15}$ ) and 50 to 80% nonvolatile diterpene acids ( $C_{20}$ ) (5). Successful resistance to bark beetle attack depends on a complex relationship involving the quantity and composition of the resin produced, the physiological condition of the tree, the virulence of the fungus and the number of attacks (6-8).

Alterations in monoterpene content can determine the degree of resistance to bark beetles and, thus, whether or not a tree is colonized. Resin monoterpenes (Fig. 1) may be either repellents or attractants to insects or fungistatic or chemotropic stimulants of fungal growth, depending on the precise monoterpene composition and the particular herbivore or pathogen (9-13).

0097-6156/87/0325-0076\$06.00/0  
© 1987 American Chemical Society

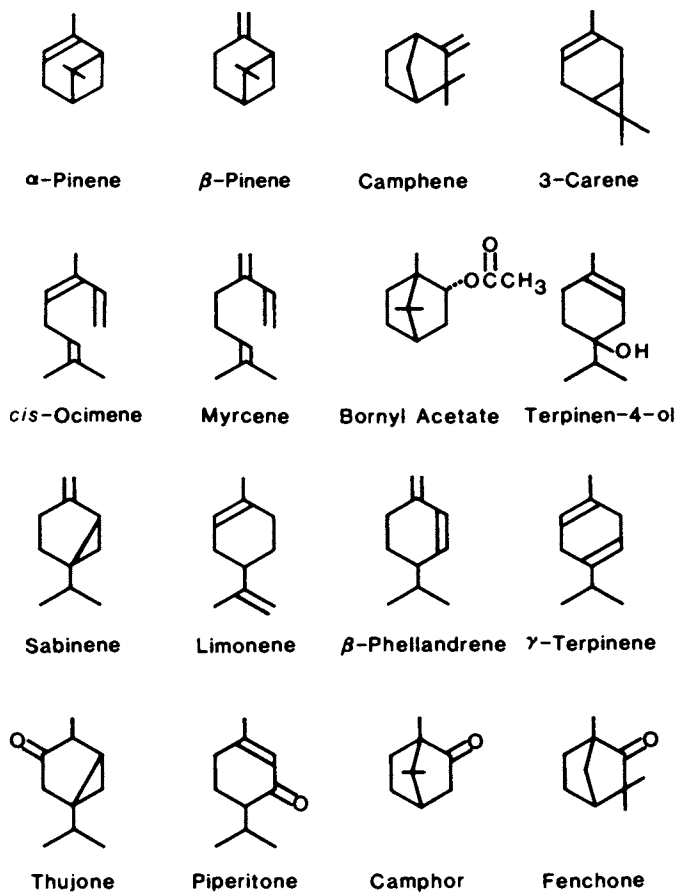


Figure 1. Major components of the monoterpene fraction of conifer resins.



Monoterpenes are derived from the classical mevalonate pathway, and control of monoterpene composition is presumed to occur, at least in part, at the latter stages of the pathway, during which the trans C<sub>10</sub> prenyl pyrophosphate (geranyl pyrophosphate (GPP)) is converted to acyclic and cyclic products (14). The conversion of geranyl pyrophosphate to parent compounds of the various cyclic types is catalyzed by enzymes collectively referred to as monoterpene cyclases. The cyclases of conifers have received little experimental attention thus far, but these enzymes are probably similar to the numerous monoterpene cyclases which have been isolated from herbaceous species (15,16). In general, monoterpene cyclases are soluble enzymes with molecular weights in the range of 50,000-100,000. They require a divalent cation (Mg<sup>++</sup> or Mn<sup>++</sup>) for activity, have pH optima between 6 and 7, and are capable of cyclizing geranyl pyrophosphate as well as the cis-isomer, neryl pyrophosphate (NPP), and the tertiary allylic isomer, linalyl pyrophosphate (LPP) (17). Studies with partially purified cyclases have focused on the mechanism and stereochemistry of the cyclization process, and offer promising insights into the manner by which the monoterpene composition in conifer wood resins may be controlled.

Detailed reviews of the literature on terpene biochemistry can be found in two volumes edited by Porter and Spurgeon (18,19). A volume on plant terpenes has been edited by Nes and associates (20), and similar reviews by Loomis and Croteau (21) and Banthorpe and Charlwood (22) are available. Terpenes occurring in trichomes and in wood extractives have been described by Croteau and Johnson (15,23). Specific accounts of monoterpene biochemistry can be found in reviews by Croteau (16,17,24), Cori (25), Charlwood and Banthorpe (26) and Bernard-Dagan and co-workers (27).

### Bark Beetle Attack

The role of conifer resins in resistance to infestation by bark beetles and their fungal symbionts has been discussed extensively by Shrimpton (1), Raffa and Berryman (6), Cates and Alexander (8) and others (28,29), and a brief summary of the interactions between conifers, beetles and fungi is presented here. Tree-killing bark beetles, which attack conifers by boring into the phloem, belong primarily to the genera Dendroctonus, Ips and Scolytus of the Scolytidae (30). Death follows successful colonization of the tree which is brought about by fungal invasion of xylem tissue. Tree mortality is usually necessary if the beetles and their progeny are to survive, since excavation of egg galleries and subsequent larval development can only take place after host defensive reactions have stopped.

Pioneer beetles which initiate an attack may encounter constitutive (preformed) resin in cortical blisters or when resin ducts are severed. Contact with the resin leads to emission of volatile aggregation pheromones, which in many cases are derived from host monoterpenes, and this in turn leads to a mass attack by flying beetles. Mass attack may deplete the flow of

constitutive resin in 1-2 days, and in the absence of further resinosis, the beetles will successfully colonize the tree. Healthy trees react to wounding and fungal invasion by a series of well-documented responses. First, the wound site develops a layer of autolysed cells (necrotic lesion) which serves to deprive the pathogen of nutrients and compartmentalize the damage (this is the hypersensitive response which is common in higher plants (3,31)). Second, adjacent wood parenchyma differentiate into traumatic resin ducts and secrete a secondary resin into the wound site and sapwood, creating a reaction zone around the wound (3,6). Bark beetles respond to resinosis either by attempting to clear resin from the wound or by abandoning the wound site. In the absence of resinosis, beetles easily bore through the necrotic lesion, allowing the fungus to spread unhindered.

Secondary resinosis and the hypersensitive response are also important factors in fungal development. If the flow of resin is sufficiently intensive, fungal growth can be prevented (1,32,33). However, since secondary resin is derived from xylem carbohydrate reserves, a pervasive fungal attack, mediated by numerous beetle penetrations, will deplete this carbon source, leading to reduced secondary flow and increased tree vulnerability (6).

#### Resin Composition and Host Resistance

While other resin components, such as diterpene acids, show little intraspecific variation, monoterpene content varies greatly from tree to tree even within small stands. This variability has been attributed to selection for resistance to bark beetle infestation. Monoterpenes of conifer oleoresins may have opposing functional effects as both insect repellents and as pheromone precursors and synergists. For example, Sturgeon and Mitton (34) have detailed the complex balance between the repellent role of limonene in ponderosa pine oil and the attractant role of  $\alpha$ -pinene as the aggregation pheromone precursor of the western pine beetle (*Dendroctonus brevicomis*). A high concentration of limonene alone is insufficient to deter attack by the pine beetle; however, trees bearing both high limonene and low  $\alpha$ -pinene content avoid colonization.

Bark beetles may encounter oleoresin having different monoterpene compositions at three different stages of colonization. Constitutive resin stored in cortical blisters and resin ducts may serve to deter pioneer beetles, and cortical monoterpene content has often been used as an indicator of insect resistance (13,35). Beetle attack initiates a hypersensitive wound response in which parenchyma around the wound site differentiates and secretes new resin. Subsequent fungal invasion elicits a strong resinous infusion (fungal-elicited resin) from the sapwood at a considerable distance from the wound site, and this process continues until either the fungus or the tree dies (6). Thus, a careful measure of resistance must account for resin quantity and composition at each infection stage.

Studies on the interactions between grand fir (*Abies grandis*), the fir engraver beetle (*Scolytus ventralis*) and the

associated fungus Trichosporium symbioticum have shown that monoterpenes which are particularly repellent to the beetle and toxic to the fungus occur principally in the secondary (induced) resin rather than in constitutive resin (9,36). Components which are found to specifically increase in the induced resin include myrcene, limonene,  $\alpha$ -pinene and 3-carene. (The total monoterpene content increases 25-fold over pre-infestation resin levels.) In a related study, resistant lodgepole pine (P. contorta) was shown to produce particularly high levels of  $\alpha$ -pinene and limonene in the secondary resin. In this case, however, the major factor in resistance to the mountain pine beetle (Dendroctonus ponderosae) and associated fungus (Euophium clavigerum) appeared to be a rapid and vigorous secondary resinosis with increased production of all constitutive monoterpenes (6,32,37). This finding correlates well with field studies in which only stressed, weakened or old trees, which are unlikely to mount a vigorous defensive response, were observed to be successfully colonized by endemic bark beetle populations.

Survival of healthy trees during epidemics, however, depends on a highly variable intraspecific resin composition which increases the probability that some trees will have the right combination of resin components to be effective against the attacking beetles (34). Thus, genetic effects which determine subtle differences in resin composition are probably the critical factors in conifer survival during epidemics, since both physiologically weak trees and healthy trees which produce the precursors of insect attractants are likely to be eliminated.

The optical purity of resin terpenoids can have a significant effect on the success or failure of beetle colonization since generally only one enantiomer is effective as a pheromone precursor or synergist. Ips typographis bark beetles produce the aggregation pheromone (-)-cis-verbenol from host resin (-)- $\alpha$ -pinene. This insect also produces the biologically inactive (+)-trans-verbenol from host resin (+)- $\alpha$ -pinene. Since both enantiomers of  $\alpha$ -pinene often co-occur in wood resins, the proportion of (-)- $\alpha$ -pinene would be expected to be a factor in tree vulnerability to mass attack. This expectation was confirmed by Klimetzek and Francke (38) in a study of all the verbenol stereoisomers produced by beetles exposed to natural oleoresins containing varying proportions of (+)- and (-)- $\alpha$ -pinene. The proportion of (-)- $\alpha$ -pinene in the resin was shown to be directly related to the amount of (-)-cis-verbenol produced, providing a chemical basis for beetle-host specificity. The isomeric compound (-)-trans-verbenol is produced from host-derived (-)- $\alpha$ -pinene by Dendroctonus brevicomis and functions as a repellent to female beetles, presumably to reduce intraspecific competition. The antipodal (+)-trans-verbenol produced from (+)- $\alpha$ -pinene, is without biological effect (39). The effects of enantiomeric monoterpenes on bark beetle activity have been reviewed by Wood (30), Francke and Vite (40) and Silverstein (41). In some cases the presence of as little as 2-5% of the enantiomeric product completely inhibits the effect of the active antipodal form (30).

Optical purity has also been shown to influence the fungistatic properties of oleoresin monoterpenes. The growth of blue-stain fungi (Ceratocystis spp.) and Fomes annosus is inhibited by chiral monoterpenes from ponderosa pine resin (e.g., limonene,  $\beta$ -pinene and  $\beta$ -phellandrene and  $\alpha$ -pinene). The greatest enantiomeric effect is observed with (+)- $\alpha$ -pinene which is three times more inhibitory against Ceratocystis than the (-)-antipode (42,43). In another study using Pinus radiata monoterpenes, (+)-limonene was shown to be a potent inhibitor of Diplodea pinea spore germination while (-)-limonene was inactive (44).

The role of monoterpene enantiomers in imparting differential conifer resistance to bark beetle attack has received little attention because of the difficulties in purifying the individual monoterpenes and in quantitating the optical antipodes. Techniques which have been used to determine optical purity include preparative gas-liquid chromatography (GLC) followed by polarimetry or NMR analysis using chiral shift reagents, GLC analysis of diastereomeric derivatives, and, more recently, direct separation of enantiomers by chiral phase capillary GLC (30,40,45,46). The latter technique is both convenient and very sensitive, and with this development, new information concerning the roles of antipodal monoterpenes should be forthcoming.

#### Fungal Elicitors

Wounding alone, caused by insects or mechanical damage, will induce a resinous wound response that includes formation of traumatic resin ducts in the sapwood (1,47). However, an early study by Hepting showed that conifers respond more vigorously to fungal infection than to simple wounding (48). Live canker fungus (Fusarium sp.) induced 30 times the resin flow from wounds on Pinus virginiana than wounds where dead fungus was applied. Later studies, using artificial inoculations of bark beetle fungal symbionts (Ceratocystis, Europhium and Trichosporium) have clearly supported the role of the fungus in induction of secondary resin production in conifers (1,6,7,32,37). Further support for fungal elicitation has been obtained recently from tissue culture studies. Laszlo and Heinstejn (49) have shown that heat inactivated Fusarium sp. induce a 4- to 10-fold increase in diterpene resin acid production (especially dehydroabietate) by pine cell suspension cultures. Further consideration of conifer resistance mechanisms must take into account the nature of the chemical signals involved (fungal elicitors), their origin and their effects on plant metabolism and cell differentiation.

Darvill and Albersheim (50), Dixon and associates (51), Hadwiger and co-workers (52,53) and West (54) have reviewed the extensive literature on elicitors responsible for the biosynthesis of phytoalexins (newly-synthesized compounds which impart resistance). It is now clear that elicitors of physiological origin are soluble carbohydrates derived by enzymatic hydrolysis of either plant cell walls

(polygalacturonates) or fungal walls (chitosans) and that these signals arise from pathogen attack or plant counterattack. Elicitors from mycelial walls of a pathogenic fungus induce the rapid appearance of chalcone synthetase mRNA in bean cells, thus showing that phytoalexin synthesis is controlled at the transcriptional level (55). The postulated mechanism of elicitor action involves binding to a specific cell membrane receptor which triggers the release of an intracellular second message (possibly  $\text{Ca}^{++}$ ) and initiates new mRNA synthesis for the enzyme(s) of phytoalexin biosynthesis (51).

In a preliminary study, the presumptive induction of monoterpene cyclases, and the resulting changes in monoterpene content, were examined in lodgepole pine saplings in response to fungal infection (56). Two year-old lodgepole pine were inoculated with *Europhium clavigerum*, a symbiont of the mountain pine beetle, and total monoterpene content was monitored over a 12-day period. Infected stems accumulated three times the monoterpene level of untreated controls (Fig. 2, Table I). Aseptic wounds led to a lesser increase (1.5-fold) in monoterpene level which is a characteristic wound response in conifers. Specific increases in the levels of the monoterpene olefins  $\alpha$ -pinene,  $\beta$ -pinene, 3-carene and  $\beta$ -phellandrene, which are toxic to this fungus, were found in infected tissues compared to controls. Two weeks after infection, monoterpene cyclase levels were measured and compared to untreated controls. (Crude stem homogenates were assayed with [ $^3\text{H}$ ]geranyl pyrophosphate as substrate under standard conditions for monoterpene cyclases (57).) Infected tissue exhibited a corresponding 3-fold increase in monoterpene hydrocarbon cyclase activity. Thus, both monoterpene cyclase activities and monoterpene levels increased in lodgepole pine in response to fungal infection, in a manner reminiscent of the typical phytoalexin response.

Elicitor-stimulated resin production is ultimately limited by the availability of carbohydrate reserves, which serve as resin precursors (58). The death of sapwood parenchyma due to fungal toxins or other physiological malfunction (59,60) will obviously also limit resin production. The relationship between the extent of fungal attack and the quantity of monoterpenes synthesized has been examined by Raffa and Berryman (32). Lodgepole pine exhibited a 6-fold increase in monoterpene content over constitutive levels at the wound site when subjected to up to 10 inoculations of *Europhium* per 0.3 m<sup>2</sup>, and a 30-40% decrease in this maximum when subjected to 20-35 inoculations per 0.3 m<sup>2</sup>. Given these inherent limitations of tree physiology (due to age, vigor and size), an understanding of the origin and mechanism of action of fungal elicitors is essential to the development of protective strategies against bark beetle attack and subsequent fungal invasion.

### Biosynthesis of Monoterpenes in Conifers

The variation in monoterpene skeletal types found in conifer oleoresins is determined to a large degree by differential expression of the corresponding monoterpene cyclases. These

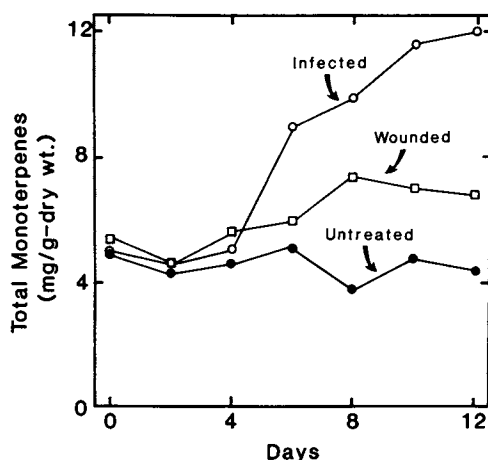


Figure 2. Effect of time on the accumulation of monoterpenes in infected or wounded lodgepole pine stems. Two year-old lodgepole pine (*P. contorta*) were infected with the vegetative form of *Europhium clavigerum* or wounded aseptically. Monoterpenes were quantified following GLC analysis of steam distillates of stem tissue.

TABLE I. Effect of Wounding and Infection on the Monoterpene Composition of Lodgepole Pine Stems<sup>a</sup>

Monoterpene <sup>b</sup>	Control	Aseptic Wound	Infected Wound
$\alpha$ -Pinene	0.44	0.69	1.68
$\beta$ -Pinene	1.55	2.79	5.97
3-Carene	0.27	0.34	1.33
$\beta$ -Phellandrene	1.22	2.14	3.14
$\alpha$ -Phellandrene	0.14	0.14	0.08
Limonene	0.85	0.70	0.22
Camphene	tr	tr	0.09
Total Monoterpenes	4.47	6.79	12.51

<sup>a</sup>Two year-old Lodgepole pine saplings were infected with *Europhium clavigerum* or wounded aseptically. Monoterpene content of pine stems was determined 10 days after treatment following steam distillation and quantitation of peak areas obtained from gas-liquid chromatography.

<sup>b</sup>Composition is given in mg/g dry weight.

enzymes catalyze the crucial ring-forming reactions in monoterpene biosynthesis by exploiting a variety of electrophilic cyclizations of geranyl pyrophosphate which involve carbonium ion attack on a double bond as the primary C-C bond-forming event (61). Geranyl pyrophosphate (Fig. 3) is derived by ligation of two 5-carbon isoprenoid units, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), under the influence of the enzyme prenyl transferase (specifically GPP synthetase) (15,62).

Monoterpene biosynthesis has been studied in conifers using labeled precursors such as carbon dioxide, acetate and mevalonate (63,64). Specifically labeled precursors have been employed to deduce mechanistic features of  $\alpha$ -pinene (65,66) and 3-carene (67,68) biosynthesis in pine species. Gleizes and co-workers (69) have argued, by way of time-course studies, that the initial formation of acyclic hydrocarbons (ocimene, myrcene) from  $^{14}\text{CO}_2$  in Pinus pinaster needles indicated that these olefins serve as precursors to cyclic monoterpenes ( $\alpha$ -pinene,  $\beta$ -pinene) by a reversible protonation mechanism. These suggestions, however, are without precedent, and run counter to direct evidence demonstrating that the cyclization of geranyl pyrophosphate occurs without the involvement of free intermediates (17).

Studies on monoterpene biosynthesis using cell-free extracts from conifers have been few in number, due in large part to the limitations in propagating conifers (i.e., slow growth) and to the difficulties in extracting enzymes from woody tissue. Cyclic terpenes ( $\text{C}_{10}$ ,  $\text{C}_{15}$ ) are reportedly synthesized from IPP or NPP in particulate fractions of Pinus pinaster and Douglas fir needles (70,71); yet Cori (72) has demonstrated that soluble enzymes from Pinus radiata seedlings convert GPP and NPP to cyclic monoterpene olefins ( $\alpha$ -pinene,  $\beta$ -pinene and limonene). In none of these studies have the products or the responsible enzymes been adequately characterized. Recently developed procedures for isolating terpene cyclases (57), and methods for obtaining active enzymes from sapwood extracts (73), should facilitate studies on the enzymology of monoterpene biosynthesis in conifers.

#### Mechanism of Monoterpene Cyclization

Monoterpene cyclization processes have been studied primarily in herbaceous species, and cyclase preparations have been obtained which catalyze the cyclization of geranyl pyrophosphate to essentially all major structural classes. Multiple cyclases, each producing a different skeletal arrangement from the same acyclic precursor co-occur in higher plants, while single cyclases which synthesize a variety of stereochemically distinct skeletal types are also known (24,57). Individual cyclases, each generating a simple derivative or positional isomer of the same skeletal type, have been described, as have distinct cyclases catalyzing the synthesis of enantiomeric products (74). The mechanistic outlines of the cyclization of geranyl pyrophosphate have been the subject of some controversy (17) because GPP is precluded from direct cyclization (to cyclohexanoid products) by the C2-trans double bond. Various isomerization schemes have

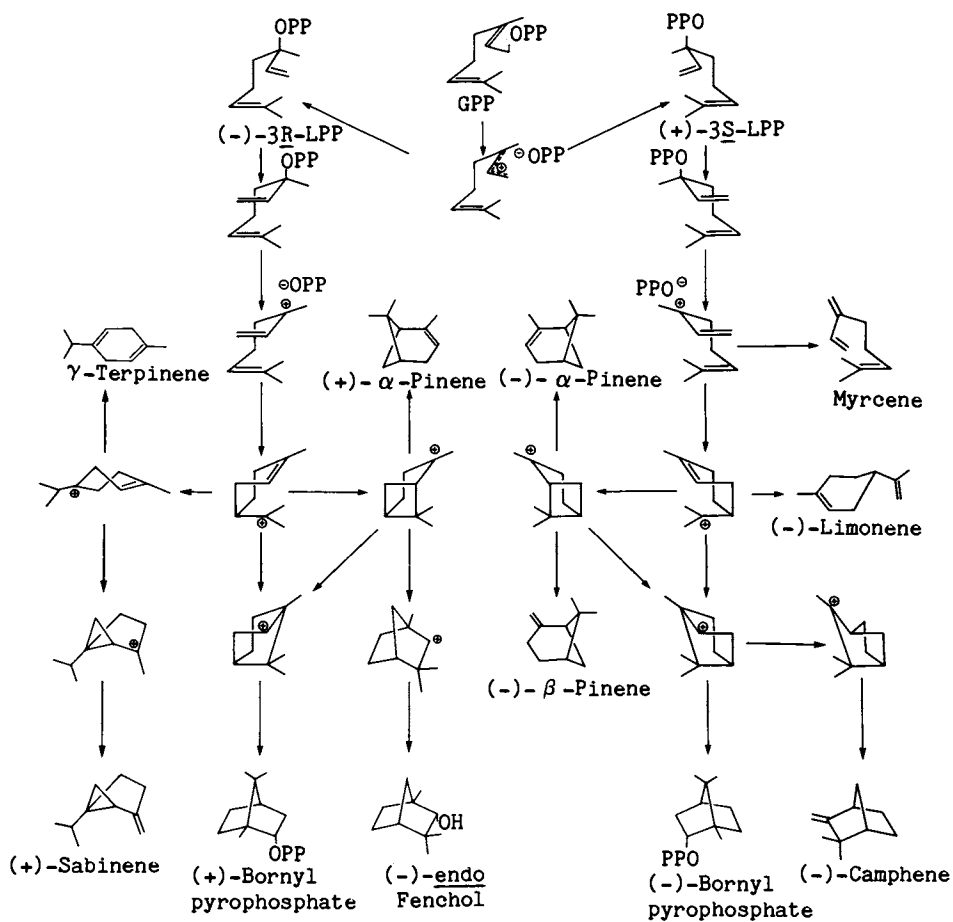


Figure 3. Unified stereochemical mechanism of monoterpene cyclization from geranyl pyrophosphate.



been proposed for the preliminary conversion of geranyl pyrophosphate to either NPP or LPP (or for the independent origin of these compounds), both of which can cyclize directly. However, since it is now clear that GPP is efficiently cyclized without formation of free intermediates, it follows that monoterpene cyclases must be capable of catalyzing both the required isomerization to a bound intermediate competent to cyclize and the cyclization reaction itself.

The mechanism of cyclization of geranyl pyrophosphate is now considered to involve the initial ionization of the pyrophosphate moiety, in which a divalent metal ion is presumed to assist, followed by stereospecific syn-isomerization to a linalyl intermediate (i.e., linalyl pyrophosphate, the corresponding ion-pair, or other bound equivalent), with rotation about the C2-C3 single bond and subsequent cyclization of the cisoid rotamer in the anti-endo conformation. The chiral  $\alpha$ -terpinyl cation so produced, may then undergo electrophilic addition to the remaining double bond, as well as hydride shifts and rearrangements, to provide the various other cationic cyclic parents. The initially formed cyclization products, consisting of one or more rings, are generated as olefins (via deprotonation) or simple alcohol or ether derivatives (via capture by a nucleophile). Subsequent transformations, frequently oxidation, ultimately give rise to the vast number of monoterpene metabolites. This unified isomerization-cyclization scheme is completely consistent with the results of numerous model studies of terpenoid cyclizations, readily allows for the observed direct cyclization of both LPP and NPP as alternate substrates, and, most importantly, accounts for the formation of essentially all cyclic types as well as the regio- and enantioselectivities inherent in cyclase catalysis (75). Implicit in this stereochemical model is the postulated folding of GPP by the enzyme on binding, which determines the absolute configuration of the intermediate LPP, which in turn dictates the stereochemical outcome of the subsequent cyclization. Accordingly, antipodal monoterpenes would be derived from different enzymes which generate opposite enantiomers of LPP in the isomerization step of the coupled sequence (74,75).

Direct evidence for the above proposal was obtained with the demonstration that separable cyclases derived from sage (*Salvia officinalis*) synthesize monoterpene olefins of opposite stereochemistry (74,76). Thus, cyclase I, of MW 96,000, converted GPP to (+)- $\alpha$ -pinene, (+)-camphene and (+)-limonene of related configuration, whereas cyclase II, of MW 55,000, transformed the same achiral precursor to (-)- $\beta$ -pinene in addition to (-)- $\alpha$ -pinene, (-)-camphene and (-)-limonene. Extensive purification of each enzyme and differential inactivation studies ensured that each set of stereochemically related products was synthesized by a single, distinct enzyme (74). Since ( $\pm$ )-LPP had been shown to serve as a precursor for both enzymes (76) it was possible to directly assess the absolute configuration of the tertiary intermediate cyclized, by the preparation and separate testing of each enantiomer. As predicted by the general model (Fig. 3), 3R-LPP preferentially

gave rise to the (+)-olefins generated by cyclase I, whereas (3S)-LPP preferentially gave rise to the (-)-olefin series generated by cyclase II. The cyclization scheme posits the formation of  $\alpha$ - and  $\beta$ -pinene by alternate deprotonations of a pinyll cation (76). Banthorpe and associates have suggested, on the basis of *in vivo* studies (65,66), that (-)- $\alpha$ -pinene could be formed indirectly via the thermodynamically favorable isomerization of (-)- $\beta$ -pinene. No support for this proposal was obtained with the cell-free systems described above (74,76), nor could an alternate proposal by Gleizes and associates (69) for the reversible protonation of ocimene and myrcene to  $\alpha$ - and  $\beta$ -pinene, respectively, be substantiated in cell-free preparations.

Support for the crucial role of the isomerization step of the reaction sequence was also obtained from studies on geranyl pyrophosphate:(-)-endo-fenchol cyclase from fennel (Foeniculum vulgare) (77-79). Direct testing of (3R)-linalyl pyrophosphate afforded a  $K_m$  value lower than that obtained with geranyl pyrophosphate, and a relative velocity nearly three-times higher. These results are clearly consistent with the proposed stereochemical model and further suggest that the isomerization step is rate limiting in the coupled isomerization-cyclization of geranyl pyrophosphate to (-)-endo-fenchol (Fig. 3). (3S)-Linalyl pyrophosphate was not an effective substrate for (-)-endo-fenchol biosynthesis but did, by an anomalous cyclization, give rise to low levels of the enantiomeric (+)-(1R)-endo-fenchol. It was therefore concluded that enzymatic recognition of the tertiary intermediate in an approach from solution was not sufficient to completely discriminate between the similar overall profiles presented by the linalyl pyrophosphate enantiomers. While the formation of (+)-endo-fenchol from (3S)-linalyl pyrophosphate is clearly an aberrant reaction sequence, the stereochemical outcome is still entirely consistent with the cyclization model. More importantly, the complete absence of the (+)-isomer with the achiral geranyl pyrophosphate as substrate serves to confirm the absolute regio- and stereochemical control over the initial isomerization step as well as the subsequent cyclization of the normal, coupled reaction sequence.

Thorough stereochemical analysis of the cyclizations of geranyl pyrophosphate to (+)- and (-)-bornyl pyrophosphate (Fig. 3) (by enzymes from Salvia officinalis and Tanacetum vulgare, respectively) have also been carried out (80,81). These reactions, which generate the cyclic parents of (+)- and (-)-camphor (82-84), are unique among monoterpene cyclizations in that the pyrophosphate moiety of the acyclic precursor is retained in the cyclic product. In this instance it was demonstrated that geranyl pyrophosphate (chirally labeled) was cyclized to bornyl pyrophosphate with net retention of configuration at C1 of the acyclic precursor, whereas similarly labeled neryl pyrophosphate was cyclized to product with inversion of configuration at C1 (85). The observed stereochemistry is a consequence of the reaction mechanism whereby geranyl pyrophosphate is first stereospecifically isomerized to linalyl pyrophosphate which, following rotation

about C2-C3 to the cisoid conformer, cyclizes from the anti-endo configuration. Neryl pyrophosphate cyclizes either directly or via the linalyl intermediate without the attendant rotation.

The role of ion pair formation in monoterpene cyclizations was revealed by additional studies with the (+)- and (-)-bornyl pyrophosphate cyclases in which the migrations of the pyrophosphate moiety were examined (86,87). Separate incubation of [ $1-^3\text{H}_2$ ;  $\alpha$ - $^{32}\text{P}$ ] and [ $1-^3\text{H}_2$ ;  $\beta$ - $^{32}\text{P}$ ]geranyl and ( $\pm$ )-linalyl pyrophosphates with partially purified preparations of each enantiomer-generating cyclase gave [ $^3\text{H}$ ;  $^{32}\text{P}$ ]bornyl pyrophosphates which were selectively hydrolyzed to the corresponding bornyl phosphates. Measurement of  $^3\text{H}$ : $^{32}\text{P}$  ratios of these monophosphate esters established that the two ends of the pyrophosphate moiety retained their identities in the cyclization of both precursors to both products, and also indicated that there was no appreciable exchange with exogenous inorganic pyrophosphate in the reaction. Subsequent incubations of each cyclase with [ $^{14}\text{C}$ ;  $1-^{18}\text{O}$ ]geranyl pyrophosphate and with [ $^3\text{H}$ ;  $3-^{18}\text{O}$ ]linalyl pyrophosphate gave the appropriate (+)- or (-)-bornyl pyrophosphates which were hydrolyzed in situ to the corresponding borneols. Analysis of the derived benzoates by mass spectrometry demonstrated each of the product borneols to possess an  $^{18}\text{O}$  enrichment essentially identical to that of the respective acyclic precursor. The absence of  $\text{P}_\alpha$ - $\text{P}_\beta$  interchange and the complete lack of positional  $^{18}\text{O}$ -isotope exchange of the pyrophosphate moiety are compatible with tight ion-pairing of intermediates in the coupled isomerization-cyclization of geranyl pyrophosphate and establish a remarkably tight restriction on the motion of the transiently generated pyrophosphate anion with respect to its cationic terpenyl reaction partner.

Most information now available on the regio- and stereochemistry of monoterpene cyclizations has been obtained from studies on the origin of bicyclic monoterpenes of the pinane, fenchane and bornane type. Progress is also being made with cyclases which transform geranyl pyrophosphate to common monocarbocyclic products such as  $\alpha$ -terpinene (88),  $\gamma$ -terpinene (89) and 1,8-cineole (90). Examination of the mechanisms leading to these achiral products has proven to be rather more challenging, however, since few stereochemical deductions can be made a priori, as with the chiral bicyclic types. The cyclases which generate the simple monoterpene types are usually more readily available, and are often more active and easier to purify, advantages that should ultimately lead to a better understanding of the physical and chemical nature of these protein catalysts; areas in which information is now sorely lacking.

### Conclusion

The biochemical bases for the terpenoid-mediated interactions of conifers with bark beetles and their fungal symbionts is understood in broad outline. Resistance to colonization ultimately resides in the species-specific arsenal of terpenoids which can be mustered in response to an attack. Resistant

conifers exhibit an induced wound response in which greater quantities of the more toxic monoterpenes are generated through catalysis of cyclases. Resistance, therefore, depends upon the genetic inheritance of cyclases which produce terpenoid products that are effective against specific insect and fungal pests, and upon the ability of the tree to respond vigorously to the appropriate chemical signal (elicitor) by induction of the cyclases and the generation of the requisite acyclic precursor. Through an understanding of the enzymology, stereochemistry and mechanism of monoterpene cyclases, it may be possible to modify or otherwise manipulate these unusual catalysts to improve conifer resistance.

#### Literature Cited

1. Shrimpton, D.M. in "Theory and Practice of Mountain Pine Beetle Management in Lodgepole Forests"; Berryman, A.A.; Amman, G.D.; Stark, R.W.; Kibbee, D.L., Eds.; College of Forest Resources, University of Idaho: Moscow, 1978, p. 64.
2. Berryman, A.A. *BioScience* 1972, **63**, 1194.
3. Shigo, A.L. *Ann. Rev. Phytopathol.* 1984, **22**, 189.
4. Dell, B.; McComb, A.J. in "Advances in Botanical Research"; Woolhouse, H.W., Ed.; Academic Press: New York, 1978, p. 227.
5. Squillace, A.E. in "Modern Methods of Forest Genetics"; Miksche, J.P., Ed.; Springer-Verlag: Berlin, 1976, p. 120.
6. Raffa, K.F.; Berryman, A.A. *Ecol. Monographs* 1983, **53**, 27.
7. Christiansen, E.; Horntvedt, R. *Z. Ang. Ent.* 1983, **96**, 110.
8. Cates, R.G.; Alexander, H. in "Bark Beetles in North American Conifers"; Mitton, J.B.; Sturgeon, K.B., Eds.; University of Texas Press: Austin, 1982, p. 212.
9. Russell, C.E.; Berryman, A.A. *Can. J. Bot.* 1976, **54**, 14.
10. Flodin, K.; Fries, N. *Eur. J. For. Pathol.* 1978, **8**, 300.
11. Franich, R.A.; Gaskin, R.E.; Wells, L.G.; Zabkiewicz, J.A. *Physiol. Plant Pathol.* 1982, **21**, 55.
12. Mowe, G.; King, B.; Senn, S.J. *J. Gen. Microbiol.* 1983, **129**, 779.
13. Harris, L.J.; Borden, J.H.; Pierce, H.D. Jr.; Oehlschlager, A.C. *Can. J. For. Res.* 1983, **13**, 350.
14. White, E.E. *Phytochemistry* 1983, **22**, 1399.
15. Croteau, R.; Johnson, M.A. in "Biosynthesis and Biodegradation of Wood Components"; Higuchi, T., Ed.; Academic Press: New York, 1985, p. 379.
16. Croteau, R. in "Herbs, Spices and Medicinal Plants - Recent Advances in Botany, Horticulture and Pharmacology"; Craker, L.E.; Simon, J.E., Eds.; Orynx Press: New York, in press.
17. Croteau, R. in "Isopentenoids in Plants"; Nes, W.D.; Fuller, G.; Tsai, L.S., Eds.; Marcel Dekker: New York, 1984, p. 31.
18. Porter, J.W.; Spurgeon, S.L., Eds. "Biosynthesis of Isoprenoid Compounds"; Vol. 1; Wiley Interscience: New York, 1981.
19. Porter, J.W.; Spurgeon, S.L., Eds. "Biosynthesis of Isoprenoid Compounds"; Vol. 2; Wiley Interscience: New York, 1983.

20. Nes, W.D.; Fuller, G.; Tsai, L.S., Eds. "Isopentenoids in Plants"; Marcel Dekker: New York, 1984.
21. Loomis, W.D.; Croteau, R. in "The Biochemistry of Plants"; Vol. 4; Stumpf, P.K., Ed.; Academic Press: New York, 1980, p. 364.
22. Banthorpe, D.V.; Charlwood, B.V. in "Encyclopedia of Plant Physiology, New Series"; Vol. 8; Bell, E.A.; Charlwood, B.V.; Eds.; Springer-Verlag: Berlin, 1980, p. 185.
23. Croteau, R.; Johnson, M.A. in "Biology and Chemistry of Plant Trichomes"; Rodriguez, E.; Healey, P.; Mehta, I., Eds.; Plenum Press: New York, 1984, p. 133.
24. Croteau, R. in "Biosynthesis of Isoprenoid Compounds"; Vol. 1; Porter, J.W.; Spurgeon, S.L., Eds.; Wiley Interscience: New York, 1981, p. 225.
25. Cori, O. Phytochemistry 1983, 22, 331.
26. Charlwood, B.V.; Banthorpe, D.V. in "Progress in Phytochemistry"; Vol. 5; Reinhold, L.; Harborne, J.B.; Swain, T., Eds.; Pergamon Press: Oxford, 1978, p. 65.
27. Bernard-Dagan, C.; Pauly, C.; Marpeau, A.; Gleizes, M.; Carde, J.; Baradat, P. Physiol. Veg. 1982, 20, 775.
28. Hodges, J.D.; Elam, W.W.; Watson, W.F.; Nebeker, T.E. Can. Ent. 1979, 111, 339.
29. Ferrell, G.T. Can. Ent. 1983, 115, 1421.
30. Wood, D.L. Ann. Rev. Entomol. 1982, 27, 411.
31. MacLean, D.J.; Sargent, J.A.; Tommerup, I.C.; Ingram, D.S. Nature 1974, 249, 186.
32. Raffa, K.F.; Berryman, A.A. Can. Ent. 1983, 115, 723.
33. Ried, R.W.; Whitney, H.S.; Watson, J.A. Can. J. Bot. 1967, 45, 1115.
34. Sturgeon, K.B.; Mitton, J.B. in "Bark Beetles in North American Conifers"; Mitton, J.B.; Sturgeon, K.B., Eds.; University of Texas Press: Austin, 1982, p. 350.
35. Wilkinson, R.C. For. Sci. 1980, 26, 581.
36. Raffa, K.F.; Berryman, A.A. Can. Ent. 1982, 114, 797.
37. Raffa, K.F.; Berryman, A.A. Environ. Entomol. 1982, 11, 486.
38. Klimetzek, D.; Francke, W. Experientia 1980, 36, 1343.
39. Byers, J.A. Science 1983, 220, 624.
40. Francke, W.; Vité, J.P. Z. Ang. Ent. 1983, 96, 146.
41. Silverstein, R.M. in "Chemical Ecology: Odor Communication in Animals"; Ritter, F.J., Ed.; Elsevier/North Holland Biomedical Press: Amsterdam, 1979, p. 133.
42. De Groot, R.C. Mycologia 1972, 64, 863.
43. Cobb, F.W. Jr.; Krstic, M.; Zavarin, E.; Barker, H.W. Jr. Phytopathology 1968, 58, 1327.
44. Chou, C.K.S.; Zabkiewicz, J.A. Eur. J. For. Pathol. 1976, 6, 354.
45. Schurig, V. Agnew. Chem. Int. Ed. Engl. 1984, 23, 447.
46. Schurig, V.; Weber, R. J. Chromatography 1984, 289, 321.
47. Kuroda, K.; Shimaji, K. For. Sci. 1983, 29, 653.
48. Hepting, G.H. Science 1947, 105, 209.
49. Laszlo, J.A.; Heinstejn, P.F. J. Cell Biochem. 1984, (8 part B, suppl.), 261.
50. Darvill, A.G.; Albersheim, P. Ann. Rev. Plant Physiol. 1984, 35, 243.

51. Dixon, R.A.; Dey, P.M.; Lamb, C.J. Adv. Enz. Related Areas Mol. Biol. 1983, 55, 1.
52. Hadwiger, L.A.; Fristensky, B.; Rigglesman, R.C. in "Chitin, Chitosan and Related Enzymes"; Zikakis, J.P., Ed.; Academic Press: Orlando, Florida, 1984, p. 291.
53. Hadwiger, L.A.; Loschke, D.C. Phytopathology 1981, 71, 756.
54. West, C.A. Naturwissenschaften 1981, 68, 447.
55. Ryder, T.B.; Cramer, C.L.; Bell, J.N.; Robbins, M.P.; Dixon, R.A.; Lamb, C.J. Proc. Natl. Acad. Sci. USA 1984, 81, 5724.
56. Croteau, R.; Gurkewitz, S. unpublished.
57. Croteau, R.; Cane, D.E. Methods Enzymol. 110, in press.
58. Wright, L.C.; Berryman, A.A.; Gurusiddaiah, S. Can. Ent. 1977, 111, 1255.
59. Paine, T.D. Can. J. Bot. 1984, 62, 556.
60. Ballard, R.G.; Walsh, M.A.; Cole, W.E. Can. J. Bot. 1984, 62, 1724.
61. Cane, D.E. Tetrahedron 1980, 36, 1109.
62. Poulter, C.D.; Rilling, H.C. in "Biosynthesis of Isoprenoid Compounds"; Porter, J.W.; Spurgeon, S.L., Eds.; Vol. 1; Wiley Interscience: New York, 1981, p. 161.
63. Stanley, R.G. Nature 1958, 182, 738.
64. Valenzuela, P.; Cori, O.; Yudelevich, A. Phytochemistry 1966, 5, 1005.
65. Banthorpe, D.V.; Le Patourel, G.N.J. Biochem. J. 1972, 130, 1055.
66. Banthorpe, D.V.; Ekundayo, O.; Njar, V.C.O. Phytochemistry 1984, 23, 291.
67. Banthorpe, D.V.; Ekundayo, O. Phytochemistry 1976, 15, 109.
68. Akhila, A.; Banthorpe, D.V. Phytochemistry 1980, 19, 1691.
69. Gleizes, M.; Marpeau, A.; Pauly, G.; Bernard-Dagan, C. Phytochemistry 1982, 21, 2641.
70. Bernard-Dagan, C.; Gleizes, M.; Pauly, G.; Carde, J.P.; Marpeau, A. in "Biogenesis and Function of Plant Lipids"; Mazliak, P.; Benveniste, P.; Costes, C.; Douce, R., Eds.; Elsevier/North Holland Biomedical Press: Amsterdam, 1980, p. 437.
71. Croteau, R.; Karp, F. Arch. Biochem. Biophys. 1976, 176, 734.
72. Cori, O. Arch. Biochem. Biophys. 1969, 135, 416.
73. Haissig, B.E.; Schipper, A.L. Anal. Biochem. 1972, 48, 129.
74. Gambliel, H.; Croteau, R. J. Biol. Chem. 1984, 259, 740.
75. Croteau, R. in "Model Building in Plant Physiology/Biochemistry"; Newman, D.W.; Wilson, K.G., Eds.; CRC Press: Boca Raton, Florida, in press.
76. Gambliel, H.; Croteau, R. J. Biol. Chem. 1982, 257, 2335.
77. Satterwhite, D.M.; Wheeler, C.J.; Croteau, R., J. Biol. Chem., in press.
78. Croteau, R.; Felton, M.; Ronald, R.C. Arch. Biochem. Biophys. 1980, 200, 524.
78. Croteau, R.; Karp, F. Arch. Biochem. Biophys. 1977, 184, 77.
79. Croteau, R.; Shaskus, J. Arch. Biochem. Biophys. 1985, 236, 535.

80. Croteau, R.; Karp, F. Arch. Biochem. Biophys. 1979, 198, 523.
81. Croteau, R.; Hooper, C.L.; Felton, M. Arch. Biochem. Biophys. 1978, 188, 132.
82. Croteau, R.; Felton, N.M. Phytochemistry 1980, 19, 1343.
83. Croteau, R.; Felton, N.M.; Wheeler, C.J. J. Biol. Chem., 1985, 260, 5956.
84. Cane, D.E.; Saito, A.; Croteau, R.; Shaskus, J.; Felton, M. J. Amer. Chem. Soc. 1982, 104, 5831.
85. Croteau, R.B.; Shaskus, J.J., Renstrøm, B., Felton, N.M.; Cane, D.E.; Saito, A.; Chaug, C. Biochemistry, in press.
86. Johnson, M.; Croteau, R. Plant Physiol. 1980, 65, (suppl.), 96.
87. Poulouse, A.J.; Croteau, R. Arch. Biochem. Biophys. 1978, 191, 400.
88. Croteau, R.; Karp, F. Arch. Biochem. Biophys. 1977, 179, 257.

RECEIVED February 3, 1986

## Chapter 7

# Sesquiterpenes as Phytoalexins and Allelopathic Agents

Stella D. Elakovich

Department of Chemistry, University of Southern Mississippi, Hattiesburg,  
MS 39406-5043

The plant origin and structure are given for those sesquiterpenes which have been shown to be active as phytoalexins or allelopathic agents. Potato (Solanum tuberosum), pepper (Capsicum annuum), tobacco (Nicotiana species), eggplant (Solanum melongena), tomato (Lycopersicon esculentum) and jimsonweed (Datura stramonium), all members of the Solanaceae, are rich sources of sesquiterpenoid phytoalexins. Over twenty furanosesquiterpenoid phytoalexins have been isolated from sweet potato (Ipomoea batatas). Both elm (Ulmus glabra) and cotton (Gossypium species) have also been sources of sesquiterpenoid phytoalexins. Allelopathic sesquiterpenoids have been implicated in a limited number of investigations. Potential sesquiterpenoid allelochemicals have been found in Artemisia absinthium, Ambrosia psilostachya, Cyperus serotinus and Lippia nodiflora.

Sesquiterpenes frequently occur in the steam volatile essential oils of higher plants. They occur less frequently in lower plants and in the animal kingdom although a number of marine organisms have proved to be abundant sources of a novel group of both halogenated and nonhalogenated sesquiterpenes (Ref. 1). A large class of highly oxygenated sesquiterpene lactones has been isolated, many from the Compositae, and shown to be bioactive. Stevens (Ref. 2) has recently reviewed these compounds. Plant derived sesquiterpenes include hydrocarbons as well as alcohols, ketones, aldehydes and carboxylic acids. Robinson (Ref. 3) suggests the term "sesquiterpenoid" to better describe both hydrocarbon and oxygenated compounds while "sesquiterpene" refers only to hydrocarbons. Many sesquiterpenoids possessing interesting biological properties have been detected. This chapter will discuss those which have been shown to be active as phytoalexins or allelopathic agents. The structures of these compounds are given in Figures 1-8 in the text.

0097-6156/87/0325-0093\$06.00/0  
© 1987 American Chemical Society



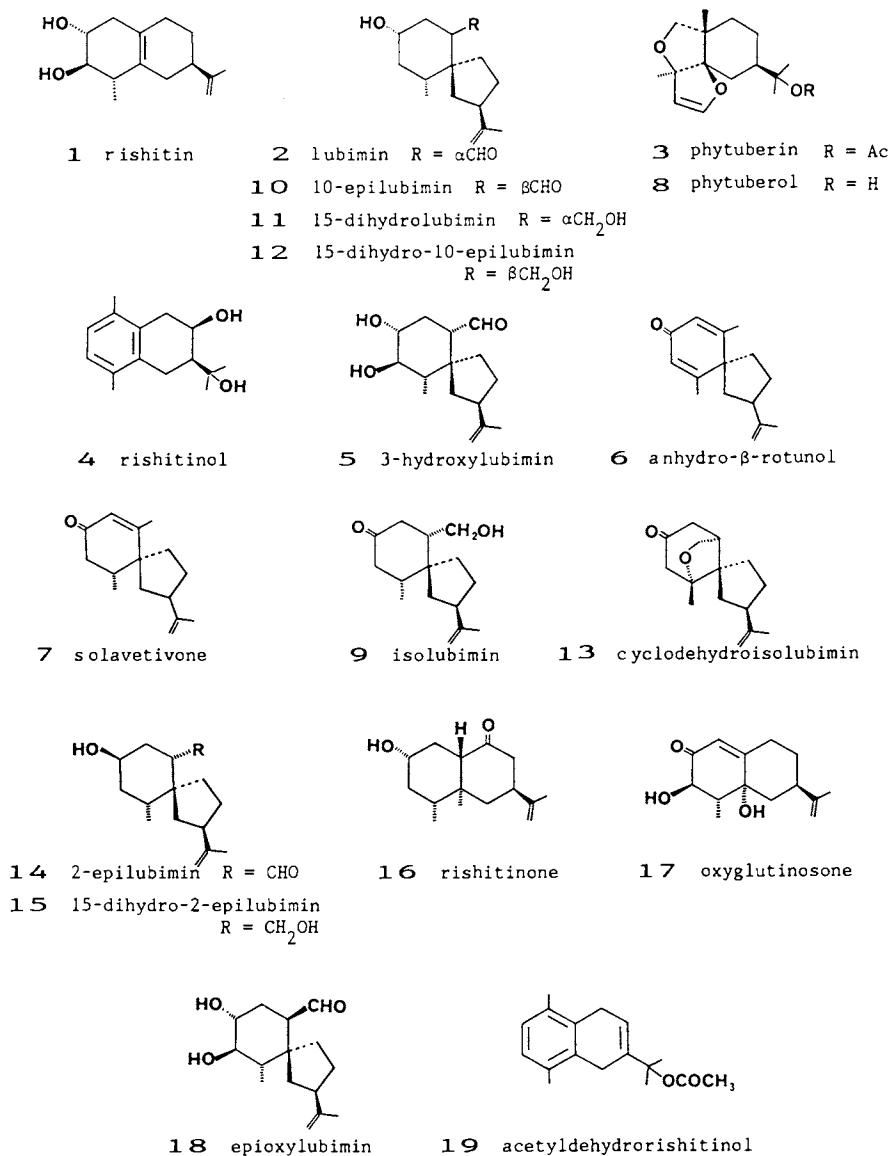


Figure 1. Sesquiterpenoid Stress Metabolites from Potato.

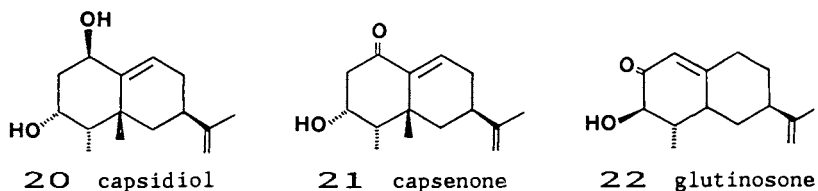


Figure 2. Sesquiterpenoid Stress Metabolites from Sweet Pepper (20 and 21) and Tobacco (20) and (22).

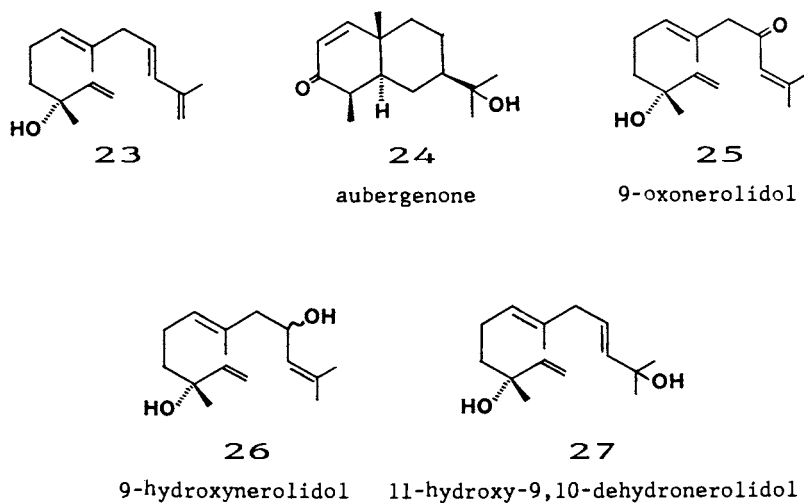


Figure 3. Sesquiterpenoid Stress Metabolites from Eggplant.

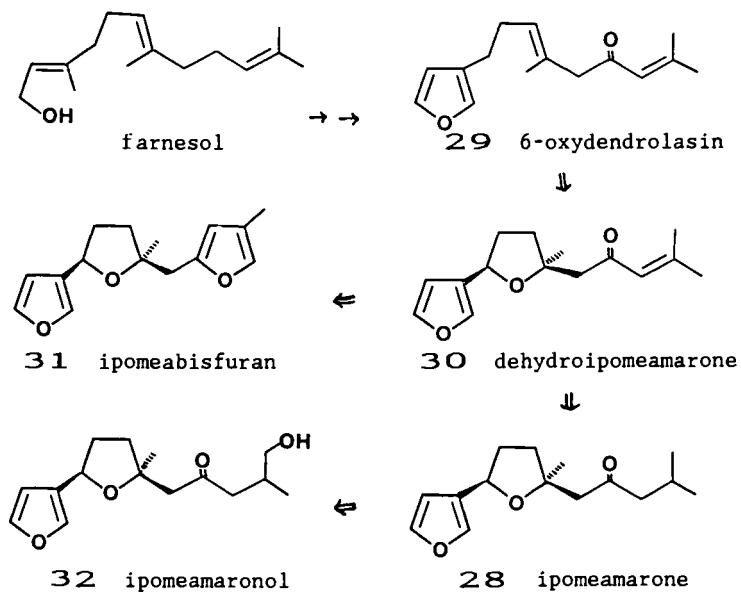


Figure 4. Biosynthetic Relationship of Furanosesquiterpenoid Stress Metabolites from Sweet Potato.

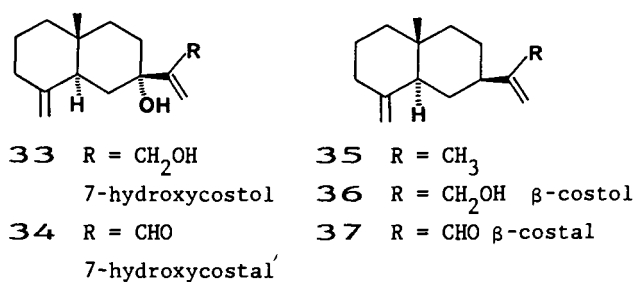


Figure 5. Selinene-type Sesquiterpenoid Stress Metabolites from Sweet Potato.



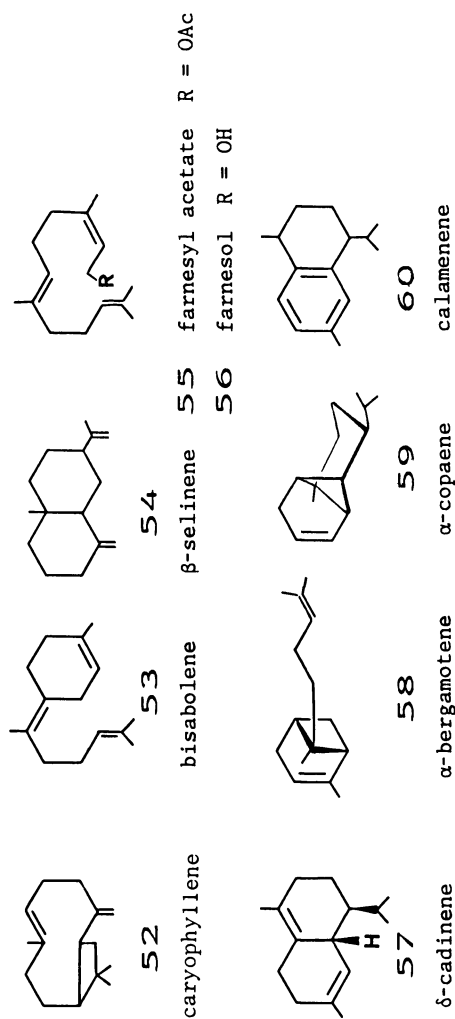


Figure 8. Sesquiterpenes Reported to be Allelopathic Agents.

### Biosynthesis

Sesquiterpenoids arise via the mevalonate-isopentyl pyrophosphate-farnesyl pyrophosphate pathway with 2-cis-6-trans- or 2-trans-6-trans-farnesyl pyrophosphate as the biological precursor for almost all sesquiterpenoids. Because biosynthetic investigations of plants have been hampered by low and nonspecific uptake of added precursors, compartmentation effects, rapid turnover of metabolites, and seasonal metabolic variations, the majority of biosynthetic investigations of sesquiterpenoid biosynthesis have been carried out with fungi (Ref. 4). The recent routine availability of <sup>13</sup>C nuclear magnetic resonance (nmr) has greatly simplified the ability to directly locate sites of labeling without recourse to laborious degradative schemes (Ref. 5). This has led to a dramatic increase in the number of reports on sesquiterpenoid biosynthesis. A number of investigations of phytoalexin biosynthesis have reported added precursor being incorporated at levels comparable to those seen in fungal cultures (Ref. 4). Thus phytoalexins may provide a rich source of plant sesquiterpenoid biosynthetic information. A recent report by Brindle, et al. (Ref. 6) established that potato-cell suspension cultures synthesize and accumulate sesquiterpenoid phytoalexins. This ability to elicit terpenoid phytoalexin formation in cell suspension culture should speed the exploitation of tissue culture techniques in the studies of terpenoid phytoalexin biosynthesis.

### Phytoalexins

Phytoalexins are substances that are absent in normal plant tissue, but are biosynthesized in response to a challenge. Originally, phytoalexins were antifungal compounds elicited upon infection of the host plant by some fungi. It is now clear that phytoalexins are produced in plants in response to other living organisms such as bacteria, viruses and nematodes, and also in response to treatment with chemicals, mechanical wounding, dehydration, cold or ultra-violet light. Stoessl et al. (Ref. 8) suggest that the term 'phytoalexin' be applied to any antifungal compound synthesized by the plant in greatly increased amounts after fungal infection, and that the term 'stress metabolite' be applied to compounds produced in response to other challenges. However, other authors use the terms almost interchangeably. The amounts of phytoalexins produced from viruses can be as large as 10 to 500 µg/g tissue. Thus virus-infected tissues have been useful for the isolation of new phytoalexins and also the provision of quantities sufficient for studies on their metabolism and toxicity (Ref. 7). A 1982 monograph edited by Bailey and Mansfield (Ref. 9) provides a thorough review of all the various aspects of phytoalexins.

### Phytoalexins from the Solanaceae

Most phytoalexin research has been conducted with plants in the Leguminosae and Solanaceae. Sesquiterpenoid phytoalexins are common in the Solanaceae, but are not apparent in the Leguminosae. Kuć (Ref. 10) has recently reviewed phytoalexins from the Solanaceae. In 1976 Stoessl et al. (Ref. 8) reviewed

sesquiterpenoid stress compounds of the Solanaceae, and in 1981 Stothers reviewed these compounds with emphasis on the biosynthetic pathways leading to their formation (Ref. 11).

Potato - *Solanum tuberosum*. Classical work by Müller and Börger (Ref. 12) in 1940 with potatoes began phytoalexin research in the Solanaceae. They were attempting to produce potato cultivars with resistance to *Phytophthora infestans*, the fungus which causes late blight disease in potatoes. They demonstrated that potato tubers infected with *P. infestans* produced low molecular weight fungitoxic compounds and that the accumulation of these compounds was related to the resistance of the tuber to the fungus. In spite of the great economic importance of cultivated potatoes, it was some 28 years before a group of Japanese workers (Ref. 13) isolated rishitin (1) as the first well-defined phytoalexin from potatoes. Reports of the two additional phytoalexins, lubimin (2) (Ref. 14) and phytuberin (3) (Ref. 15), followed quickly. The originally reported structure for lubimin was later corrected by Katsui et al. (Ref. 16) and Stoessl et al. (Ref. 17) to that shown in (2). Subsequently rishitinol (4) (Ref. 18), 3-hydroxylubimin (5) (Ref. 16a), anhydro- $\beta$ -rotunol (6) (Ref. 19), solavetivone (7) (Ref. 19) and phytuberol (8) (Ref. 20) were isolated as sesquiterpenoid stress metabolites (SSMs) of the potato. All of these sesquiterpenoids clearly belong to the same biogenetic group, but the role of each in disease resistance has not been thoroughly investigated. Rishitin, lubimin, solavetivone and phytuberin are termed phytoalexins. Rishitin, lubimin and solavetivone generally comprise 85% or more of the total potato derived SSMs with rishitin often the major SSM (Ref. 10). In different experiments using the same potato cultivar and race of *P. infestans*, Kuc (Ref. 10) has observed either rishitin, lubimin or solavetivone as the major SSM. He suggested that slight changes in the physiological state of the potato tubers and the environment can profoundly influence which of the sesquiterpenoids predominates. None of the compounds ever appears to be induced alone, but not all of them are produced in detectable amounts in any given situation.

Accumulation of small amounts of the additional sesquiterpenoids isolubimin (9) (Ref. 21), 10-epilubimin (10), 15-dihydrolubimin (11), 15-dihydro-10-epilubimin (12) (Ref. 22), cyclodehydroisolubimin (13) (Ref. 23), 2-epilubimin (14) (Ref. 24), 15-dihydro-2-epilubimin (15) (Ref. 25), rishitinone (16) (Ref. 26), oxyglutinosone (17), eploxylubimin (18) (Ref. 27), and acetyldehydrorishitinol (19) (Ref. 28) may be due to synthesis by the host or degradation by host or pathogen of host-synthesized terpenoids, or even to synthesis by a pathogen. (Ref. 10) The role of these compounds as phytoalexins has not been fully investigated.

Pepper - *Capsicum annuum*. The sesquiterpenoid phytoalexin capsidiol (20) is formed in pepper fruit after inoculation with many fungi and at least one bacterium (Ref. 22). Its skeleton is interesting because the vicinal methyl groups are trans, in contrast to all other previously described eremophilanes (Ref. 11). A second sesquiterpenoid, capsenone, is also present in peppers infected with certain fungi, but has been shown to be a fungal oxidation product of capsidiol (Ref. 29). Capsidiol accounts for

about half of the total ether extractives from the aqueous diffusates of peppers inoculated with the fungus Monilinia fructicola (Ref. 30). The diffusates from pepper are thus much less complex than those obtained from potato, or indeed, from any other members of the Solanaceae. Capsidiol may be obtained in concentrations of up to 0.75 mM in diffusates (Ref. 29). Fruits of Capsicum frutescens possess a sterile cavity into which large quantities of potential precursors can be aseptically injected together with fungal spore suspensions with little or no mechanical damage. They are thus almost ideal for host-parasite studies.

Ward et al. (Ref. 31) compared the fungitoxicity of capsidiol with that of rishitin, the major phytoalexin from potatoes, capsenone, and 20 other capsidiol-related or capsidiol-derived compounds. They used both pathogenic and non-pathogenic fungi. Capsidiol was the most active compound tested, but no correlations were found among the sensitivity to capsidiol or rishitin and pathogenicity for peppers or potatoes, nor could correlations between structure and activity be drawn.

Tobacco - Nicotiana species. Guedes et al. (Ref. 32) reported the accumulation of six sesquiterpenoid stress metabolites in foliage of Nicotiana tabacum innoculated with Pseudomonas lachrymans, a nonpathogen of tobacco. The infected tobacco foliage accumulated capsidiol (20), also reported in infected pepper, as well as rishitin (1), lubimin (2), phytuberin (3), phytuberol (8) and a trace of what was thought to be solavetivone (7), all of which are also found in infected potato. Maximum accumulation of these SSMs occurred 12-24 hours after infection whereas studies of pepper and potato showed maximum accumulation of SSMs after 48 to 96 hrs. Accumulation of the sesquiterpenoids coincided with the appearance of necrosis, was detected in and immediately around necrotic tissue, and was not detected in apparently healthy tissue 5 mm or more from the edges of lesions.

Burden et al. (Ref. 33) found the sesquiterpenoid glutinosone (22) in leaves of Nicotiana glutinosa which had been infected with tobacco mosaic virus. Although some investigators would approve the term 'phytoalexin' for a virus induced compound, glutinosone has not yet been reported in fungal infected tissue of N. glutinosa. Glutinosone could not be detected in healthy, uninoculated leaves. Both capsidiol and solavetivone could also be induced by virus infection. Capsidiol accumulated in both N. tabacum and N. clevelandii infected with tobacco necrosis virus, (Ref. 34) and solavetivone accumulated in N. tabacum infected with tobacco mosaic virus (Ref. 35).

Eggplant - Solanum melongena. Fruits of eggplants produce sesquiterpenoid phytoalexins, thus conforming to the pattern of other members of the Solanaceae (Ref. 36). Eggplants (Solanum melongena 'Black Beauty') routinely accumulated phytoalexins when innoculated with spore suspensions of Monilinia fructicola as well as with four other fungi. The ethyl ether extract of the diffusate obtained was very complex and required repeated chromatography for the separation of pure components. Seven compounds were isolated, accounting for about 30% of the extract. Of these seven, lubimin (2), which is also a potato phytoalexin, and a compound having



structure (23) were the two most active compounds in spore germination inhibition assays, but with the exception of aubergenone (24), all compounds had appreciable activity against P. infestans (Ref. 36). Compound (23) and another of the compounds isolated were almost certainly artifacts since they were not detected in fresh crude extracts. The remaining three identified compounds had structures (25), (26), and (27). All five of the fungi tested induced all of the compounds, although in differing amounts, which suggests that phytoalexin production is nonspecific.

The structure of aubergenone was revised to that of (24) by Murai and coworkers (Ref. 37). The revised structure, 11-hydroxy-4 $\alpha$ ,5 $\alpha$ -eudesm-1-en-3-one (24) is the only stress compound with a 5 $\alpha$ -eudesmane skeleton among the Solanaceae metabolites and hence stands on unique biogenetic grounds (Ref. 37).

Tomato - *Lycopersicon esculentum*. The tomato has not been investigated as thoroughly as potatoes, and the only sesquiterpenoid stress compound so far identified is rishitin (1), the main potato phytoalexin (Ref. 38). The tomato derived extracts from which rishitin can be isolated are complex. It is likely that other sesquiterpenoids will be isolated from these complex mixtures in time (Ref. 8).

Jimsonweed - *Datura stramonium*. Like other Solanaceae, *Datura stramonium* produces antifungal sesquiterpenoid compounds in response to inoculation with *Monilinia fructicola* and several other fungi (Ref. 39). Three of the four phytoalexins thus far identified are also accumulated by other Solanaceous species. Lubimin (2) is also produced both in potato and eggplant, capsidiol (20) is the main phytoalexin in sweet pepper fruit, and hydroxylubimin (5) is also found in potato. The fourth compound 2,3-dihydroxygermacrene, is unique to *Datura* and could serve as an almost direct precursor of lubimin and 4-hydroxylubimin as well as other Solanaceae phytoalexins (Ref. 39a).

#### Phytoalexins from Other Families

Convolvulaceae - Sweet Potato (*Ipomoea batatas*). In the forty years since Hiura (Ref. 40) first isolated ipomeamarone (28) from sweet potatoes, the furanosesesquiterpenoid stress metabolites of this plant have been extensively investigated and over twenty furano-sesquiterpenoids have been isolated (Ref. 41, 42). Extensive biosynthetic investigations have provided the following relationships among these phytoalexins (Ref. 43). 6-Oxodendrolasin (29) which is accumulated upon either infection by *Ceratocystis fimbriata* or HgCl<sub>2</sub> treatment, is the close precursor of dehydroipomeamarone (30) which appears to be the immediate biosynthetic precursor of ipomeamarone (28) and also the precursor of ipomeabisfuran (31), which, like (29) is accumulated upon either *C. fimbriata* infection or HgCl<sub>2</sub> treatment. Ipomeamarone (28) is the precursor of ipomeamaranol (32). All five of these compounds (28) - (32) have the properties of phytoalexins. They are produced in relatively large amounts in response to infection and they show antifungal activity. Schneider et al. (Ref. 42) recently characterized nine new minor stress metabolites from *C. fimbriata*

infected tissue and have proposed a detailed biogenic scheme which includes 19 furanoterpenoids and furanoterpenoid-related stress metabolites. At about the same time, Schneider and Nakanishi (Ref. 44) reported the presence of two new compounds of the eudesmane skeleton, 7-hydroxycostol (33) and 7-hydroxycostal (34), as well as three known selinene derivatives, compound (35),  $\beta$ -costol (36) and  $\beta$ -costal (37). These five compounds are included in the biogenic scheme of Schneider *et al.* (Ref. 42). The eudesmane skeleton is biogenically different from that of furanosesquiterpenoids, and thus the sweet potato represents a unique case of a plant which concurrently produces two skeletally different series of sesquiterpenoid phytoalexins (Ref. 44).

Ulmaceae - Elm (*Ulmus glabra*). Eight cadinane-type sesquiterpenes were isolated from Wych elm (*Ulmus glabra*) branches which had been infected with the fungi *Ceratocystis ulmi* (Causative agent of Dutch Elm disease), *Coriolus versicolor* (a white rot fungus) and *Chondrostereum purpureum* (causal agent of silver leaf disease in many trees) (Ref. 45). None of the eight compounds (38) - (45) were observed in chromatograms of sapwood of healthy branches, although all compounds (38) - (43) had been previously reported as heartwood constituents in various elm species. The mansonones E (42) and F (43) had previously been isolated from *U. hollandica* following infection by *C. ulmi*. The role of these tree phytoalexins in disease resistance has yet to be determined, but the present evidence suggests that they provide little effective fungal resistance (Ref. 45).

Malvaceae - Cotton (*Gossypium* species). Gossypol (46), a dimeric sesquiterpene of the cadalane class, is a natural pigment found in tissues of healthy cotton plants, but, because of certain properties it is often classed as a phytoalexin. Its biosynthesis was reviewed in 1979 by Heinstein *et al.* (Ref. 46). Gossypol accumulation can be induced by inoculation of cotton tissues with *Verticillium albo-atrum* (causative agent of wilt disease) or *Rhizopus nigricans*, or by chemical treatment with cupric or mercuric ions (Ref. 47). Purified gossypol proved active against fungi as measured by fungal spore germination assays, and similar amounts of gossypol were accumulated from those cotton cultivars having gossypol and those which have little or no free gossypol. Thus, gossypol may be classed as a phytoalexin. Bell *et al.* (Ref. 48) confirmed that hemigossypol (47) is the major phytoalexin formed in both *G. barbadense* and *G. hirsutum* upon *Verticillium* infection. Hemigossypol, 6-methoxy-hemigossypol (48) and 6-deoxyhemigossypol (49) were the major sesquiterpenoid stress metabolites from the infected tissue of a range of *Gossypium* species (Ref. 48). Russian workers have isolated isohemigossypol (50) and gossyvertin (51) from stem tissue of cotton plants infected with *V. dahliae* (Ref. 47). Very little gossypol was found in this same tissue. It thus appears that gossypol itself is not the most important contributor to the phytoalexin response of cotton even though it has antifungal properties.

### Allelopathic Agents

In his classical paper titled "The Influence of One Plant on Another-Allelopathy", Molisch in 1937 coined the term 'allelopathy' to refer to biochemical interactions between all types of plants including microorganisms. He included both inhibitory and stimulatory interactions (Ref. 49). Rice, (Ref. 50) in the first edition of his comprehensive monograph, Allelopathy, used the term to include only inhibitory interactions. In the recent second edition of his monograph, Rice reverts to Molisch's use of the term because the published literature convinced him that most, or perhaps all, organic compounds that are inhibitory at some concentrations may be stimulatory at some much lesser concentrations (Ref. 51).

Most secondary metabolites can be classified into five major categories: phenylpropanes, acetogenins, terpenoids, steroids, and alkaloids (Ref. 51). Higher plants produce a great variety of terpenoids, but only a very few sesquiterpenoids have been implicated in allelopathy (Ref. 51). Artemisia absinthium produces three sesquiterpene inhibitors,  $\beta$ -caryophyllene (52), bisabolene (53) and another component which forms chamazulene in the open air (Ref. 52). Both Ambrosia psilostachya and A. acanthicarpa produce several sesquiterpene lactones, but none of them have been confirmed as allelochemicals (Ref. 51). Volatile plant growth inhibitors isolated from western ragweed (Ambrosia psilostachya) may be sesquiterpenes.

Komai et al. (Ref. 53) found gc-ms evidence of the sesquiterpenes  $\beta$ -selinene (54), methyl farnesate, farnesyl acetate (55), and farnesol (56) present in an inhibitory fraction isolated from water nutgrass (Cyperus serotinus). This fraction at 300 ppm inhibited lettuce germination and also inhibited growth of lettuce and rice seedlings as well as nutgrass itself. The authors conclude that the sesquiterpenes are responsible for the observed allelopathic effects.

We (Ref. 54) have recently identified the sesquiterpenes  $\beta$ -caryophyllene (52),  $\delta$ -cadimene (57),  $\alpha$ -bergamotene (58),  $\beta$ -bisabolene (53),  $\alpha$ -copaene (59), calamenene (60) and 4,10-dimethyl-7-isopropylbicyclo[4.4.0]-1,4-decadiene from the steam volatiles of the creeping perennial herb Lippia nodiflora (family Verbenaceae) which is known for its rampant growth. Extracts of L. nodiflora reduced lettuce seedling radical length as compared to controls suggesting the presence of allelochemicals.  $\beta$ -Caryophyllene comprises almost 20% of the identified hydrocarbons. The steam volatiles of L. nodiflora also contain the monoterpenes  $\beta$ -pinene and p-cymene which Asplund (Ref.55) found inhibitory toward radish seed germination. These terpenes may well be acting as allelochemicals in L. nodiflora, contributing to its ability to grow rampantly and to inhibit lettuce seedling growth.

### Literature Cited

1. P. J. Scheuer, Ed., Marine Natural Products, Vol. 1, Academic Press, New York, 1978, pp. 125 and 177.

2. K. L. Stevens, "Biological Activity and Chemistry of Sesquiterpene Lactones", in W. D. Ness, G. Fuller, and L.-S. Tsai, eds., Isopentenoids in Plants: Biochemistry and Function, Marcel Dekker, Inc., New York, NY, 1984, pp. 65-80.
3. T. Robinson, The Organic Constituents of Higher Plants, 4th Ed., Cordus Press, North Amherst, Mass, 1980, p. 134.
4. D. E. Cane, "Biosynthesis of Sesquiterpenes", in J. W. Porter and S. L. Spurgeon, eds., Biosynthesis of Isoprenoid Compounds, Vol. I, John Wiley and Sons, NY, 1981, pp. 284-374.
5. J. R. Hanson, "The Biosynthesis of Some Sesquiterpenoids", Pure Appl. Chem., **53**, 1155-1162 (1981).
6. P. A. Brindle, P. J. Kuhn and D. R. Threlfall, "Accumulation of Phytoalexins in Potato-cell Suspension Cultures", Phytochemistry, **22**, 2719-2721 (1983).
7. J. A. Bailey and R. A. Skipp, "Toxicity of Phytoalexins", Ann. Appl. Biol., **89**, 354-358 (1978).
8. A. Stoessl, J. B. Stothers and E. W. B. Ward, "Sesquiterpenoid Stress Compounds of the Solanaceae", Phytochemistry, **15**, 855-872 (1976).
9. J. A. Bailey and J. W. Mansfield, eds., Phytoalexins, John Wiley and Sons, New York, 1982.
10. J. Kuć, "Phytoalexins from the Solanaceae", in J. A. Bailey and J. W. Mansfield, eds., Phytoalexins, John Wiley and Sons, New York, 1982, pp. 81-105.
11. J. B. Stothers, "Sesquiterpenes - Biosynthetic Studies with  $^{13}\text{C}$  and  $^2\text{H}$  Magnetic Resonance - A Synthetic Approach via Homoienolization", Pure Appl. Chem., **53**, 1241-1258 (1981).
12. K. O. Müller and H. Börger, "Experimentelle Untersuchungen über die Phytophthora - Resistenz der Kartoffel", Arb. Biol. Anst. Reichsanst. (Berl.), **23**, 189-231 (1940).
13. K. Tomiyama, T. Sakuma, N. Ishizaka, N. Sato, N. Katsui, M. Takasugi and T. Masamune, "A New antifungal Substance Isolated from Resistant Potato Tuber Tissue Infected by Pathogens", Phytopathology, **58**, 115-116 (1968).
14. O. L. Ozeretskoykaya, N. I. Vasyukova, and L. V. Metlitskii, Dokl. Bot. Sci., **187-189**, 158 (1969).
15. J. Varns, J. Kuć and E. Williams, "Terpenoid Accumulation as a Biochemical Response of the Potato Tuber to Phytophthora infestans", Phytopathology, **61**, 174-177 (1971).
16. (a) N. Katsui, A. Matsunaga, and T. Masamune, "The Structure of Lubimin and Oxylubimin, Antifungal Metabolites from Diseased Potato Tubers", Tetrahedron Lett., 4483-4486 (1974). (b) N. Katsui, A. Matsunaga, H. Kitahara, F. Yagihashi, A. Murai, T. Masamune and N. Sato, "Lubimin and Oxylubimin. The Structure Elucidation", Bull. Chem. Soc. Jpn., **50**, 1217-1225 (1977).
17. A. Stoessl, J. B. Stothers and E. W. B. Ward, "Lubimin: A Phytoalexin of Several Solanaceae. Structure, Revision and Biogenetic Relationships", J. Chem. Soc. Chem. Comm., 709-710 (1974).
18. N. Katsui, A. Matsunaga, K. Imaizumi, T. Masamune and K. Tomiyama, "The Structure and Synthesis of Rishitinol, A New Sesquiterpene Alcohol from Diseased Potato Tubers", Tetrahedron Lett., 83-86 (1971).

19. D. T. Coxon, K. R. Price, B. Howard, S. F. Osmon, E. B. Kalan and R. M. Zacharius, "Two New Vetispirane Derivatives: Stress Metabolites from Potato (*Solanum tuberosum*) tubers", Tetrahedron Lett. 2921-2924 (1974).
20. (a) K. R. Price, B. Howard and D. T. Coxon, "Stress Metabolite Production in Potato Tubers infected by *Phytophthora infestans*, *Fusarium avenaceum* and *Phoma exigua*", Physiol. Plant Pathol., 9, 189-197. (b) D. T. Coxon, K.R. Price, B. Howard and R. F. Curtis, "Metabolites from Microbially Infected Potato. Part 1, Structure of Phytuberin", J. Chem. Soc. Perkin, 53-59 (1977).
21. A. Stoessl, J. B. Stothers and E. W. B. Ward, "Sesquiterpenoid Stress Compounds of the Solanaceae", Phytochemistry, 15, 855-872 (1976).
22. A. Stoessl, E. W. B. Ward and J. B. Stothers, "Biosynthetic Relationships of Sesquiterpenoidal Stress Compounds from the Solanaceae", in P. A. Hedin, ed., Host Plant Resistance to Pests, Amer. Chem. Soc., Washington, DC, 1977, pp. 61-77.
23. D. T. Coxon, K. R. Price, J. B. Stothers and A. Stoessl, "Cyclodehydroisolibumin: A New Tricyclic Sesquiterpene from Potato Tubers Inoculated with *Phytophthora infestans*", J. Chem. Soc. Chem. Comm., 348-349 (1979).
24. N. Katsui, F. Yagihashi, A. Matsunaga, K. Orito, A. Murai, and T. Masamune, "The Structure of Epilubimin, A Stress Metabolite from Diseased Potato Tubers", Chem. Lett., 723-724 (1977).
25. A. Stoessl and J. B. Stothers, "2-Epi and 15-dihydro-2-epilubimin: New Stress Compounds from the Potato", Can. J. Chem., 58, 2069-2072 (1980).
26. N. Katsui, F. Yagihashi, A. Murai and T. Masamune", Structure of Rishitinone, a Valencane Stress Metabolite in Diseased Potato", Bull. Chem. Soc. Jpn., 55, 2428-2433 (1982).
27. N. Katsui, F. Yagihashi, A. Murai and T. Masamune, "Structure of Oxyglutinosone and Epioxylubimin, Stress Metabolites from Diseased Potato Tubers", Chem. Lett., 1205-1206 (1978).
28. L. M. Alves, R. M. Kirchner, D. T. Lodato, P. B. Nee, J. M. Zappia, M. L. Chichester, J. D. Stuart, E. B. Kalan and J. C. Kissinger, "Acetyldehydrorishitinol, A Rishitinol-Related Potato Stress Metabolite", Phytochemistry, 23, 537-538 (1984).
29. E. W. B. Ward and A. Stoessl, "Postinfectional inhibitors from Plants. III. Detoxification of Capsidiol, an Antifungal Compound from Peppers", Phytopathology, 62, 1186-1187 (1972).
30. A. Stoessl, C. H. Unwin, and E. W. B. Ward, "Postinfectional Inhibitors from Plants. I. Capsidiol, and Antifungal Compound from *Capsicum frutescens*", Phytopathology Z., 74, 141-152 (1972).
31. E. W. B. Ward, C.H. Unwin, and A. Stoessl, "Postinfectional Inhibitors from Plants. XIII. Fungitoxicity of the Phytoalexin, Capsidiol, and Related Sesquiterpenes", Can. J. Bot., 52, 2481-2488 (1974).
32. M. E. M. Guedes, J. Kuć, R. Hammerschmidt and R. Bostock, "Accumulation of Six Sesquiterpenoid Phytoalexins in Tobacco Leaves Infiltrated with *Pseudomonas lachrymans*", Phytochemistry, 21, 2987-2988 (1982).

33. R. S. Burden, J. A. Bailey and G. G. Vincent, "Glutinosone, A New Antifungal Sesquiterpene from *Nicotiana glutinosa* Infected with Tobacco Mosaic Virus", Phytochemistry, **14**, 221-223 (1975).
34. J. A. Bailey, R. S. Burden, and G. G. Vincent, "Capsidiol: An Antifungal Compound Produced in *Nicotiana tobacum* and *N. clevelandii* Following Infection with Tobacco Necrosis Virus", Phytochemistry, **14**, 597 (1975).
35. R. Uegaki, T. Fujimori, S. Kubo and K. Kato, "Sesquiterpenoid Stress Compounds from *Nicotiana* Species", Phytochemistry, **20**, 1567-1568 (1981).
36. E. W. B. Ward, C. H. Unwin, J. Hill, and A. Stoessl, "Sesquiterpenoid Phytoalexins from Fruits of Eggplants", Phytopathology, **65**, 859-863 (1975).
37. (a) A. Murai, A. Abiko, M. Ono, N. Katsui, and T. Masamune, "Structure Revision and Biogenetic Relationship of Aubergerone, a Sesquiterpenoid Phytoalexin of Eggplants", Chem. Lett., 1209-1212 (1978). (b) A. Murai, A. Abiko, M. Ono, and T. Masamune, "Synthesis of Aubergerone, a Sesquiterpenoid Phytoalexin from Diseased Eggplants", Bull. Chem. Soc. Jpn., **55**, 1191-1194 (1982).
38. N. Sato, K. Tomiyama and N. Katsui, "Isolation of Rishitin from Tomato Plants", Ann. Phytopathol. Soc. Japan, **34**, 344-345 (1968).
39. (a) E. W. B. Ward, C. H. Unwin, G. L. Rock, and A. Stoessl, "Postinfectional Inhibitors from Plants. XXIII. Sesquiterpenoid Phytoalexins from Fruit Capsules of *Datura stramonium*", Can. J. Bot., **54**, 25-29 (1976). (b) G. I. Birnbaum, C. P. Huber, M. L. Post, and J.B. Stothers "Sesquiterpenoid Stress Compounds of *Datura stramonium*: Biosynthesis of the Three Major Metabolites from [1,2-<sup>13</sup>C] Acetate and the X-ray Structure of 3-Hydroxyubimin", J. Chem. Soc. Chem. Comm., 330-331 (1976).
40. M. Hiura, "Studies in Storage and Rot of Sweet Potato", Rep. Gifu Agric. Coll., **50**, 1-5 (1943).
41. L. T. Burka and L. Kuhnert, "Biosynthesis of Furanosesquiterpenoid Stress Metabolites in Sweet Potatoes (*Ipomoea batata*). Oxidation of Ipomeamarone to 4-hydroxymyoporone", Phytochemistry, **16**, 2022-2023 (1977).
42. J. A. Schneider, J. Lee, Y. Naya, K. Nakanishi, K. Oba and I. Uritani, "The Fate of the Phytoalexin Ipomeamarone: Furanoterpenes and Butenolides from *Ceratocystis fimbriata* - Infected Sweet Potatoes", Phytochemistry, **23**(4), 759-764 (1984).
43. I. Ito, N. Kato, and I. Uritani, "Biochemistry of Two New Sesquiterpenoid Phytoalexins from Sweet Potato Roots", Agric. Biol. Chem., **48**(1), 159-164 (1984) and references therein.
44. J. A. Schneider and K. Nakanishi, "A New Class of Sweet Potato Phytoalexins", J. Chem. Soc. Chem. Commun., 353-355 (1983).
45. R. S. Burden and M. S. Kemp, "Sesquiterpene Phytoalexins from *Ulmus glabra*", Phytochemistry, **23**, 383-385 (1984).
46. P. Heinstein, R. Widmaier, P. Wegner, and J. Howe, "Biosynthesis of Gossypol", Recent Adv. Phytochem., **12**, 313-333 (1979).

47. D. T. Coxon, "Phytoalexins from Other Families", Chapter 4 in J. A. Bailey and J. W. Mansfield, eds., Phytoalexins, John Wiley and Sons, New York (1982), pp. 116-118.
48. A. A. Bell, R. D. Stipanovic, C. R. Howell and P. A. Fryxell, "Antimicrobial terpenoids of Gossypium: Hemigossypol, 6-Methoxyhemigossypol and 6-Deoxyhemigossypol", Phytochemistry, 14, 225-231 (1975).
49. H. Molisch, "Der Einfluss einer Pflanze auf die andere- Allelopathie", Fischer, Jena (1937).
50. E. L. Rice, Allelopathy, Academic Press, New York, 1974.
51. E. L. Rice, Allelopathy, 2nd Ed., Academic Press, New York, 1984.
52. G. Grummer, "The Role of Toxic Substances in the Interrelationships Between Higher Plants", in F. L. Mitthorpe, ed., Mechanisms in Biological Competition, Academic Press, New York (1961), pp. 224-225.
53. K. Komai, Y. Sugiwaka and S. Sato, "Plant-growth Retardant of Extracts Obtained from Water Nutgrass (Cyperus serotinus Rottb.)", Kinki Daigaku Nogakubu Kiyo, 1981, (14), 57-65. Chem. Abstr., 95, 162961c (1981).
54. S. D. Elakovich and K. L. Stevens, "Volatile Constituents of Lippia nodiflora", J. Nat. Prod., 48, 504-506 (1985).
55. R. O. Asplund, "Monoterpenes: Relationship Between Structure and Inhibition of Germination", Phytochemistry, 7, 1995-1997 (1968).

RECEIVED May 1, 1986

## Chapter 8

# Plant Growth Regulation by Mevinolin and Other Sterol Biosynthesis Inhibitors

Thomas J. Bach and Hartmut K. Lichtenthaler

Botanical Institute (Plant Physiology), University of Karlsruhe, Kaiserstrasse 12,  
D-7500 Karlsruhe, Federal Republic of Germany

There is conclusive evidence that sterols have essential roles in the regulation of growth and development of plants. Mevinolin, a metabolite produced by Aspergillus terreus, specifically inhibits HMG-CoA reductase, a rate limiting enzyme of the isopentenoid pathway. This compound has been used as a molecular probe to study the interrelationships of MVA and sterol biosynthesis in the developmental events of plants and animals. The accumulation of other isopentenoids, such as mitochondrial ubiquinone(s) or plastidic prenylquinones, chlorophylls and carotenoids, is less or not at all affected by mevinolin, thereby emphasizing the concept of mevalonic acid biosynthesis being present in these organelles. The effect on growth of several microbiologically produced or chemically synthesized inhibitors (including mevinolin) known or expected to interfere with enzymes that catalyze steps later in the sterol pathway were evaluated. Generally, the growth inhibitory reactions of treated radish seedlings induced by the various test compounds were quite similar, though some specific reactions induced by mevinolin, such as inhibition of tillering at higher concentrations, suggest a requirement for isopentenoid-derived factors other than sterols to ensure cell division in meristematic plant tissue or in cell cultures. The results indicate that mevinolin may have physiological importance as a plant growth regulator.

0097-6156/87/0325-0109\$08.50/0  
© 1987 American Chemical Society

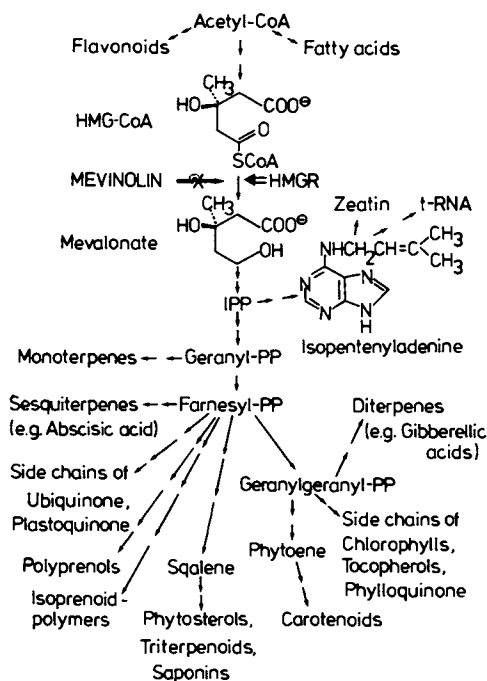


A basic question in which our laboratory has been interested over the last few years revolves around the problem of MVA synthesis and the flow of this compound to the major isopentenoid compounds in the plant cell. The pathway for the biosynthesis of polyisoprenoids and the position of the key-regulating enzyme HMG-CoA reductase is summarized in Figure 1. Our studies and those of other groups have revealed that the regulatory role of HMG-CoA reductase does not seem to be confined only to mammals (1-8), but can also be extended to plants (9-22) and fungi (23-27).

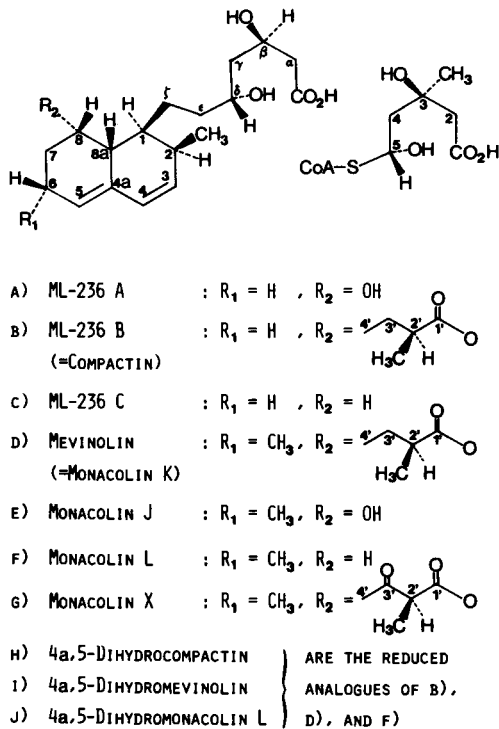
MVA is the ultimate precursor of sterols (28) and triterpenoids (29), compounds that act as architectural and functional components of endoplasmic reticulum and plasmalemma, presumably through specific interaction with fatty acids and proteins (28, 29). MVA is also the precursor of prenylquinone components of photosynthetic or oxidative electron transport chains in the chloroplast and mitochondria (30-33), of isoprenoid light harvesting accessory pigments such as carotenoids (34), and the mixed prenylpigments chlorophyll a and b (35).

That the enzyme HMG-CoA reductase plays an important ecological role in growth of plant and microbial cells is emphasized through the existence of highly specific antibiotics that bind with and regulate this enzyme. A series of microbially produced hypocholesterolemic drugs have been isolated from different strains of Ascomycetes (Figure 2). Compactin and the related ML-236A were first obtained from species of Penicillium as an antifungal metabolite (36, 37). Mevinolin has been obtained from Aspergillus terreus (38) and Monascus ruber (39). Recently, the isolation of dihydromevinolin from A. terreus (40), dihydrocompactin from P. citrinum (41), monacolins J (42) and X (43) and dihydromonacolin L from M. ruber (43) were reported. These drugs were revealed to be potent competitive inhibitors of HMG-CoA reductase and hence of mammalian cholesterol synthesis, with mevinolin being the most effective inhibitor. Compactin (ML-236B) and mevinolin have been found to be extremely useful tools in studying the regulation of vertebrate isoprenoid synthesis and it has been shown that these fungal metabolites specifically inhibit a wide variety of prokaryotic and eukaryotic HMG-CoA reductases (37-54) (Table I). It is interesting to note that the biosynthesis of mevinolin and presumably that of related polyketide derived drugs (55-58) seem to begin at a state within the fungal life cycle where the accumulation of sterols and possibly that of other essential MVA-derived products have reached their stationary phase (57).

The possible interference of the mevinolin-type compounds with microorganisms in the soil raises the question as to whether these microorganisms are able to make derivatives. In systematic studies it was recently shown that compactin could be  $\beta$ -hydroxylated (59) by Mucor hiemalis at position 6 (following the numbering system for mevinolin as introduced by Alberts et al. (38) and used in Figure 2). The nearly exclusive 6  $\alpha$ -hydroxylation of compactin by Synecephalastrum nigricans and S. racemosum (60), however, indicated a high degree of selectivity and stereospecificity of the microbial enzymes (60). In a cell-free



**Figure 1.** Role of HMG-CoA reductase-directed synthesis and its flow into various classes of isoprenoid compounds in plant cells. The inhibition of HMG-CoA reductase by mevinolin is indicated.



**Figure 2.** Structures of the free-acid forms of mevinolin and of related compounds. The correct absolute configuration is shown using the nomenclature given by Alberts et al. (38). Note that one region of the molecules closely resembles the mevaldyl moiety of (3S,5R)-mevaloyl-CoA thiohemiacetal, the enzyme-bound intermediate in the two-step reduction of (S)-HMG-CoA to (R)-MVA.

Table I. Comparison of inhibition constants ( $K_i$  values) of compactin/mevinolin-type metabolites against HMG-CoA reductase preparations from various enzyme sources

Compound	Enzyme source	$K_i$ (nM)	Reference
ML-236A	rat liver microsomes partially purified	220	Endo et al. 1976 (44)
ML-236B ( = Compactin)	rat liver microsomes, partially purified	10	Endo et al. 1976 (44)
ML-236B	rat liver microsomes, partially purified	2.66	Tanzawa & Endo 1979 (45)
ML-236B	human fibroblasts, detergent-solubilized	1.1	Brown et al. 1978 (46)
ML-236B	homogenates of the Cropora tobacco hornworm ( <u>Manduca sexta</u> )	0.9	Monger et al. 1982 (47)
ML-236B	embryonic <i>Drosophila</i> cells (microsomes)	1.0	Brown et al. 1983 (48)
ML-236B	<i>Crithidia fasciculata</i> , semi-purified	4.5	Kim & Holmlund 1985 (50)
ML-236B	yeast, partially purified	0.24	Nakamura & Abeles 1985 (49)

Continued on next page

Table I. Continued

Compound	Enzyme source	K <sub>i</sub> (nM)	Reference
Mevinolin	rat liver microsomes, partially purified	0.64	Alberts et al. 1980 (38)
Mevinolin (= Monacolin K)	rat liver microsomes, partially purified	0.50	Endo 1980 (39)
Mevinolin	yeast, partially purified	3.5	Bach & Lichtenthaler 1982, 1983 (52, 53)
Mevinolin	radish seedlings, microsome-bound	2.2	Bach & Lichtenthaler 1983 (53)
Mevinolin	<u>Halobacterium halobium</u>	20	Watson et al. 1983 (54)
4 $\alpha$ ,5-Dihydrocompactin	rat liver microsomes, partially purified	3.7	Lam et al. 1981 (41)
4 $\alpha$ ,5-Dihydro-mevinolin	rat liver microsomes, partially purified	? I <sub>50</sub> = 2.7	Albers-Schönberg et al. 1981 (40)

For other compounds (cf. Fig. 2) no K<sub>i</sub> values are available as yet. The I<sub>50</sub> values reported using rat liver HMG-CoA reductase (42, 43) appear to be about three magnitudes higher than that of 4 $\alpha$ ,5-dihydro-mevinolin (40).

system from rat liver the inhibitory activity of 6 $\alpha$ -hydroxycompactin carboxylate against cholesterol synthesis was more potent than that of the 6  $\beta$ -hydroxy-derivative (60). Strain SANK 32772 of Absidia coerulea catalized the conversion of ML-236B to 3 $\alpha$ -hydroxy-iso-ML-236B (6 $\alpha$ - in the numbering system used by the Sankyo group (61)). This latter compound, with  $\Delta^{4,4\alpha}$  and  $\Delta^{5,6}$ , was similar to the parent compound in its level of inhibition of cholesterol synthesis (61). Other microbial strains capable of 6  $\beta$ -hydroxylation of ML-236 were ascomycetes isolated from soil samples collected in Australia and were identified as substrains of Nocardia autotrophica (62).

Several fungal strains (Circinella muscae, Absidia cylindrospora and A. glauca) have been reported to biologically phosphorylate the hydroxyl group at position  $\delta$  of the open carboxylic forms of compactin as well as of monacolin K (= mevinolin), and monacolins L and X (63). The Basidiomycete Schizophyllum commune was found to transform compactin as well as mevinolin (monacolin K) to the corresponding 8- $\alpha$ -hydroxy derivatives (64). The existence of antibiotics, other than the mevinolin-type (65), produced by Cephalosporum caerulens (known inhibitors of the synthesis of polyketides and of fatty acids (66)) indicates a complex biochemical and physiological interplay may exist among organisms throughout the rhizosphere. This interplay may include higher plants in the rhizosphere. We have shown that mevinolin, applied as its water-soluble sodium salt, inhibits the root growth of intact radish and wheat seedlings by inhibiting their isopentenoid biosynthesis (67). We have utilized mevinolin as a molecular probe to study the importance of MVA and associate products to growth and development of seedlings, bearing in mind that in vivo inhibition of an enzyme, expected to be at least close to rate limiting for the pathway, should result in clear morphological and biochemical responses. A logical consequence was to establish what type of isoprenoid compound might be affected in its synthesis or accumulation in the presence of mevinolin (52, 68, 69), thereby yielding information also on the intracellular localization of HMG-CoA reductase activity in plant cells which is currently a matter of controversy in literature (70-72). Since it appeared that the accumulation of phytosterols was primarily affected by mevinolin (52, 68, 69) (which led us to hypothesize that mevinolin can easily penetrate the plant cell wall and the plasmalemma, but rather poorly the envelopes of mitochondria and plastids), we tested other inhibitors known or expected to interfere with later steps of sterol biosynthesis, such as squalene epoxidation, squalene-oxide cyclization, 14 $\alpha$ -desmethylation or side chain alkylation, in order to look for similarities and differences in the morphological response of radish seedlings upon treatment (73). A side aspect of these latter studies is to establish a versatile screening system for hypocholesterolemic drugs or fungicides.

### Materials and Methods

Chemicals. Mevinolin was kindly provided by Dr. A. W. Alberts (Merck Sharp & Dohme Res. Labs.) and was converted to its

water-soluble sodium salt as described by Kita et al. (74). Triparanol (MER-29), U 18666 A, A 25822 B were gifts from Merrell Dow Pharmaceuticals Inc., Dr. Harry Rudney (University of Ohio, Cincinnati, OH), and Eli Lilly G.m.b.H., respectively. 2,3-Epiminosqualene was kindly synthesized by Dr. Lunkenheimer (Bayer AG), Naftifine was purchased from Dr. Högenauer (Sandoz Forschungszentrum Wien). Triarimol and imminium salt ("NES") were gifts of Dr. W. D. Nes (USDA Berkeley). SC 32561 was a gift from G. D. Searle & Co. Clotrimazole, miconazole, and sodium deoxycholate were purchased from Sigma.

Cultivation of seedlings and associated test systems. Radish seeds (Raphanus sativus, cv. Saxa Knacker) were immersed in an aerated water bath for 30 min. These seeds were then placed onto a sprouting tray containing 1 l of H<sub>2</sub>O or H<sub>2</sub>O supplied with the chemicals and were allowed to germinate and develop for one week in the light (Osram Fluora lamps 65 W at 2.5 W·m<sup>-2</sup>, 25°C, 65% relative humidity). Hydrophobic drugs were usually dissolved in a system containing DMSO:Triton X-100 in the ratio 3:1 (v:v), maximum 1 ml/l in the growth solution. Controls contained the same amount of this mixture without inhibitors. In some cases, the addition of a small amount of EtOH was required to dissolve the compounds. Plant growth was measured daily (25-30 plants per condition).

Wheat seedlings were cultivated from Triticum aestivum cv. Anza. Etiolated primary leaf segments from one week-old plants were obtained and used in the test system as previously described (75, 76).

Cell Suspension Cultures of Silybum marianum. Suspension cultures were grown from primary callus cultures. Submersed cells were kept in Erlenmeyer-flasks (120 rpm; 25° C; Phillips TL 40W/47, 2000 lux) in a growth medium as described by Murashige and Skoog (77). Four to six flasks per condition were inoculated with 5 ml aliquots of a cell suspension from 8 to 10 days old cell cultures and then supplied with mevinolin to a final concentration of 0.625, 1.25, 1.5, 5 and 10 µM. At day 3 after inoculation the cells were in the early log phase of development and the plateau phase was reached between day 5 and 6. Under the culture conditions employed the cells started to degenerate between day 6 and 9. Thus, cells were analyzed at day 3 and 6.

Chemical analysis. For the extraction of lipids and prenylpigments the plant material was macerated in the presence of 100% acetone and the lipids partitioned into petrol ether (b.p. 50-70° C). Chlorophylls and total carotenoid content were determined spectrophotometrically (78). Prenylquinones were separated by TLC, HPLC and reversed phase HPLC as described in detail (69). Free 4-desmethylsterols were quantified after TLC on silica gel (solvent 84 ml petrol ether b.p. 50-70° C and 15 ml Me<sub>2</sub>CO, R<sub>f</sub> = 0.25). Plates were sprayed with saturated antimony trichloride in H<sub>2</sub>O-free CHCl<sub>3</sub>, and after a short heating period at 100° C, the pink spots indicating desmethylsterols (with pure stigmasterol as a standard) were

scanned with densimeter (550 nm) with separate standard curves being made for each plate.

### Results and Discussion

Growth inhibition by mevinolin. To ascertain the specificity of mevinolin as an enzyme inhibitor and to elucidate the role of HMG-CoA reductase activity in vivo, its effect on plant growth and development in conjunction with other isopentenoids has been examined. Mevinolin induced a strong growth inhibition of the main root of the dicotyledonous etiolated radish seedlings and of the roots of the monocotyledonous wheat seedlings (67). A significant drop in elongation growth was obtained between 10 and 100 ppb ( $2.5 \times 10^{-8}$  and  $2.5 \times 10^{-7}$  M).

We suspected that mevinolin inhibited root elongation growth via the interference with HMG-CoA reductase activity in vivo; therefore, the effect should have been overcome by the uptake of exogenously supplied MVA - the direct product of the inhibited enzyme reaction. Indeed, increasing concentrations of exogenous MVA in the presence of mevinolin gave nearly the same growth rate of roots of etiolated radish seedlings as found for the control plants (68). Exogenous MVA at an intermediate concentration of 2 mM did not stimulate root elongation growth but rather led to weakly decreased values, probably due to a secondary pH effect.

A main branch point within the isoprenoid pathway is located at the site of farnesyl pyrophosphate (Figure 1). This key position of C<sub>15</sub>-prenyltransferase makes it a likely candidate for fine tuning or rate limitation of MVA flux into the various end-products, some of which might contribute to normal root (and hypocotyl) growth. Farnesol was revealed to enhance root growth in barley (79, 80). This accelerated root elongation, however, was correlated with increased cytokinin activity as a secondary effect (79). This appeared to be to some extent contradictory to the results of Buschmann and Lichtenthaler (81) who demonstrated an inhibitory effect of exogenously supplied kinetin or benzylaminopurine on root elongation of dark- and light-grown radish seedlings. Other related isoprenoid alcohols, however, failed to enhance the growth of barley roots (80). Furthermore, other plant species tested for this farnesol-effect did not exhibit any positive reaction (80).

It is possible that mevinolin might interfere with the synthesis of other isopentenoid phytohormones such as the gibberellins or the brassinosteroids which are known to induce strong growth promotion ((82-87) and elsewhere in this volume). We have determined that the growth inhibition of the roots of etiolated radish seedlings induced by mevinolin could not be reversed by the addition of gibberellic acid (GA<sub>3</sub>). Exogenously supplied concentrations of GA<sub>3</sub> up to 37 μM could not overcome the effect of mevinolin at 2.5 μM or at 0.25 μM (52, 68). Gibberellic acid itself seemed to slightly stimulate root elongation growth of etiolated radish seedlings, especially in the later stages of development [68].

To gain further information about the mode of action of mevinolin, we extended our experiments to light-grown radish



seedlings. In this system, mevinolin proved to be a highly potent inhibitor of root elongation growth (Figures 3, 4). In etiolated seedlings the elongation growth of hypocotyls needs comparably high concentrations of mevinolin to be affected, but in light-cultivated seedlings it induces a clear dwarfing growth response.

Radish seeds were incubated in Petri dishes on filter paper with 10 ml solutions of 0, 2.5  $\mu\text{M}$ , 0.25  $\mu\text{M}$  and .025  $\mu\text{M}$  mevinolin (68). Even in the presence of the inhibitor at its highest concentration, there was no clear effect on the germination rate (68). The fact that even very high inhibitor concentrations could not prevent a minimal root growth in radish seedlings (Figure 3) led us to conclude that this minimal root growth is a function of MVA-derivatives already present in the seeds and therefore independent from de novo mevalonate biosynthesis.

It is evident that increasing concentrations of mevinolin inhibit the elongation growth of main roots in etiolated or light-grown radish seedlings and also block the formation and development of lateral roots (Figure 4). This might reflect, as already discussed, an interaction of mevinolin with the synthesis of certain isopentenoid phytohormones. In addition to the examples cited above, Geuns (88) demonstrated that, besides the stimulation of hypocotyl growth of etiolated mung bean seedlings, the lateral root number could be increased to 50 and/or 250%, respectively, by rather high concentrations (25 to 50  $\text{mg.l}^{-1}$ ) of corticosterone and cortisol. Cortisol was found to stimulate root elongation by about 100% and hypocotyl elongation by when added to the growth medium at a concentration of 0.41  $\mu\text{M}$  (89). Due to the low uptake of cortisol by the roots, the concentration in the plant itself was thought to be in the range of 0.1 to 1 nM. The growth stimulation was found to be a result of cell elongation rather than the production of more cells (89).

Effect of mevinolin on isoprenoid accumulation in radish seedlings. Under the conditions in which mevinolin is usually applied to the seedlings (see Materials and Methods) it would first be taken up by the roots exposed to the watery solution of the inhibitor and then distributed to the remaining seedling parts. The effect of mevinolin on fresh and dry weights, sterol content, and ubiquinone content in different parts of the seedlings upon treatment has been determined (Table II). At a maximum concentration of 5  $\mu\text{M}$ , mevinolin reduces the content of free 4-desmethylsterols in roots to about 20 per cent of the control. At 0.625  $\mu\text{M}$ , sterol content is reduced by about 20 per cent, thus indicating a very fast response of sterol accumulation to inhibition of MVA biosynthesis. These effects are less dramatic in hypocotyls, and especially in cotyledons. This result implies that there might exist a gradient in the concentration of mevinolin present in upper parts of the seedlings. The possibility of a low transport rate of mevinolin within the tissue was indicated when excised dark-grown radish seedlings were used to study the effect of inhibitor on the light-induced accumulation of isoprenoid compounds (68); the effect on sterol synthesis was

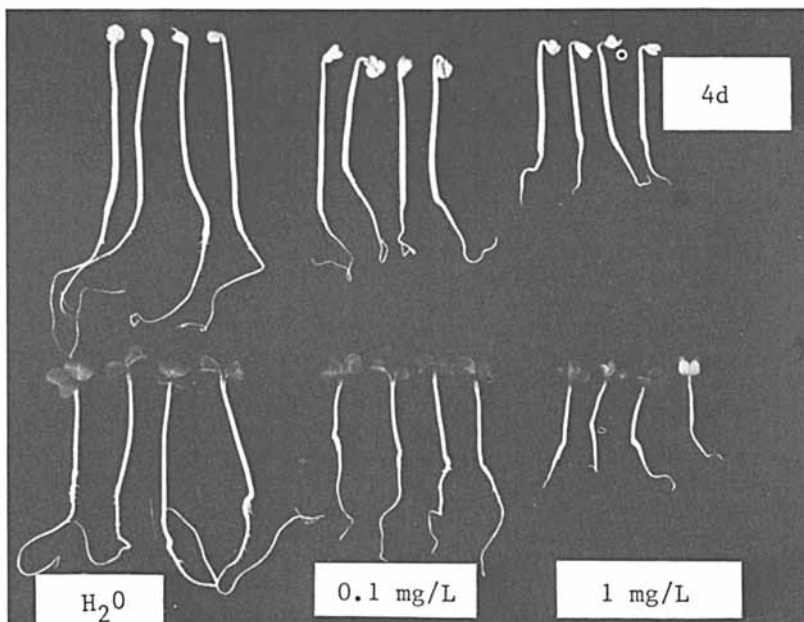


Figure 3. Radish seedlings (4 days old) grown in the dark (upper part) or in the light (lower part) in the presence of mevinolin from onset of germination. Note the lack of lateral root growth at the high inhibitor concentration.

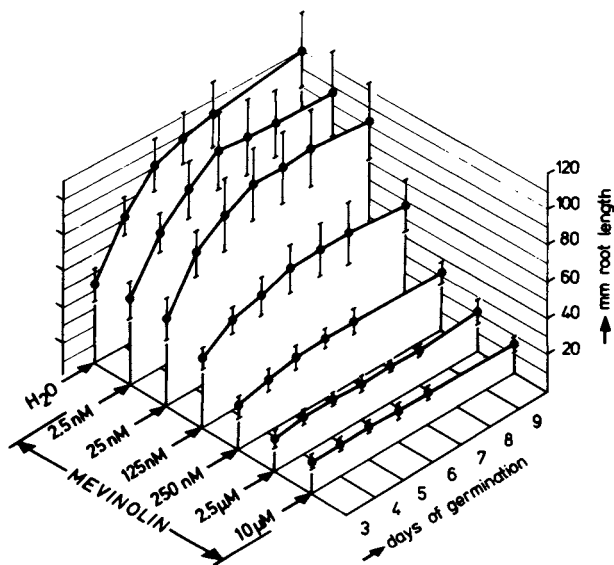


Figure 4. Mevinolin-induced growth inhibition of the main root of light grown radish seedlings. Mean values  $\pm$  SD from 30 to 50 plants per condition. (Reprinted with permission from Ref. 68. Copyright 1983 *Physiologia Plantarum*.)

Table II. Fresh/dry weight, sterol content and accumulation of the light for 6 days in the presence of mevinolin<sup>a</sup>

Plant Part, Treatment	g FW (100 parts)	g DW (100 parts)	Free Sterols ( $\mu\text{g}/100$ parts)
<b>Cotyledons<sup>b</sup></b>			
0 $\mu\text{M}$ Mevinolin	4.7	0.62	1640
0.25 $\mu\text{M}$ Mevinolin	4.9	0.66	1647
0.625 $\mu\text{M}$ Mevinolin	4.6	0.63	1569
2.5 $\mu\text{M}$ Mevinolin	3.9	0.63	1453
5.0 $\mu\text{M}$ Mevinolin	3.4	0.63	1091
<b>Hypocotyls<sup>c</sup></b>			
0 $\mu\text{M}$ Mevinolin	3.9	0.23	593
0.25 $\mu\text{M}$ Mevinolin	4.2	0.25	571
0.625 $\mu\text{M}$ Mevinolin	4.1	0.26	521
2.5 $\mu\text{M}$ Mevinolin	3.3	0.25	426
5.0 $\mu\text{M}$ Mevinolin	2.2	0.18	276
<b>Roots<sup>c</sup></b>			
0 $\mu\text{M}$ Mevinolin	2.8	0.15	522
0.25 $\mu\text{M}$ Mevinolin	2.5	0.14	416
0.625 $\mu\text{M}$ Mevinolin	2.2	0.12	274
2.5 $\mu\text{M}$ Mevinolin	1.8	0.11	200
5.0 $\mu\text{M}$ Mevinolin	1.3	0.09	112

<sup>a</sup> Mean values of three independent experiments

<sup>b</sup> 100 Cotyledon pairs analyzed per single experiment

<sup>c</sup> 100 Hypocotyls per analysis

total ubiquinone (Q-9 + Q-10) in radish seedlings grown in

% of controls	Q-9 + Q-10 ( $\mu\text{g}/100$ parts)	% of controls	% Q-9 of total Q-9 + Q-10
100	61.1	100	8.6
100	59.2	97	8.6
96	52.7	86	10.1
89	46.8	77	11.2
67	23.7	39	12.5
100	12.2	100	7.2
96	11.6	95	8.6
88	10.3	84	10.5
72	8.5	69	12.8
47	4.8	39	14.3
100	17.0	100	6.3
79	15.9	94	9.5
52	11.8	69	11.4
39	8.0	47	13.0
22	7.0	41	16.6

paralleled by an inhibition of elongation of hypocotyl segment growth. Total ubiquinone content was about 40% of the control at the maximal inhibitor concentration regardless of what part of the seedling was analyzed (Table II). However, in radish seedlings mevinolin treatment did cause a shift towards the synthesis of Q-9 at the expense of Q-10, the predominant homologue in radish (Table II). Interestingly, only the fresh weight, but not the dry weight, of radish cotyledons was affected, whereas in hypocotyls, and more clearly in roots, fresh weight and dry weight were diminished upon mevinolin treatment (Table II).

Even though a clear inhibition of sterol accumulation in cotyledons by mevinolin can be achieved at higher concentrations as compared to the roots, there was no inhibition of chlorophyll and carotenoid biosynthesis under these conditions ((68) and Table III). At low mevinolin concentrations chlorophyll a+b and carotenoid content was even enhanced as compared to untreated controls (Table III). The synthesis of other isopentenoid compounds of plastidic origin such as phyloquinone and plastoquinone appeared to be slightly enhanced at low inhibitor concentrations and hardly inhibited even at 5  $\mu$ M (Table III).  $\alpha$ -Tocopherol (Table III), which was considered earlier to be synthesized in the chloroplast as well as in the cytoplasm (90, 91), does not react like sterols (Table II), which are clearly cytoplasmic products, but rather like phyloquinone, known to be exclusively synthesized in the plastid (92, 93). This result agrees with recent findings (94), demonstrating the capacity for  $\alpha$ -tocopherol synthesis in envelope membranes from spinach chloroplasts.

It appears that sterols, as they are mainly affected, might be involved in physiological processes governing water uptake and cell elongation growth (cf. (95)). Evidently, the diminished synthesis of sterols, needed for biomembrane formation (28, 29), makes a strong contribution to the growth-retardant effect of mevinolin. The drastic inhibition of sterol biosynthesis by mevinolin indicates MVA biosynthesis to be rate-limiting, as has already been demonstrated in the case of animal cells (cf. 1, 3-7). This may also be true for ubiquinone, but apparently not for other compounds investigated here. Even though conclusive evidence is available to indicate an independent MVA-synthesizing machinery being present in plant mitochondria (10, 14), possibly regulated differentially from that assayed in the ER (96, 97), HMG-CoA reductase activity is clearly inhibited, which led us to conclude that mevinolin can penetrate the mitochondrial envelope. Of course, we do not exclude the possibility that mitochondrial MVA utilization - and thereby ubiquinone biosynthesis - might additionally be linked to cytoplasmic MVA-synthesis, depending on the need of the organelle for additional IPP units (cf. 72). The mevinolin-induced shift in the Q-pattern toward homologues containing shorter isopentenoid side chains (Table II) might reflect the ability of mitochondria to adjust the usage of isopentenyl units to the available substrate inside or outside of the organelle, thereby maintaining a basic rate of synthesis needed for a functional respiratory electron transport. The slight increase of phyloquinone and, to a somewhat lower extent,

Table III. Effect of mevinolin on plastidic prenyllipids and prenylquinones<sup>a</sup>

Plant Part, Treatment	Chlorophylls a+b ( $\mu\text{g}/100$ ) (%)	Carotenoids X+c ( $\mu\text{g}/100$ ) (%)	Plastoquinone ( $\mu\text{g}/100$ ) (%)	Phylloquinone ( $\mu\text{g}/100$ ) (%)	$\alpha$ -Tocopherol ( $\mu\text{g}/100$ ) (%)
<b>Cotyledons<sup>b</sup></b>					
0 $\mu\text{M}$ Mevinolin	3970	100	154	100	252
0.25 $\mu\text{M}$ Mevinolin	—	—	157	102	259
0.625 $\mu\text{M}$ Mevinolin	4930	111	192	124	268
1.25 $\mu\text{M}$ Mevinolin	4670	113	—	—	—
2.5 $\mu\text{M}$ Mevinolin	4770	114	158	103	240
5.0 $\mu\text{M}$ Mevinolin	4470	104	137	89	212
<b>Hypocotyls<sup>c</sup></b>					
0 $\mu\text{M}$ Mevinolin	—	—	14.0	100	100
0.25 $\mu\text{M}$ Mevinolin	—	—	16.8	120	118
0.625 $\mu\text{M}$ Mevinolin	—	—	19.0	135	141
2.5 $\mu\text{M}$ Mevinolin	—	—	15.5	113	159
5.0 $\mu\text{M}$ Mevinolin	—	—	13.5	98	96

<sup>a</sup> Mean values of three independent experiments.<sup>b</sup> 100 Cotyledon pairs analyzed per single experiment<sup>c</sup> 100 Hypocotyls per analysis

of plastoquinone, upon mevinolin treatment at concentrations below 2.5  $\mu\text{M}$  might be explained by an increased availability of acetate to be routed towards plastidic isopentenoid biosynthesis. This would require that the acetate from the cytoplasm penetrates the chloroplast envelope.

Effect of mevinolin on isoprenoid synthesis in primary leaves of wheat. Mevinolin can to some extent inhibit the biosynthesis and accumulation of chlorophylls and carotenoids when it sufficiently penetrates the leaf, as shown in experiments with leaf segments of etiolated wheat seedlings floated on a mevinolin solution of 2 mm thickness in Petri dishes and then exposed to light. Despite the extreme in vitro efficacy of mevinolin in inhibiting microsomal plant HMG-CoA reductase (see Table I), high concentrations were needed to affect the light-induced in vivo formation of carotenoids and chlorophylls (Table II). The chlorophyll b accumulation was inhibited to a higher degree than that of chlorophyll a. This can be explained by assuming a preceding formation of chlorophyll-protein complex containing chlorophyll a present in reaction centers of photosystem I and II followed by the formation of (under these experimental conditions where no supply of storage products from the seeds is possible) rudimentary light harvesting complexes containing chlorophyll b (12, 98). The light-induced change in percent composition of carotenoids (especially the increase of  $\beta$ -carotene levels) appeared to be partially blocked only at the higher mevinolin level. Because  $\beta$ -carotene is mainly located in the photosynthetic reaction centers (99) and known to protect chlorophyll a molecules from photooxidation; this lower  $\beta$ -carotene level may also account for the apparently diminished accumulation of chlorophylls.

In contrast to pigments, the sterol accumulation (defined as increase over dark control) is completely blocked by the lower mevinolin concentration (Table IV). With primary leaves of wheat, incubated under comparable conditions but supplied with [ $^{14}\text{C}$ ]-acetate and [ $^3\text{H}$ ]-mevalonate, precursors able to enter the isoprenoid pathway before or after the HMG-CoA reductase step, respectively, it was shown (75, 76) that mevinolin could completely prevent acetate incorporation into phytosterols. Incorporation of tritium from labeled MVA was unaffected. This elimination of [ $^{14}\text{C}$ ]-acetate incorporation into phytosterols was observed at a mevinolin concentration which had no effect on chlorophyll and carotenoid accumulation in controls; virtually identical accumulation  $^{14}\text{C}$  in bands of TLC-plates identified as pheophytins or  $\beta$ -carotene was found for controls and mevinolin treatment (Bach & Nes, unpublished). The limited ability of mevinolin to prevent pigment accumulation in chloroplasts favors the assumption that plastids contain their own independent enzyme system for MVA production. The plastidic envelope is apparently not at all or only poorly permeable to mevinolin. The ability of plastids to synthesize MVA has been questioned (70, 71). Our observations, together with in vitro measurements of enzyme activity (16, 21), support the view that plastids possess their own HMG-CoA reductase.

Table IV. Differential inhibition of sterol accumulation and of the light-induced prenyllipid accumulation at high mevinolin concentration in excised 9-day-old etiolated primary wheat leaves during 18 h of continuous white light. Prenyllipids in  $\mu\text{g}$  per 40 leaf segments. Mean of 2 runs with SD < 10%. X = Xanthopylls; c =  $\beta$ -carotene; x+c = sum of carotenoids. Chlorophylls and carotenoids were separated by TLC (1) and determined photometrically.

Parameter	Initial Value	Final value control	Final value + Mevinolin ( $\mu\text{m}$ )	
			125	500
<b>a) Prenyllipid content</b>				
Chlorophyll a	0	273	134	72
Chlorophyll b	0	24	6.6	1.5
Ratio a/b	-	11	20	48
Carotenoids	134	165	114	90
Ratio x/c	29	7.1	11.3	19.4
Ratio a+b/x+c	-	1.8	1.2	0.8
Free desmethylsterols	230	302	232	229
<b>b) % composition of carotenoids</b>				
$\beta$ -carotene	3.3	12.4	8.1	4.9
Violaxanthin	18.6	17.7	17.8	17.0
Antheraxanthin + Lutein epoxide	17.8	11.2	13.1	15.2
Lutein	58.2	54.8	57.3	59.5
Neoxanthin	3.7	3.9	3.7	3.4



Effect of mevinolin on the growth and chemical composition of cell suspension cultures of *Silybum marianum*. In order to test the inhibitor in a system where extracellular transport from the site of application to the expected site of action is not an important factor, we used cell suspension cultures of *Silybum marianum* (100). Cell suspension cultures were initiated in flasks containing growth medium supplemented with mevinolin at various concentrations. After 3 and 6 days the cells were analyzed (Table V). The data demonstrate that the effect of mevinolin becomes more evident at a later stage of growth. Also, in this system the inhibition by mevinolin of isoprenoid synthesis follows a gradient: Sterols  $\geq$  ubiquinones  $\geq$  plastoquinone  $\approx$  carotenoids  $\approx$  chlorophylls, thereby emphasizing the results obtained with radish and wheat seedlings. Besides an overall inhibition of ubiquinone accumulation to about 50% (on the basis of dry weight), mevinolin induced a shift in the homologue pattern towards a shorter side-chain, e.g., the appearance of Q-8 at the expense of Q-10 (with Q-9 being the by far predominant Q-homologue formed in *Silybum marianum* cells). Ryder and Goad (101) have demonstrated using suspension cultures of sycamore (*Acer pseudoplatanus* L.) that in the presence of 5 mg/l compactin the incorporation of [ $^{14}$ C]-leucine and [ $^{14}$ C]-acetate into 4-desmethylsterols was inhibited to 95% and 99%, respectively, while  $^{14}$ C-MVA incorporation was unaffected. However, sterol synthesis from endogenous precursors, measured by incorporation of [Me- $^{14}$ C]-methionine into the side chain, continued at a reduced rate for at least 6 h after addition of the inhibitor. This result suggested the presence of a pool of precursors which was presumably gradually depleted by the process of sterol biosynthesis but could not be replenished from acetate in the presence of compactin (101). Compactin was also revealed to inhibit growth of callus cultures of tobacco (102). At 5  $\mu$ M the average inhibition (fresh weight of callus) was between 45 and 80%, at 10  $\mu$ M around 95%. Cytokinins such as N<sup>6</sup>-isopentenyladenine or kinetin at concentrations from 0 to 1.0 and 0 to 5  $\mu$ M, respectively, could not substantially counteract the growth inhibition by compactin (102). Mevinolin as well as compactin were recently tested using explants of *Helianthus tuberosus* (103). The effects on growth parameters (fresh and dry weight) were well within the range determined with cell cultures of *Silybum*.

Aspects of secondary physiological responses of seedlings and cell cultures upon mevinolin treatment. Some observations that were made by the use of our radish growth system provide further support for the importance of MVA synthesis for normal development of seedlings. In earlier work (81) it was documented that kinetin, an artificial cytokinin, induced in radish seedlings a growth response comparable to mevinolin, e.g., shortened main roots. Cytokinins have been considered to be senescence regulation factors in many plant systems. By measuring fast and slow fluorescence kinetics, it was revealed that mevinolin can inhibit radish seedling senescence, e.g., as indicated by the maintenance of a functional photosynthetic apparatus (68). Thus,

Table V. Effect of mevinoлин on a cell suspension culture of *Silybum marianum*

$\mu\text{M}$ Mevinoлин	100 mg dw mg susp.	mg protein g dw	mg sterols g dw	$\mu\text{g}$ Chl.a+b g dw	$\mu\text{g}$ carot. g dw	$\mu\text{g}$ PQ-9 g dw	$\mu\text{g}$ total Q-n g dw	molar ratio Q-8 / Q-10
Initial value	11	364	2.9	146.9	99.4	199	57	
<u>day 3:</u>	(%)	(%)	(%)	(%)	(%)	(%)	(%)	
0 (control)	277	100	3.4	100	63.9	100	211	1 / 6
0.625	290	105	3.0	88	54.6	85.4	203	1 / 3
1.25	310	110	1.7	50	84.2	89.5	192	89
2.5	247	89	32.8	59.0	1.2	35	173	82
5.0	173	63	24.3	43.7	1.1	33	144	67
10.0	180	65	23.3	41.9	0.9	26	133	53
<u>day 6:</u>								
0 (control)	400	100	3.8	100	65.1	100	216	100
0.625	340	85	34.4	90.5	2.9	76	210	88
1.25	300	75	23.3	61.3	2.0	53	234	101
2.5	260	65	21.9	57.6	1.3	34	207	86
5.0	140	35	19.3	50.8	0.8	21	183	72
10.0	110	28	16.4	43.2	0.3	8	105	49

the root growth inhibiting affect by cytokinin was perplexing since, in plants, roots are regarded to be the main source of cytokinins (104, 105), cytokinins are then transported to the shoot via the xylem (106). However, for the complete inhibition of, say, N<sup>6</sup>-isopentenyladenine or zeatin synthesis, concentrations of mevinolin might be required that are magnitudes higher than that needed to completely knock out de novo sterol synthesis. Thus root growth may be inhibited by a lack of sterol synthesis in the treated roots while the cytokinin synthesis continues indicating differential inhibition by mevinolin of the accumulation of various end-products of the multibranched isoprenoid pathway.

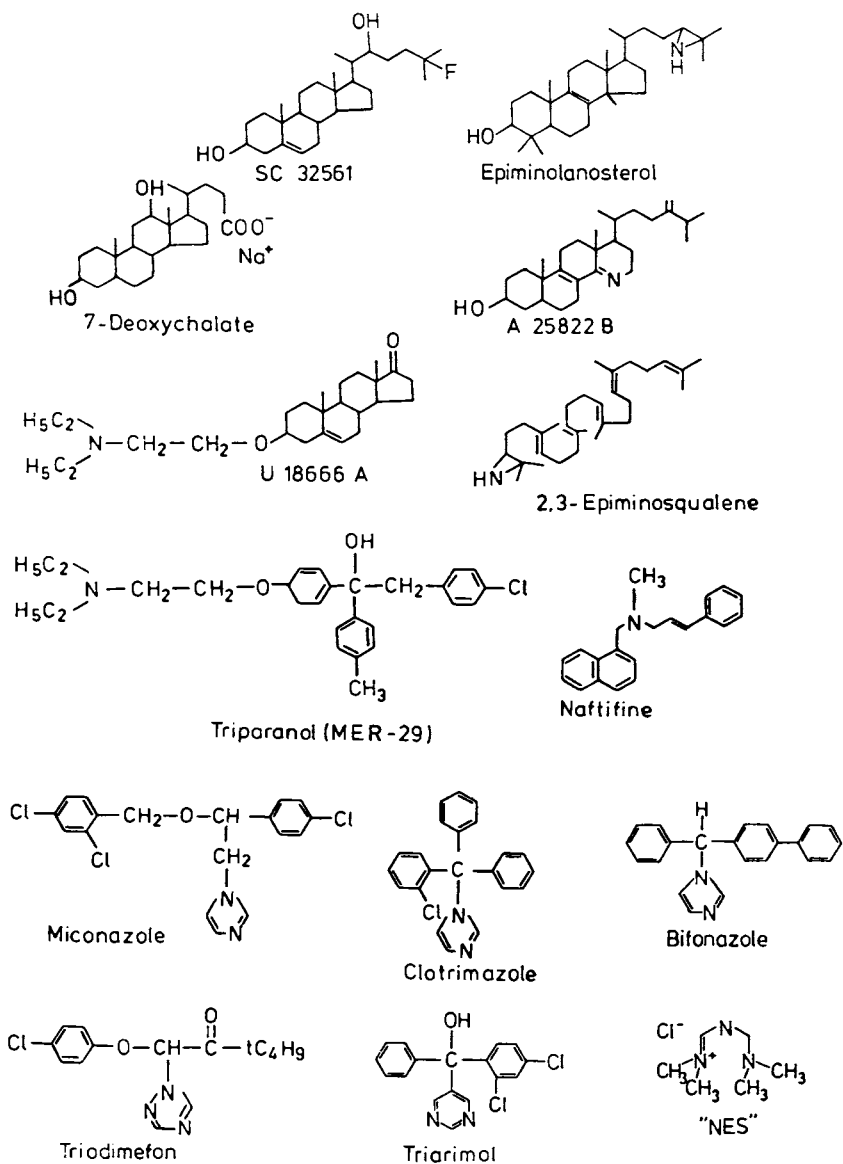
It has been reported (84) that the effect of brassinolide treatment on senescence of dark-maintained discs of Xanthium leaves was opposite to equimolar concentrations of kinetin, promoting senescence (loss of chlorophyll) as compared to the control. It may well be that the synthesis of brassinolide-type steroid hormones requires the de novo synthesis of a phytosterol as has been suggested by work that has been done in Dr. W. David Nes' lab at Berkeley (107) which supports the thesis of ascribing different functional or metabolic roles for different sterols by measuring individual turnover rates. In this regard it is interesting to note that application of steroid hormones to etiolated mung beans (88) resulted, in the formation of longer main roots and in a strongly increased number of side roots. In radish, at very high concentrations of mevinolin ( $\geq 1$  mg per liter) the formation of lateral roots (see Figure 4) was completely prevented, an effect that could be observed over the full cultivation period of 10 days. Mevinolin may induce changes in the endogenous phytohormone balance: a small amount of biologically synthesized MVA might be sufficient for the supply of the isopentenyl derived moiety of isopentenyl-adenine or zeatin (-riboside?) or the isopentenylation of t-RNA but not for that of steroid phytohormones, thereby leading to a promotion of kinetin-like growth responses.

The increased accumulation of anthocyanins in hypocotyls of mevinolin-treated radish seedlings (69) certainly does not simply reflect the routing away of acetate units from sterols (cf. Figure 1) but also reflects a more general response of plants upon treatment with chemicals (95). Inhibitors (see also below) affecting later steps in phytosterol synthesis seem to affect various other products of the isoprenoid pathway before and after squalene formation (95). Secondary effects of such growth regulators like disruption of the functional integrity of the endoplasmic reticulum or the plasmalemma, thus leading to changes in water and ion transport capacity (108) or interaction with isoprenoid carrier proteins, as suggested by Nes et al. (95), may additionally account for their mode of action. Since both sterols and GA<sub>3</sub> can independently reverse the retardant action of these biocides on stem growth (109, 110) it was suggested by Nes et al. (95) that several end-products of the isoprenoid pathway may act independently on developmental processes, but produce the same end response. This emphasizes the more general concept of a shade-type or sun-type growth response of plants upon biocide

treatment as proposed by Lichtenthaler (111). Treatment of yeast cell cultures with systemic fungicides such as clotrimazol and triadimefon resulted in an accumulation of lanosterol that, in a secondary process, proved to be a highly specific feedback inhibitor of HMG-CoA reductase activity within the yeast cells (23). Since there is some evidence that plant HMG-CoA reductase activity might also be regulated by feedback mechanisms, as demonstrated with pea seedlings (13, 15), similar effects may account for the inhibitory potency of several biocides which cause an accumulation of intermediary products of the brached isopentenoid pathway, a problem which needs further investigation.

Effect of inhibitors affecting late steps in phytosterol synthesis on growth of radish seedlings. HMG-CoA reductase and enzymes catalyzing late steps of phytosterol synthesis are membrane-bound. In cells, membrane-bound enzymes within a biosynthetic pathway, such as phytosterol synthesis, might be less abundant than soluble ones. In addition, the methods of regulating their activity include membrane effects such as induced changes in the lipid composition in the microspheres around various enzyme molecules. The results obtained with mevinolin support the view that HMG-CoA reductase plays a key role at least in the coarse control of phytosterol synthesis. However, this does not exclude the possibility that membrane-bound enzymes catalyzing later steps of phytosterol synthesis might be responsible for fine-tuning substrate flux. Therefore, site-specific inhibitors of such regulating enzymes should exhibit in situ, a clear effect on growth.

The compounds (Figure 5) tested in the radish system are arranged to indicate some structural relationships. However, this does not indicate per se what enzyme is the target of these chemicals. It appears reasonable to classify them according to the enzyme(s) inhibited. Most of these compounds, in particular the series of substituted imidazoles Clotrimazole, Miconazole and Bifonazole (typical systemic fungicides) are reported to interfere with the oxidative 14  $\alpha$ -desmethylation by binding nitrogen to the haem iron of cytochrome P-450 (112, 113, 114). In susceptible fungi the accumulation of ergosterol precursors retaining the 14  $\alpha$ -methyl group may have led to their being unsatisfactorily packed with the fatty acyl chains of the phospholipids of the fungal membranes (115). This could have led to an altered membrane fluidity (116) which then causes a decreased activity of the membrane-bound desaturase, resulting in an increase of saturated fatty acids (115). This group of 14  $\alpha$ -desmethylation inhibitors also includes Triarimol and Triadimefon. The 14  $\alpha$ -desmethylation involves several reactions, including  $\Delta^{14}$  reduction (28). Since the azasterol A 25822 B, a natural antibiotic originally isolated from Geotrichum flavobrunneum and characterized by Chamberlain et al. (117), was revealed to affect  $\Delta^{14}$ -reductase in yeast thereby causing the accumulation of ignosterol (118), this compound may be included in the same group. It has also been shown that in bramble cells, cultured in the presence of A 25822 B,  $\Delta^{8,14}$ -sterols accumulated at the expense of  $\Delta^5$ -sterols (119). Another group (U 18666 A,

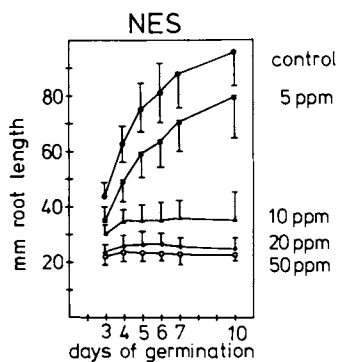


**Figure 5. Structures of sterol synthesis inhibitors used and arranged to indicate some structural relationships.**

Triparanol, epiminolanosterol is comprised of compounds that affect the reduction of (120-122) or the  $C_1$ -transfer to the  $\Delta^{24}$  of lanosterol and cycloartenol (123). A third group contains inhibitors of oxidosqualene cyclization (epiminosqualene (124, 125), U 18666 A (126, 127), "NES"). A known antimycotic agent, Natifine, was found to inhibit fungal squalene epoxidase (128). At this point, however, it has to be noted that most of the compounds mentioned above are able to inhibit more than one enzyme, including HMG-CoA reductase (113), depending on the concentrations used or organisms tested, in particular in algae and plant cell cultures (129, 130 and literature cited therein). Another drug, SC-32561, has been reported to prevent cholesterol ester accumulation and induction of HMG-CoA reductase in mammalian tissue cultures and intact animals, presumably through indirect feedback regulation (131).

Among those compounds being completely inactive in the radish root test was SC-32561, presumably indicating that oxygenated sterols or their derivatives might not be as efficient as feedback regulators as they are in mammalian systems (for a recent review see ref. (132)), even though conclusive data on this topic are lacking. Iminolanosterol was also completely inactive (data not shown), but this inability to inhibit growth might be due to its low solubility in water, thereby reflecting the main limitation of the radish root system which works best if the compounds are a) readily water-soluble or b) can be solubilized and kept in solution by the aid of reasonable concentrations of emulsifying agents. Bifonazole (data not shown) was less active than Miconazole, a very hydrophobic compound, and this was less inhibitory than Clotrimazole, which induced a similar growth reaction (73) as was found for Triadimefon; both caused a dwarfing response in radish (111). U 18666 A, Triparanol, and "NES" (Figure 6) were comparably effective growth inhibitors with 10 mg per liter being the critical concentration where no further root growth over the initial value at day 3 of germination was observed. Triarimol reached this level at about 50 mg per liter and was more active than Naftifine (75). Iminosqualene was inhibitory at > 5 mg per liter, a concentration that is at the limit of its solubility. In addition, the preparation used contained impurities (inner epimino groups) and was probably unstable at room temperature. The "best" agent - even though less effective than mevinolin - was the naturally produced azasterol (A 25822 B) which reached the forementioned level between 1 and 5 mg per liter (73). The experiments give further evidence that inhibition of phytosterol synthesis can cause similar morphological changes in radish, e. g., a decrease in root elongation growth.

However, one difference between the effects of mevinolin as compared to that of all other compounds tested has to be noted: The complete lack of lateral root formation in the presence of high concentrations of mevinolin was never observed with other chemicals. It frequently appeared that lateral root growth was even stimulated as compared to control plants. This may indicate that at least one further MVA-derivative, other than sterols, is involved not only in regulation of growth but, more basically, in



**Figure 6.** Growth inhibition of the main root of light cultivated radish seedlings by imminium salt ("NES"). Mean values  $\pm$  SD from 25 to 30 plants per condition.

cell cycle dynamics. The formation of lateral roots might require particularly meristematic initials within the root cambium where cells are rapidly dividing, and mevinolin, at concentrations higher than 1 mg per liter, presumably inhibits cell division through arresting the cells within the cell cycle as it does in mammalian cells (133-136). It should be mentioned that in crown gall tumors caused by Agrobacterium high concentrations of isopentenyladenine are synthesized (for literature see ref. (137) and one gene product of the TI plasmid obviously is coding for a dimethylallylphosphosphate transferase which is involved in the formation of isopentenyladenosine from 5'AMP (138, 139). In this tissue the rate of cell division is high and requires an overproduction of cytokinins (and auxin (137, 140). In addition to isopentenyl-derived cytokinins, dolichols are involved in the regulation of the cell cycle of eukaryotes as cofactors in the glycosylation cycle of proteins (141) and in cell wall biosynthesis (142). At high concentrations, mevinolin might affect their synthesis, thereby leading to an inhibition of regulatory events in the cell cycle which are independent from sterol synthesis.

#### Conclusions and Outlook

From our results and those of other groups, there is strong evidence that the key-regulating role of HMG-CoA reductase seems not to be confined only to the animal kingdom, but can also be extended to plants and fungi. Since mammalian cells contain fewer classes of isopentenoid compounds and even fewer different cell compartments, the regulation of mevalonate and isopentenoid biosynthesis at the step committed by the HMG-CoA reductase reaction is far more extensively studied and might not be so complex as compared to plant cells. Nevertheless, the models of regulation valid for mammalian systems might serve as a basis for understanding the features of regulation of mevalonate biosynthesis and substrate flow into various classes of isoprenoids possibly synthesized within different organelles of the plant cell (Figure 1).

The possible interference of mevinolin with the synthesis of recently described brassinolide-type steroid phytohormones (see literature cited above) might open new aspects for experimental designs to elucidate steroid phytohormone-dependent regulation processes in plants. Moreover, the interaction of mevinolin with HMG-CoA reductase activity and possibly with the balance of several growth hormones and how these phytohormones interact with the regulation of isoprenoid synthesis at the enzyme level, may account for typical growth responses of plants upon biocide treatment and needs further investigation.

The specificity of the inhibition by mevinolin through its high affinity for HMG-CoA reductase may also serve as a model to develop highly effective and specific artificial biocides and to stimulate research in this topic. For example, citrinin, another antibiotic having a bicyclic structure produced by Penicillium citrinum has been shown to inhibit sterol biosynthesis (143) at the site of acetoacetyl-CoA thiolase (EC 2.3.2.9) and HMG-CoA



reductase (144), but rather high concentrations were required. This compound was reported to inhibit plant growth (145) which we suppose is also a result of interference with HMG-CoA reductase activity. This is further evidence, besides our studies using mevinolin, to propose HMG-CoA reductase to be a good enzymic target for biologically active biocides. In any case, in our attempt to shed new light on the physiological role of mevalonate biosynthesis and metabolism in plants, mevinolin has proved to be a very suitable tool and can help to further elucidate the role of a functional isopentenoid and prenyllipid pathway in the regulation of plant growth and development.

#### Acknowledgments

Part of this work was supported by the Deutsche Forschungsgemeinschaft. We are indebted to Mrs. Ute Sieber for assistance in the preparation of the manuscript.

#### Legend of symbols

GA, Gibberellic acid;  
HMG-COA, 3-hydroxy-3-methylglutaryl-coenzyme A;  
MVA, mevalonic acid

#### Literature Cited

1. Rodwell, V. W.; Nordstrom, J. L.; Mitschelen, J. J. Adv. Lipid Res. 1976, 14, 1-74.
2. Brown, W. E., Rodwell, V. W. In "Dehydrogenases Requiring Nicotinamide Co-enzymes"; Jeffery, J., Ed.; Birkhäuser Verlag: Basel - Boston - Stuttgart, 1980; pp. 232-72.
3. Qureshi, N.; Porter, J. W. In "Biosynthesis of Isoprenoid Compounds"; Porter, J. W., Spurgeon, S. L., Eds.; J. Wiley: New York, 1981; Vol. I, pp. 47-94.
4. Dugan, R. E. In "Biosynthesis of Isoprenoid Compounds"; Porter, J. W., Spurgeon, S. L., Eds.; J. Wiley: New York, 1981; Vol. I, pp. 95-159.
5. Schroepfer, G., Jr. Ann. Rev. Biochem. 1981, 50, 585-621.
6. Chang, T.-Y. The Enzymes. Academic Press, New York, 1983, Vol. XVI, pp. 491-521.
7. Sabine, J. R. (Ed.) In "HMG-COA REDUCTASE"; Monographs on Enzyme Biology, 1983, CRC Press: Boca Raton.
8. Preiss, B. (Ed.) Regulation of HMG-CoA Reductase, 1985, Academic Press: New York.
9. Brooker, J. D.; Russell, D. W.. Arch. Biochem. Biophys. 1975, 167, 723-9.
10. Suzuki, H.; Uritani, I.; Oba, K. Physiol. Plant Pathol. 1975, 7, 265-276.
11. Grumbach, K. H.; Bach, T. J. Z. Naturforsch. 1979, 34 c, 941-3.
12. Ito, R.; Oba, K.; Uritani, I. Plant Cell Physiol. 1979, 20, 867-74.

13. Brooker, J. D.; Russell, D. W. Arch. Biochem. Biophys. 1979, 198, 323-34.
14. Bach, T. J.; Lichtenthaler, H. K.; Rétey, J. In "Biogenesis and Function of Plant Lipids Mazliak, P., Benveniste, P., Costes, C.; Douce, R., Eds.; Elsevier: Amsterdam, 1980, pp. 355-62.
15. Russell, D. W.; Davidson, H. Biochem. Biophys. Res. Commun. 1982, 104, 1537-43.
16. Wong, R. J.; McCormack, D. K.; Russell, D. W. Arch. Biochem. Biophys. 1982, 216, 631-8.
17. Sipat, A. B. Phytochemistry 1982, 21, 2613-18.
18. Nishi, A.; Tsuritani, I. Phytochemistry 1983, 22, 399-401.
19. Bach, T. J.; Rudney, H. J. Lipid Res. 1983, 24, 1401-05.
20. Bach, T. J.; Rogers, D. H.; Rudney, H. In "Structure, Function and Metabolism of Plant Lipids; Siegenthaler, W., Eichenberger, W., Eds.; Elsevier: Amsterdam, 1984; pp. 221-4.
21. Arebalo, R. E.; Mitchell, E. D., Jr. Phytochemistry 1984, 23, 13-18.
22. Bach, T. J.; Rogers, D. H.; Rudney, H. Eur. J. Biochem., in press.
23. Berg, J. D.; Draber, W. von Hugo, H.; Hummel, W.; Mayer, D. Z. Naturforsch. 1981, 36c, 798-803.
24. Quain, D. E.; Haslam, J. M. J. Gen. Microbiol. III, 1979, 343-51.
25. Quain, D. E.; Haslam, J. M. J. Gen. Microbiol. 1982, 128, 2653-60.
26. Tada, M.; Shiroishi, M. Plant Cell Physiol. 1982, 23, 615-21.
27. Boll, M. In "HMG-COA REDUCATSE"; Sabine, J., Ed.; Monographs in Enzyme Biology: CRC Press, 1983, pp. 39-53.
28. Nes, W. R.; McKean, M. L. Biochemistry of Steroids and other Isopentenoids, University Park Press: Baltimore, London, Tokyo, 1977.
29. Nes, W. D.; Heftman, E. J. Nat. Prod. 44, 377-400.
30. Lichtenthaler, H. K. In "Lipids and Lipid Polymers in Higher Plants"; Tevini, M.; Lichtenthaler, H. K., Eds.; Springer-Verlag: Berlin - Heidelberg, 1977.
31. Lichtenthaler, H. K. In "Advances in the Biochemistry and Physiology of Plant Lipids; Appelqvist, L.-A.; Liljenberg, C., Eds.; Elsevier: Amsterdam, 1979.
32. Lichtenthaler, H. K. In "Biogenesis and Function of Plant Lipids"; Mazliak, P.; Benveniste, P.; Costes, C.; Douce, R., Eds.; Elsevier: Amsterdam, 1980; pp. 299-309.
33. Rudney, H. In "Biomedical and Clinical Aspects of Coenzyme Q; Folkers, K.; Yamamura, Y., Eds.; Elsevier: Amsterdam, 1977; pp. 29-45.
34. Davies, B. H. In "Lipids and Lipid Polymers in Higher Plants; Tevini, M.; Lichtenthaler, H. K., Eds.; Springer-Verlag: Berlin - Heidelberg, 1977; pp. 199-217.
35. Schneider, H. A. W. Ber. Deutsch. Bot. Ges. 1975, 88, 83-123.
36. Brown, A. G., Smale, T. C.; King, T. J.; Hasenkamp, R.; Thompson, R. H. J. Chem. Soc. Perkin I 1976, 1165-70.
37. Endo, A.; Kuroda, M.; Tsujita, Y. J. Antibiotics 1976, 29, 1346-48.

38. Alberts, A. W.; Chen, J.; Kuron, G.; Hunt, V.; Huff, J.; Hoffman, C.; Rothrock, J.; Lopez, M.; Joshua, H.; Harris, E.; Patchett, A.; Monagan, R.; Currie, S.; Stapley, E.; Albers-Schönberg, G.; Hensens, O.; Hirschfield, J.; Hoogsteen, K.; Liesch, J.; Springer, J. Proc. Natl. Acad. Sci. USA, 1980, 77, 3957-61.
39. Endo, A. J. Antibiotics, 1979, 32, 852-54.
40. Albers-Schönberg, G.; Joshua, H.; Lopez, M.; Hensens, O.; Springer, J.; Chen, J.; Ostrove, S.; Hoffman, C.; Alberts, A.; Patchett, A. J. Antibiotics 1981, 34, 507-12.
41. Lam, Y. K. T.; Gullo, V. P.; Goegelman, R. T.; Jorn, D.; Huang, L.; De Riso, C.; Monaghan, R. L.; Putter, I. J. Antibiotics 1981, 614-16.
42. Endo, A.; Hasumi, K.; Negishi, S. J. Antibiotics 1985, 38, 420-22.
43. Endo, A.; Hasumi, K.; Nakamura, T.; Hunishima, M.; Masuda, M. J. Antibiotics 1985, 38, 321-27.
44. Endo, A.; Kuroda, M.; Tanzawa, K. FEBS Lett. 1976, 72, 323-26.
45. Tanzawa, K.; Endo, A. Eur. J. Biochem. 1979, 98, 195-201.
46. Brown, M. S.; Faust, J. R.; Goldstein, J. L.; Kaneko, I.; Endo, A. J. Biol. Chem. 1978, 253, 1121-28.
47. Monger, D. J.; Lim, W. A.; Kézdy; Law, J. H. Biochem. Biophys. Res. Commun. 1982, 105, 1374-80.
48. Brown, K.; Havel, C. M.; Watson, J. A. J. Biol. Chem. 1983, 258, 8512-18.
49. Nakamura, C. E.; Abeles, R. E. Biochemistry USA 1985 24, 1364-76.
50. Kim, K.; Holmlund, C. E. Fed. Proc. 44, 657.
51. Endo, E. J. Antibiotics 1980, 33, 334-6.
52. Bach, T. J.; Lichtenthaler, H. K. In "Biochemistry and Metabolism of Plant Lipids; Wintermans, J. F. G. M.; Kuiper, P. J. C., Eds.; Elsevier: Amsterdam, 1982, pp. 515-22.
53. Bach, T. J.; Lichtenthaler, H. K. Z. Naturforsch. 1983, 38c, 212-19.
54. Watsdon, J. A.; Havel, C. M.; Caberra, J.; Shields, P.; Bolds, J.; Poster No. 463, 74th Annual Meeting Amer. Soc. Biol. Chem.; San Francisco, California, June 5-9, 1983.
55. Chan, J. K.; Moore, R. N.; Nakashima, T. T.; Verderas, J. C. J. Amer. Chem. Soc. 1983, 105, 3334-6.
56. Moore, R. N.; Bigam, G.; Chan, J. K.; Hogg, A. M.; Nakashi, T. T.; Veojeras, J. C. J. Amer. Chem. Soc. 1985, 107, 3694-701.
57. Greenspan, M. D.; Yudkovitz, J. B. J. Bacteriol. 1985, 162, 704-7.
58. Endo, A.; Negishi, Y.; Iwashita, T.; Mizukawa, K. HIRAMA, M. J. Antibiotics 1985, 38, 444-8.
59. Serizawa, N.; Serizawa, S.; Nakagawa, K.; Furuya, K.; Okazaki, T.; Terahara, A. J. Antibiotics 1983, 36, 887-91.
60. Serizawa, N.; Nagakawa, K.; Tsujita, Y.; Terahara, A.; Kuwano, H. J. Antibiotics 1983, 36, 608-10.
61. Serizawa, N.; Nagakawa, Y.; Tsujita, Y.; Terahara, A.; Kuwano, H.; Tanaka, M. J. Antibiotics 1983, 36, 918-20.

62. Okazaki, T.; Serizawa, N.; Enotika, R.; Torikata, A.; Terahara, A. J. Antibiotics 1983, 36, 1176-83.
63. Endo, A.; Yamashita, H.; Naoki, H.; Iwashita, T.; Mizukawa, Y. J. Antibiotics 1985, 38, 328-32.
64. Yamashita, H.; Tsubokawa, S.; Endo, A. J. Antibiotics 1985, 38, 605-9.
65. Hata, T.; Sano, Y.; Matsumae, A.; Kamio, Y.; Nomura, S.; Sugawara, R. Jpn. J. Bacteriol 1960, 15, 1075-77.
66. Omura, S. Bacteriol. Rev. 1976, 40, 681-97.
67. Bach, T. J.; Lichtenthaler, H. K. Z. Naturforsch 1982, 37c, 46-50.
68. Bach, T. J.; Lichtenthaler Physiol. Plant. 1983, 59, 50-60.
69. Schindler, S.; Bach, T. J.; Lichtenthaler, H. K. Z. Naturforsch 1985, 40c, 208-14.
70. Kreuz, K.; Beyer, P. Kleinig, H. Planta 1982, 154, 66-9.
71. Kreuz, K.; Kleinig, H. Eur. J. Biochem. 1984, 141, 531-35.
72. Lütke-Brinkhaus, F.; Liedvogel, B.; Kleinig, H. Eur. J. Biochem. 1984, 537-41.
73. Bach, T. J. Plant Sci. Lett. 1985, 39, 183-7.
74. Kita, T.; Brown, M. S.; Goldstein, J. L. J. Clin. Invest. 1980, 66, 1094-100.
75. Bach, T. J.; Nes, W. D. In "Structure, Function and Metabolism of Plant Lipids"; Siegenthaler, P.-A.; Eichenberger, W., Eds.; Elsevier: Amsterdam, 1984; pp. 217-20.
76. Nes, W. D.; Bach, T. J. Proc. Roy. Soc., London 1985, B225, 425-44.
77. Murashige, T.; Skoog, F. Physiol. Plant 1962, 15, 473-98.
78. Lichtenthaler, H. K.; Wellburn, A. R. Biochem. Soc. Transact. 1983, 603, 591-2.
79. Wardle, K.; Short, K. C. Z. Pflanzenphysiol. 1981, 102, 183-8.
80. Wardle, K.; Short, K. C. Biochem. Physiol. Pflanzen 1982, 177, 210-15.
81. Buschmann, C.; Lichtenthaler, H. K. Photochem. Photobiol. 1982, 35, 217-21.
82. Gregory, L. E. Amer. J. Bot. 1981, 68, 586-8.
83. Yopp, J. H.; Mandava, N. B.; Sasse, J. M. Physiol. Plant. 1981, 53, 445-52.
84. Mandava, N. B.; Sasse, J. M.; Yopp, J. H. Physiol. Plant 1981, 53, 453-61.
85. Gregory, L. E.; Mandava, N. B. Physiol. Plant 1982, 54, 239-43.
86. Mandava, N. B.; Thompson, M. J. In "Isopentenoids in Plants, Biochemistry and Function"; Nes, W. D.; Fuller, G.; Tsai, L.-S., Eds.; Marcel Dekker Inc.: New York - Basel, 1984; pp. 401-31.
87. Meudt, W. J., this volume.
88. Geuns, J. M. C. Z. Pflanzenphysiol. 1974, 74, 42-51.
89. Geuns, J. M. C. Trends Biochem. Sci. 1982, 7, 7-9.
90. Threlfall, D. R. Vitamines and Hormones 1971, 29, 153-200.
91. Pennock, J. F.; Threlfall, D. R. In "Biosynthesis of Isoprenoid Compounds"; Porter, J. W.; Spurgeon, S. L., Eds.; J. Wiley: New York, 1983; Vol. 2, pp. 291-303.

92. Schultz, B.; Ellerbrock, B. H.; Soll, J. Eur. J. Biochem. 1981, 117, 329-32.
93. Kaiping, S.; Soll, J.; Schultz, G. Phytochemistry 1984, 23, 89-91.
94. Soll, J.; Schultz, G.; Joyard, J.; Douce, R.; Block, M. A. Arch. Biochem. Biophys. 1985, 238, 290-99.
95. Nes, W. D.; Douglas, T. J.; Lin, J.-T.; Heftman, E.; Paleg, L. G. Phytochemistry 1982, 21, 575-79.
96. Bach, T. J.; Lichtenthaler, H. K. Biochim. Biophys. Acta. 1984, 794, 152-61.
97. Lichtenthaler, H. K.; Kuhn, G.; Prenzel, U.; Buschmann, C.; Meier, D. Z. Naturforsch 1982, 37c, 464-75.
98. Anderson, J. M.; Andersson, B. Trends Biochem. Sci. 1982, 7, 288-92.
99. Lichtenthaler, H. K.; Prenzel, U.; Kuhn, G. Z. Naturforsch. 1982, 37c, 10-12.
100. Döll, M.; Schindler, S.; Lichtenthaler, H. K.; Bach, T. J. In "Structure, Function and Metabolism of Plant Lipids"; Siegenthaler, P.-A.; Fischenberg, W., Eds.; Elsevier: Amsterdam, 1984.
101. Ryder, N. S.; Goad, L. J. Biochim. Biophys. Acta. 1980, 619, 424-27.
102. Hashizume, T.; Matsubara, S.; Endo, A. Agric. Biol. Chem. 1983, 47, 1401-3.
103. Ceccarelli, M.; Lorenzi, R. Plant Sci. Lett. 1984, 34, 269-76.
104. Weiss, C.; Vaadia, Y. Life Sci. 1965, 4, 1323-26.
105. Kende, H. Proc. Natl. Acad. Sci. USA 1965, 53, 1302-7.
106. Carr, D. J.; Burrous, W. J. Life Sci. 1966, 5, 2061-77.
107. Heupel, R. C.; Sauvaire, Y.; Le, P. H.; Parish, E. J.; Nes, W. D. Lipids, 1985, 21, 69-75.
108. Fabijan, D. M.; Dhinda, P. O.; Reid, D. M. Planta 1981, 152, 481-6.
109. Douglas, T. J.; Paleg, L. G. J. Expt. Bot. 1981, 32, 59-68.
110. Buchenauer, H.; Röhner, E. Pesticide Biochem. Physiol. 1981, 15, 58-70.
111. Lichtenthaler, H. K. Z. Naturforsch. 1979, 34c, 936-40.
112. Baldwin, B. C. Biochem. Soc. Transact. 1983, 11, 659-63.
113. Berg, D.; Regel, H. E.; Harenberg, H. E.; Plempel, M. Arzneim Forsch./Drug Res. 1984, 34, 139-46.
114. Henry, M. J.; Sisler, H. D. Pesticide Biochem. Physiol. 1984, 22, 262-75.
115. Bloch, K. CRC Crit. Rev. Biochem. 1979, 9, 1-5.
116. van den Bossche, H.; Wilemsens, G.; Cools, W.; Marichal, P.; Lauwers, W. Biochem. Soc. Transact 1983, 11, 665-7.
117. Chamberlain, J. W.; Chaney, M. O.; Chen, S.; Demarco, P. V.; Jones, M. D.; Occolowitz, J. L. J. Antibiotics 1974, 27, 992-3.
118. Parks, L. W.; Rodriguez, R. J. Biochem. Soc. Transact. 1980, 19, 525-30.
119. Schmitt, P.; Scheid, F.; Benvenieste, P. Phytochemistry 1980, 19, 525-30.

120. Avigan, J. Proc. Soc. Exp. Biol. Med. 1963, 112, 233.
121. Volpe, J. J.; Obert, K. A. J. Neurochem. 1983, 38, 931-8.
122. Avigan, J.; Steinberg, D.; Vroman, H. E.; Thompson, M. J.; Mosettig, E. J. Biol. Chem. 1969, 235, 3123-9.
123. Malhotra, H. C.; Nes, W. R. J. Biol. Chem. 1971, 246, 4934-7.
124. Corey, E. J.; Ortiz de Montellano, P. R.; Lin, K.; Dean, P. D. G. J. Amer. Chem. Soc. 1967, 89, 2797-8.
125. Nes, W. D. In "Isopentenoids in Plants, Biochemistry and Dekker"; Nes, W. D.; Fuller, G.; Tsai, L.-S., Eds.; Marcel Dekker, Inc.: New York - Basel, 1984, pp. 325-47.
126. Cenedella, R. J. Biochem. Pharmacol. 1980, 29, 2751-54.
127. Sexton, R. C.; Panini, S. R.; Azran, F.; Rudney, H. Biochemistry USA, 1983, 22, 5687-92.
128. Paltauf, F.; Daum, G.; Zuder, G.; Högenauer, G.; Schulz, G. Seidel, G. Biochim. Biophys. Acta 1982, 712, 268-73.
129. Chan, J. T.; Patterson, G. W. Plant Physiol. 1973, 52, 246-7.
130. Hosokawa, G.; Patterson, G. W.; Lusby, W. R. Lipids 1984, 19, 449-56.
131. Bates, S. R.; Jett, C. M.; Miller, J. E. Biochim. Biophys. Acta 1983, 281-93.
132. Gibbons, G. G. Biochem. Soc. Transact. 1983, 11, 649-51.
133. Brown, M. S.; Goldstein, J. L. J. Lipid Res. 1980, 21, 505-17.
134. Quesney-Huneus, V.; Wiley, M. H.; Siperstein, M. D. Proc. Natl. Acad. Sci. USA 1979, 76, 5056-60.
135. Quesney-Huneus, V.; Wiley, M. H.; Siperstein, M. D. Proc. Natl. Acad. Sci. USA 1980, 77, 5842-46.
136. Faust, J. R.; Brown, M. S.; Goldstein, J. L. J. Biol. Chem. 1980, 255, 6546-48.
137. Amrhein, N. Progress in Botany 1983, 45, 136-165.
138. Barry, G. F.; Rogers, S. G.; Fraley, R. T.; Brand, L. Proc. Natl. Acad. Sci. USA 1984, 81, 4776-80
139. Thomashow, L. S.; Reeves, S.; Thomashow, M. F. Proc. Natl. Acad. Sci. USA 1984, 81, 5071-75.
140. Akiyoshi, D. E.; Klee, H.; Amasino, R. M.; Nester, E. W.; Gordon, M. P. Proc. Natl. Acad. Sci. 1984, 81, 5994-98.
141. Lehle, L.; Tanner, W. Biochem. Soc. Trans. 1983, 11, 568-74.
142. Hemming, F. W. Biochem. Soc. Transact. 1983, 11, 497-504.
143. Kuroda, M.; Hazama-Shimada, Y.; Endo, E. Biochim. Biophys. Acta 1977, 486, 254-9.
144. Tanzawa, K.; Kuroda, M.; Endo, A. Biochim. Biophys. Acta 1977, 488, 97-101.
145. Skorobogätova, R. A.; Mirchink, T. G. Sel-skokhozyaistvennaya Biologiya 1976, 9, 865, cited in: Bauer, K.; Bischoff, E.; von Hugo, H.; Berg, D.; Kraus, P. "Pflanzenschutzpräparate mikrobieller Herkunft"; CHEMIE DER PFLANZENSCUTZ- UND SCHÄDLINGSBEKÄMPFUNGSMITTEL; Wegler, R., Ed.; Springer-Verlag: Berlin, 1980; Vol. 6, pp. 215-328.

RECEIVED May 29, 1986

## Chapter 9

# Synthesis and Fungistatic Activity of Podocarpic Acid Derivatives

Edward J. Parish<sup>1</sup>, Susan Bradford<sup>1</sup>, Victoria J. Geisler<sup>1</sup>, Patrick K. Hanners<sup>2</sup>,  
Rick C. Heupel<sup>2</sup>, Phu H. Le<sup>2</sup>, and W. David Nes<sup>2</sup>

<sup>1</sup>Department of Chemistry, Auburn University, Auburn, AL 36849

<sup>2</sup>Plant and Fungal Lipid Group, Plant Development and Productivity Research Unit,  
Western Regional Research Center, Agricultural Research Service, U.S. Department of  
Agriculture, Albany, CA 94710

As a class, octahydrophenanthrene lactones, podolactones, and related podocarpic acid derivatives have been reported to possess a wide variety of biological activities, including antileukemic activity, inhibition of plant cell growth, insect toxicity and antifungal properties. In the present study, a series of synthetic derivatives of podocarpic acid have been prepared by chemical synthesis and evaluated with respect to their ability to inhibit fungal growth. These compounds were evaluated against the Oomycetes- Phytophthora cactorum, Saprolegnia ferax, and Achlya bisexualis and the Ascomycetes- Gibberella fujikuroi. The results of these studies indicate that several of these new synthetic derivatives possess significant antifungal properties.

Podocarpic acid (I) was first isolated from the resin of Podocarpus cupressins, an important timber tree which is endemic to Java, and later from Podocarpus dacrydioides ("Kahikatea") and Dacrydium cupressinum ("Rimu"), trees which are found in the timber regions of New Zealand (1). Since 1968, more than forty oxygenated metabolites of podocarpic acid have been isolated from various species of Podocarpus (2,3).

Interest in these naturally occurring and synthetic lactones, podolactones, and related podocarpic acid derivatives has been mainly due to the novel structures of these compounds and the various types of biological activity possessed by them. Octahydrophenanthrene lactones (II) and related podocarpic acid derivatives (III) have been reported to possess hormonal and anti-inflammatory properties (4). Other similar podolactones have been shown to inhibit the expansion and division of plant cells (IV) (5-10), to have antileukemic activity (V) (11), to have antibacterial activity (12), to have insect toxicity properties (13-15), and to exhibit antitumor activity (16-19).

0097-6156/87/0325-0140\$06.00/0  
© 1987 American Chemical Society

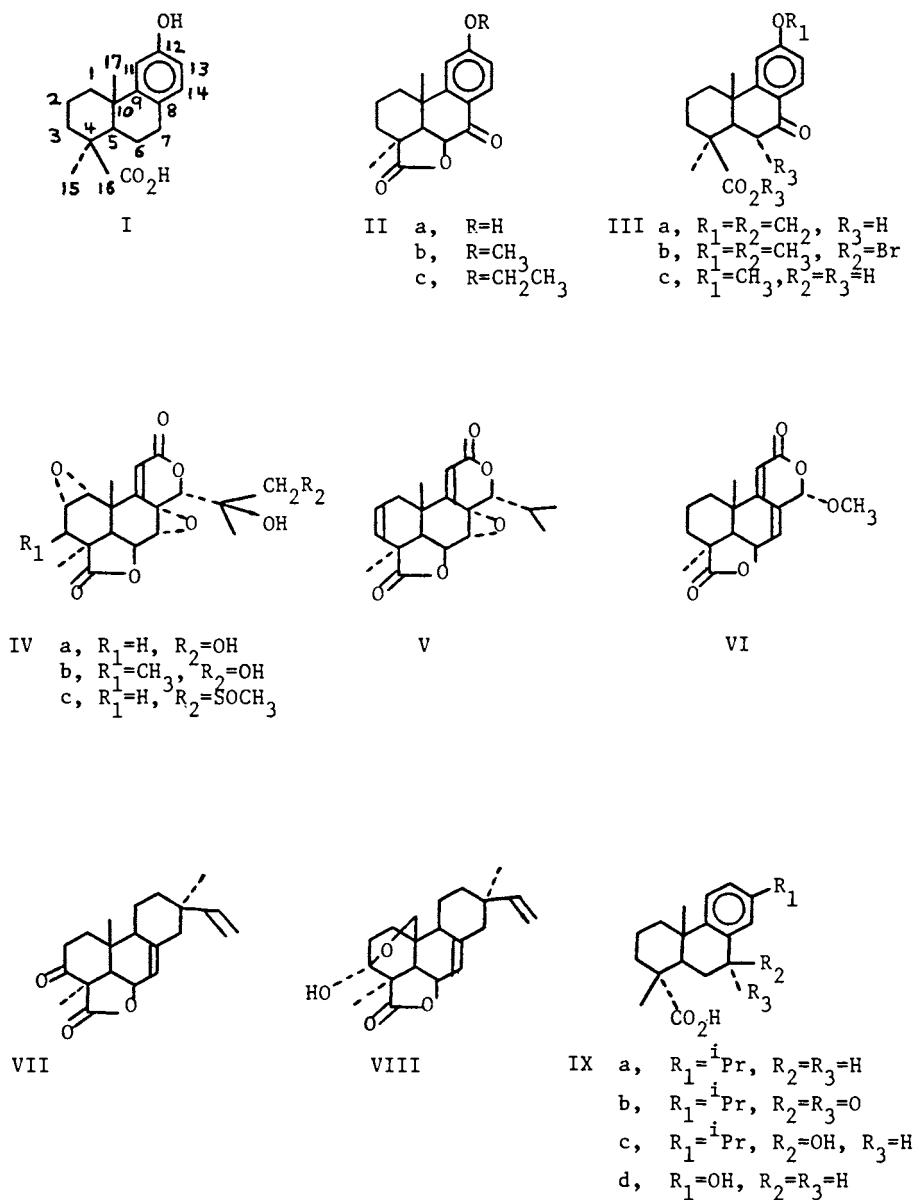


Figure 1. Structures I-IX.



Other reports have indicated that these types of compounds, as a class, possess significant antifungal properties. The lactone (VI), first isolated as a mold metabolite, was found to have significant activity against a number of fungi (20). The momilactones A (VII) and B (VIII) have been shown to be fungitoxic towards C. cucumerinum (21,22). In a recent report several oxidized resin acid derivatives of dehydroabietic acid (IX a-c) and 13-hydroxypodocarpic acid (IX d) were found to be highly fungistatic against P. pini, a conifer pathogenic fungi (23). It was observed that mature trees were more resistant to fungal infection and contained a greater quantity of oxidized resin acid derivatives in their resin suggesting greater resistance.

In view of their documented biological properties, it appeared worthwhile to evaluate a series of synthetic intermediates derived from podocarpic acid for fungistatic activity against other plant pathogens. This report describes the preparation of these derivatives and the results obtained from incubations of each compound with cultures of selected species of oomycetous and ascomycetous fungi.

#### Chemical Synthesis of Podocarpic Acid Derivatives

Commercial podocarpic acid is derived from natural sources. Several recent studies have been directed towards the total synthesis of this resin acid to assure adequate future supplies of this material for use in agriculture and medicine (24,25).

The goal of the present study was to prepare a series of derivatives related to podocarpic acid for use in structure/activity studies designed to reveal functional groups responsible for the molecules fungistatic properties. Four specific modifications were planned:

1. Substitution of electron-withdrawing groups onto C (13) of the aromatic C ring (Scheme 1).
2. Variation of the halogen at C (6) (Scheme 2).
3. Formation of the lactones from each 6  $\alpha$  - bromo methyl ester derivative (Scheme 3).
4. Substitution of an acetate group for the methyl ester group at C (16) (Scheme 4).

The first modification, substitution of the electron-withdrawing halogen and nitro groups onto C (11) and/or C (13) of the aromatic ring, was based upon the well-known observation that the antiseptic properties of phenols are enhanced by the introduction of these groups onto the phenolic ring (26).

Nitration was accomplished by reacting podocarpic acid (I, Scheme 1) with nitric acid in acetic acid (27-30). The number of nitro groups introduced onto the aromatic ring was controlled by the amount of nitric acid used in the reaction (one or two equivalents). The 13-nitro derivative X was methylated with dimethyl sulfate under basic condition to yield XIV. A similar methylation of I has been shown to produce methyl o-methyl podocarpate (XII) (4,16). Bromine was introduced at C (13) by the electrophilic substitution of bromine into the aromatic ring of XII using bromine in acetic acid. The fact that this reaction gives

only the monosubstituted 13-bromo derivative is probably due to steric hindrance resulting from the angular methyl group in the axial orientation at position 10 and the large size of the bromine atom which would prevent substitution at position 11, the other ortho position on the ring.

In Scheme 2, benzylic oxidation of XII, XIII, and XIV using chromium trioxide produced the corresponding ketone derivatives (4,16). It has also recently been shown that XII may be oxidized to the ketone XV under conditions of ozonolysis (31). Ketones XV and XVI were brominated using an adapted procedure derived from the work of Bible and Grove (4,16) to yield the mono- and di-bromoketones XVIII and XIX. In order to effect the bromination of XVII, an alternate method was utilized which gave ample quantities of bromoketone XX (32). The corresponding chloride derivatives (XXI and XXII) of XV and XVI were prepared by reaction with copper chloride and lithium chloride in N,N-dimethylformamide (33). The assignments of the  $\alpha$ -configuration to the halogen atoms at C (6) were verified using known coupling constants from the  $^1\text{H}$  NMR spectra which are correlated to the x-ray structure determination of XVIII (16,34-37).

In Scheme 3, the  $\alpha$ -bromoketones XVIII and XIX were converted to lactones XXIII and XXIV by refluxing in collidine (4,16,38). By-products of this reaction include the  $\alpha$ ,  $\beta$ -unsaturated ketone XXV which results from the dehydrobromination (38,39) of XVIII. Ketone XXVI results from a one-step dehydrobromination-decarbomethoxylation (40-43) of XIX.

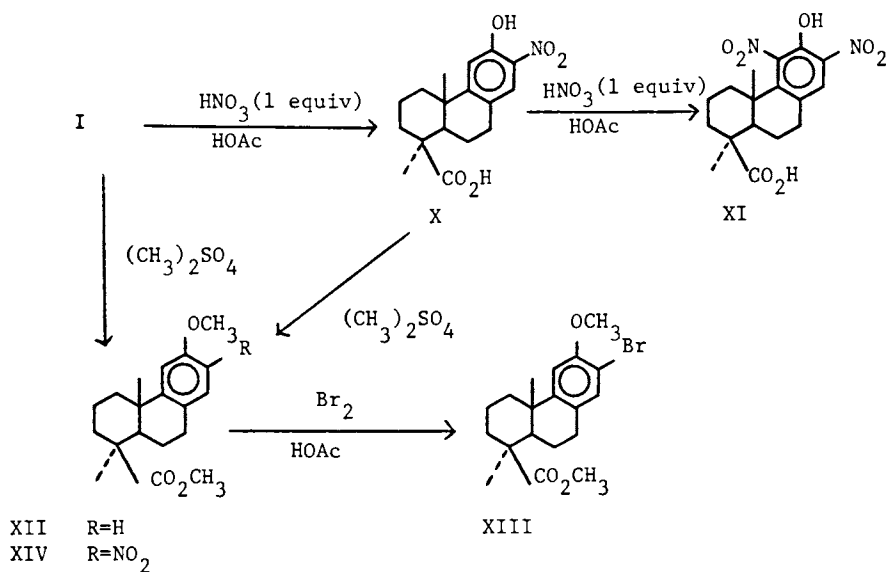
The acetate series of compounds (Scheme 4) was synthesized by hydride reduction of methyl O-methyl podocarpate (XII) followed by acetylation of the resulting alcohol XXVII (44). The acetate XXVIII was then oxidized at the benzylic position to ketone XXIX. In contrast to the methyl ester derivatives, halogenation at position 6 (using methods described previously) of the corresponding keto acetates resulted in two epimers, the 6  $\alpha$ - and 6  $\beta$ -halogenated compounds, as well as the dehydrohalogenation product XXXIV. The assignments of the  $\alpha$ - and  $\beta$ -configuration to the halogen atoms at C (6) were determined from the  $^1\text{H}$  NMR coupling constants at C (5) and C (16) as described previously.

#### Biological Evaluation of Potential Fungistatic Agents

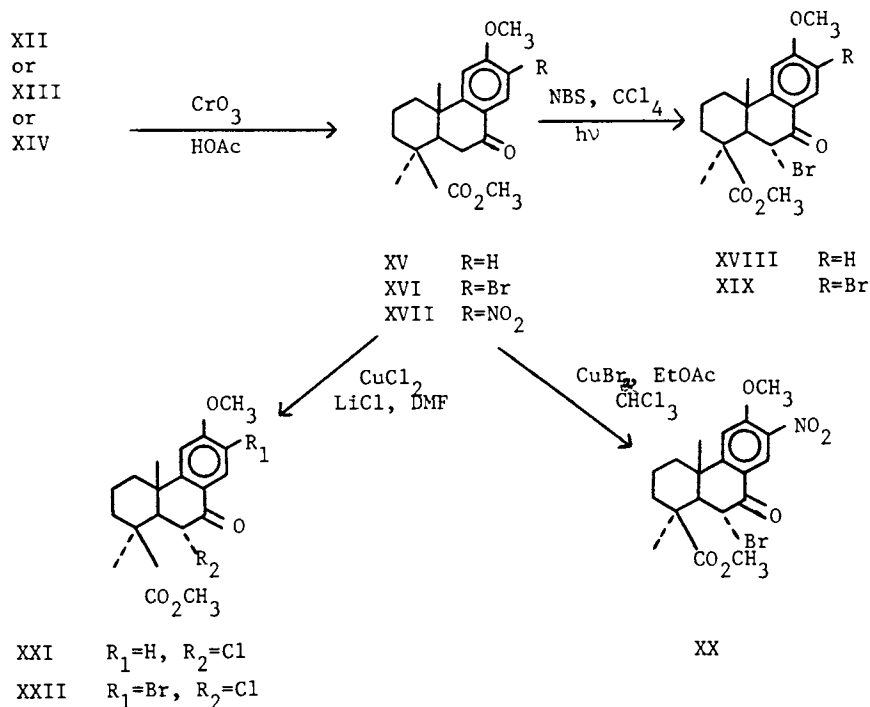
Podocarpic acid (I) and a number of its chemical derivatives (X-XXXIV) were evaluated for their potential fungistatic activity as measured by their effects on the growth of the fungi on solid media (Table 1). All compounds evaluated were of 98% or greater purity (tlc and glc analysis). Each structure was consistent with its spectral analysis ( $^1\text{H}$  NMR,  $^{13}\text{C}$  NMR, ir, and ms). Each compound was evaluated against Phytophthora cactorum (both with (B) and without (A) added cholesterol), Gibberella fujikuroi (C), Saprolegnia ferax (D), and Achlya bisexualis (both male (E) and female (F) strains). The fungi were cultured as described in references 45-47. P. cactorum, unlike the other fungi, fails to synthesize sterols (48) and requires sterol to complete the reproductive phase of its life cycle (49).

Compounds (10  $\mu\text{g/ml}$ ) evaluated were dissolved in a minimal amount of ethanol and introduced aseptically into the sterilized

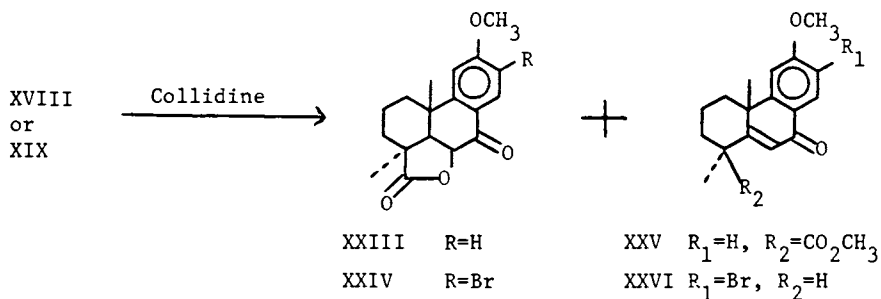
Scheme 1



Scheme 2



Scheme 3



Scheme 4

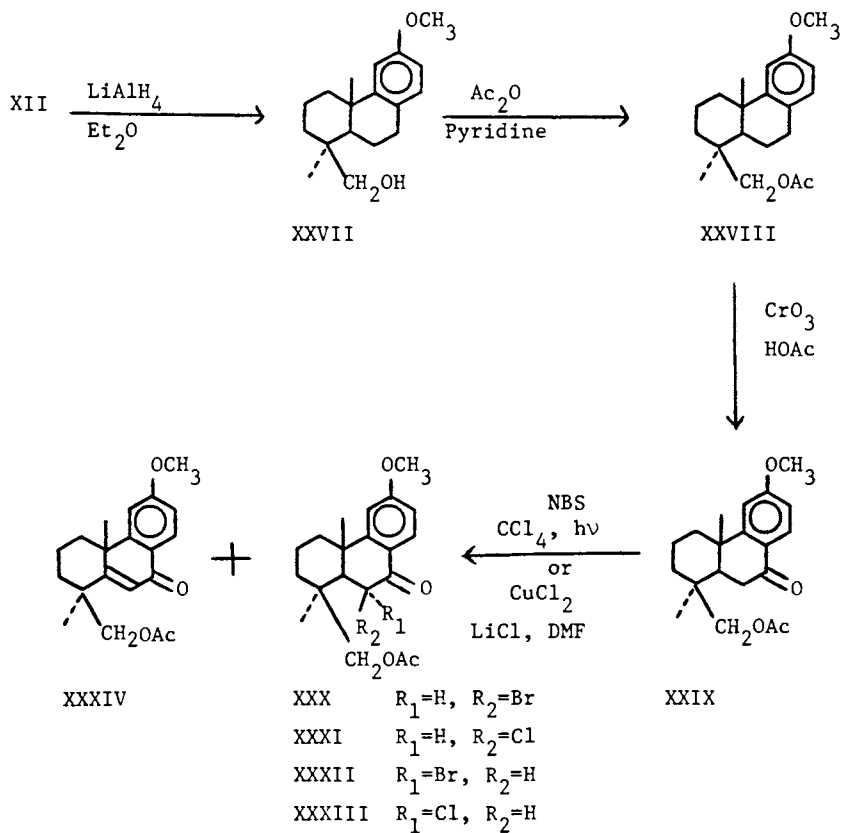


Table I. Comparison of the fungistatic properties of podocarpic acid and its derivatives<sup>1</sup>

Compound	Fungal Species					
	A	B	C	D	E	F
I	66	82	94	62	68	74
X	75	97	94	96	96	76
XI	47	50	68	27	0	0
XIII	84	110	97	93	100	100
XIV	97	105	97	73	93	81
XV	97	97	95	79	76	66
XVI	92	104	90	96	88	88
XVII	91	101	100	93	99	--
XVIII	68	83	100	78	78	76
XIX	120	104	94	93	96	88
XX	58	86	102	100	78	80
XXI	72	86	96	91	88	76
XXII	107	110	93	91	96	88
XXIII	94	104	99	73	81	84
XXIV	68	85	94	100	54	74
XXV	115	98	96	8	84	75
XXVI	77	88	90	100	45	56
XXVII	125	84	87	51	49	60
XXVIII	122	101	93	84	71	87
XXIX	90	99	95	62	65	69
XXX	67	73	100	67	61	71
XXXI	143	99	98	91	74	77
XXXII	121	93	97	18	75	78

<sup>1</sup> The values are expressed as a percentage of control's radial diameter obtained 3 to 12 days (depending on species) following inoculation with a 5mm plug. A - P. cactorum without added cholesterol, B - P. cactorum with added cholesterol, C - Gibberella fujikuroi, D - Saprolegnia ferax, E - Achlya bisexualis, male strain, F - Achlya bisexualis, female strain.

agar-supplemented culture medium. The inhibition values obtained are expressed as percent of control by measurement of the radial diameter of fungal growth. Values greater than 100% represent an enhancement or stimulation of growth.

Many of these compounds demonstrated varying degrees of significant fungistatic (defined as inhibition of growth on solid media) activity against one or more of the species in the study (Table I). In particular, dinitro derivative XI demonstrated potent activity in all assays. This may be due, in part, to its resemblance to picric acid (2,4,6-trinitrophenol), a substance which is known to complex with, and cause the irreversible precipitation of protein (50,51). The lack of fungistatic properties of a specific compound to some but not all fungi tested may be due to the lack of mycelial uptake, a possibility which is currently under study. Interestingly, cholesterol supplemented to *P. cactorum* was protective to the fungistatic properties resulting from the inhibition induced by some podocarpic acid derivatives. An increase, however, in radial diameter induced by other derivatives does not necessarily imply a beneficial effect, since these mycelia appeared abnormal (cf. 49).

In conclusion, these studies have indicated that chemical modification of the basic podocarpic acid structure can produce new compounds with antifungal activity. The results derived from this study represent preliminary findings which are subject to further investigation. We anticipate that more detailed studies will reveal information concerning the mechanism and mode of action of many of these compounds. In the meantime, we continue to develop new antifungal agents using selected natural products as model compounds.

#### Literature Cited

1. Sherwood, I.R.; Sholt, W.F. J. Chem. Soc. 1938, 1006-13, and references therein.
2. Hayashi, Y.; Matsumoto, T. J. Org. Chem. 1982, 47, 3421 - 29.
3. Cassady, J.M.; Lightner, T.K.; McCloud, T.G.; Hembree, J.R.; Blym, S.R.; Chang, C. J. Org. Chem. 1984, 49, 942-45.
4. Bible, R.H.; Grove, M. U.S. Patent 2 753 357, 1956; Chem. Abstr. 1957, 51, 2869.
5. Galbraith, M.N.; Horn, D.H.S.; Sasse, J.M.; Adamson, D. Chem. Commun. 1970, 170-71.
6. Hayashi, Y.; Yokoi, J.; Watanabe, Y.; Sakan, T.; Masuda, Y.; Yamamoto, R. Chem. Lett. 1972, 759-62.
7. Hayashi, Y.; Sakan, T. Proc. 8th Int. Conf. Plant Growth Substances 1974, p. 525.
8. Sasse, J.M.; Galbraith, M.N.; Horn, D.H.S.; Adamson, D.A. In "Plant Growth Substances, 1970".
9. Call, D.J., Ed.; Springer Verlag, Berlin, 1972; p. 430.
10. Galbraith, M.N.; Horn, D.H.S.; Sasse, J.M. Chem. Commun. 1971, 1362-63.
11. Bryan, R.F.; Smith, P.M. J. Chem. Soc., Perkin Trans. II 1975, 148-52.

American Chemical Society  
Library

1155 16th St., N.W.

Washington, D.C. 20036

12. Sacki, I.; Sumimoto, M.; Kondo, T. Holzforschung 1970, 24, 83-87.
13. Russell, G.B.; Fenemore, P.G.; Singh, P. Chem. Commun. 1973, 166-67.
14. Singh, P.; Fenemore, P. G.; Russell, G.B. Aust. J. Biol. Sci. 1973, 26, 911-15.
15. Singh, P. Russell G.B.; Hayashi, Y.; Gallagher, R.T.; Fredricksen, S. Entomol. Exp. Appl. 1979, 25, 121-125.
16. Parish, E.J.; Miles, P.H. J. Pharm. Sci. 1984, 73,694-98.
17. Hayashi, Y.; Matsumoto, T.; Tashiro, T. Gann. 1979, 70, 369-72.
18. Hayashi, Y.; Matsumoto, T.; Sakurai, Y.; Tashiro, T. Gann. 1975, 66, 587-90.
19. Kupchan, S.M.; Baxter, R.L.; Ziegler, M.R.; Smith, P.M.; Bryan, R.F. Experientia 1975, 31,137-41.
20. Ellestad, G.A.; Evans, R.H., Jr.; Kunstmann, M.P. J. Am. Chem. Soc. 1969, 91, 1234-36.
21. Cartwright, D.W.; Russell, G.E. Trans. Br. Mycol. Soc. 1981, 78, 323-26.
22. Fuchs, A.; Davicse, L.C.; de Waard, M.A.; de Wit, P.G.M. Pestic. Sci. 1983, 14, 272-96, and references therein.
23. Franich, R.A.; Gadgil, P.D. Physiol. Plant Path. 1983, 23, 183-95.
24. Snider, B.B.; Mohan, R.J. Kates, S.A. J. Org. Chem. 1985, 50, 3661-63.
25. Welch, S.C.; Hayah, C.P.; Kim, J.H., Chu, P.S. J. Org. Chem. 1977, 42, 2879-82.
26. Gisvold, O. In "Textbook of Organic Medicinal and Pharmaceutical Chemistry"; Wilson, C.O.; Gisvold, O.; Doerge, R.F.; Eds.; J.B. Lippincott; New York, 1971; p. 255.
27. Niedeli, J.B.; Vogel, H.J. J. Am. Chem. Soc. 1949, 71, 2566-69.
28. Werbin, J.; Holoway, C. J. Biol. Chem. 1956, 223, 651-56.
29. Utne, T.; Jobson, R.B.; Babson, R.D. J. Org. Chem. 1968, 33, 2469-72.
30. Santaniello, R.; Ravasi, M.; Ferraboschi, P. J. Org. Chem. 1983, 48, 739-41.
31. Parish, E.J.; McKeen, G.G.; Miles, D.H. Org. Prep. Proc. Intern. 1985, 17, 143-46.
32. King, L.C.; Ostrum, G.K. J. Org. Chem. 1964, 29, 3459-61.
33. Kosowel, E.M.; Cole, W.J.; Wu, G.-S.; Cardy, D.E.; Meisters, G., J. Org. Chem. 1963, 28, 630-34.
34. Lickei, A.E.; Rieke, A.C.; Wheeler, D.M.S. J. Org. Chem. 1967, 32, 1647-52.
35. Cutfield, J.F.; Waters, T.N.; Clark, G.R. J. Chem. Soc., Perkin Trans. II 1974, 150-55.
36. Cambie, R.C.; Clark, G.R.; Crump, D.R.; Waters, T.N. Chem. Comm. 1968, 183-85.
37. Clark, G.R.; Waters, T.N. J. Chem. Soc., C 1970, 887-90
38. Wenkert, E.; Beak, P.; Carney, R.W.J.; Chamberlain, J.W.; Johnston, D.B.R.; Roth, C.D., Taharce, A. Can. J. Chem. 1963, 41, 1924-40.
39. Wenkert, E.; Fuchs, A.; McChesney, J.M. J. Org. Chem. 1965, 30, 2931-34.

40. Miles, D.H.; Parish, E.J. Tetrahedron Lett. 1972, 3987-90.
41. Parish, E.J.; Miles, D.H. J. Org. Chem. 1973, 38, 1223-1225.
42. Parish, E.J.; Mody, N.V., Hedin, P.A.; Miles, D.H. J. Org. Chem. 1974, 39, 1592-93.
43. Parish, E. J.; Haung, B.-S.; Miles, D.H. Synth. Commun. 1975, 5, 341-45.
44. Parish, E.J. Ph.D. Thesis, Mississippi State University, Mississippi, 1984.
45. Nes, W.D.; Heupel, R.C. Arch. Biochem. Biophys. 1985 in press.
46. Nes, W.D.; Le, P.H.; Berg, L.; Patterson, G.W., Kerwin, J. Experientia 1985, in press.
47. Berg, L.; Ph.D. Thesis Univ. of Md. 1983.
48. Nes, W.D.; Stafford, A.E. Proc. Natl. Acad. Sci. 1983, 80, 3227-31
49. Nes, W.D.; Stafford A.E. Lipids 1984, 19, 544-49.
50. Haurowitz, F. "Chemistry and Biology of Proteins"; Academic Press: New York, 1950; p.11.
51. White, A.; Handler, P.; Smith, E.L.; Hill, R.L.; Lehmann, I.R. "Principles of Biochemistry"; McGraw-Hill: New York, 1978; 6th Ed., p. 107.

RECEIVED May 1, 1986



## Chapter 10

# The Role of Cutin, the Plant Cuticular Hydroxy Fatty Acid Polymer, in the Fungal Interaction with Plants

P. E. Kolattukudy, Mark S. Crawford, Charles P. Woloshuk, William F. Ettinger, and Charles L. Soliday

Institute of Biological Chemistry and Biochemistry/Biophysics Program, Washington State University, Pullman, WA 99164

Cutin, the structural component of plant cuticle, is a polyester composed of  $\omega$ -hydroxy-C<sub>16</sub> and C<sub>18</sub> fatty acids, dihydroxy-C<sub>16</sub> acid, 18-hydroxy-9,10-epoxy-C<sub>18</sub> acid and 9,10,18-trihydroxy-C<sub>18</sub> acid. This insoluble polymer constitutes a major physical barrier to the penetration of pathogenic fungi into plants. Pathogenic fungi produce an extracellular cutinase when grown on cutin as the sole source of carbon. Cutinase has been isolated and characterized from many pathogenic fungi. This enzyme is a "serine hydrolase" containing the characteristic catalytic triad. With the use of ferritin-labeled antibodies and electron microscopy it was shown that cutinase is produced by the penetrating fungus during the actual infection of its host. Inhibition of this enzyme prevents fungal penetration into the plant and thus prevents fungal infection. Cutinase cDNA has been cloned from Fusarium f. sp. solani pisi and Colletotrichum capsici. The complete primary structure of the Fusarium enzyme and a partial structure of the Colletotrichum enzyme have been determined. These two enzymes show conservation of certain structural features. Cutinase mRNA synthesis is rapidly induced in fungal spores by the unique monomers of cutin initially generated by the small amount of the enzyme carried by the spores. The induction of cutinase is followed by the induction of polygalacturonase which is presumably used to degrade the pectin barrier which lies under the cuticle.

Plant cuticle, the boundary layer at which microbial pathogens come into contact with the plant, is derived entirely from lipids. Obviously, the cuticle can play an important role in the interaction between the microbe and the host plant. The cuticle is composed of an insoluble structural polymer called

0097-6156/87/0325-0152\$06.75/0  
© 1987 American Chemical Society

cutin which is derived from interesterified hydroxy and hydroxy epoxy fatty acids. This polymer is embedded in a complex mixture of soluble lipids collectively called wax which is also secreted onto the plant surface. The soluble components of the cuticle may play a chemical role in influencing the interaction between microbes and plants whereas cutin would play a physical role in that the organism must penetrate this barrier before it can enter the plant. Although there are many reports that various wax components may have inhibitory effects on microbial growth, the specific roles of such components have not been elucidated. It is possible, if not likely, that some of the wax components play specific roles in the interaction between pathogens and plants. On the other hand, the role of cutin as the first barrier which the pathogen must penetrate to infect the plant is rather obvious and in recent years the mechanism by which fungal pathogens penetrate this barrier has been elucidated. In this paper we shall review the progress made in this area and discuss the recent findings concerning how a fungal spore senses that it is resting on the plant surface so that it can induce the appropriate enzymes required to disrupt the cuticular and underlying barrier layers.

#### Structure of Cutin

Cutin is a polyester composed of 16-hydroxy-C<sub>16</sub> acid, 9 or 10,16-dihydroxy-C<sub>16</sub> acid, 18-hydroxy-C<sub>18</sub> acid, 18-hydroxy-9,10-epoxy-C<sub>18</sub> acid and 9,10,18-trihydroxy-C<sub>18</sub> acid (1-3). The C<sub>18</sub> acids containing an additional double bond at C-12 are also usually present whereas components containing additional double bonds at C-12 and C-15 are less common. The double bond at C-12 is also epoxidized and subsequently hydrated generating 9,10,12,13,18-pentahydroxy-C<sub>18</sub> acids in some plants. In some cases further oxidation products of the above monomers are also found. For example, 16-oxo-9- or 10-hydroxy-C<sub>16</sub> acid was found as a major component of *Vicia faba* embryo cutin (4), and 9,16-dihydroxy-10-oxo-C<sub>16</sub> acid was found in citrus cutin (5). Besides these common major hydroxy and epoxy acids, a variety of other fatty acids have been found as minor components in many cutins as indicated in other reviews (1-3).

The hydroxy acids are interesterified to generate the insoluble polyester. The precise details of how the monomers are linked are not understood. Indirect chemical studies have been conducted to determine the nature of the linkages involved and the extent of involvement of the secondary hydroxyl groups in ester linkages. Mesylation of the free hydroxyl groups of the polymer followed by depolymerization by LiAlD<sub>4</sub> resulted in the substitution of a D atom for each free hydroxyl group in the polymer whereas the hydroxyl groups involved in ester linkages retained the hydroxyl groups during depolymerization. Thus GC/MS analysis of the depolymerization products revealed the degree of involvement of the alcohol groups in ester linkages (6). A similar approach was recently used on cutin from *Quercus suber* (7). Another approach to determine the amount and nature of free hydroxyl groups present in the polymer was oxidation of the free hydroxyl groups in the

polymer followed by depolymerization by hydrolysis or transesterification and analysis of the products by GC/MS (8). Selective reduction of the ester bonds by  $\text{LiBH}_4$  was used to determine the amount of free carboxyl groups present in the polymer. The limited amount of such indirect chemical studies conducted on cutin showed that about one-half of the mid-chain hydroxy groups and most of the primary hydroxyl groups and carboxyl groups are involved in ester linkages, suggesting the presence of branches and/or cross-links in the polymer.

Most plants contain a mixture of the 16- and 18-carbon family of cutin monomers. Even though there appears to be a degree of species specificity in the cutin composition, whether the polymer structure can vary sufficiently to affect major functional properties of the polymer is not known. The polyester also contains covalently attached minor components such as phenolic acids and flavanoids (9,10) which, if released by the attacking pathogen, can play important roles in the interaction between the pathogens and the host. However, such areas have not been explored adequately to draw firm conclusions.

#### Isolation and Characterization of Cutinase

How pathogenic fungi penetrate the cuticular barrier has been debated for the better part of a century (11,12). The penetration process was considered by some to be mediated entirely by the physical force of growth of the germinating spore whereas others argued that the cuticular barrier is breached by an extracellular enzyme that catalyzes degradation of cutin. Electron microscopic examination of the penetration areas suggested that at least in some cases the penetration involved enzymatic degradation of the cuticle (13-15). Such ultrastructural evidence constituted apparent dissolution of cuticle with no obvious signs of physical force such as depression of the barrier in the penetration area. However, such an approach could not provide direct proof for the involvement of a cutin degrading enzyme and the controversy continued.

To determine whether fungal pathogens can generate enzymes capable of degrading the plant cutin, Fusarium solani pisi, a pathogen of Pisum sativum was provided apple cutin as the sole source of carbon. Since the fungus grew on this carbon source, it became obvious that this fungus had the ability to produce extracellular cutinase. This extracellular enzyme was purified from the extracellular fluid of cutin-grown cultures of Fusarium solani pisi. Since then a variety of fungal pathogens have been grown on cutin as the sole source of carbon and cutinases have been purified from many of the pathogens (3). The procedure that yields pure cutinase from most fungi is summarized in Table I. The fungal pathogens so far examined for their ability to produce cutinase are listed in Table II.

The properties of cutinase obtained from the various fungal sources appear to be quite similar. The molecular weight is near 25,000 and the amino acid composition appears to be quite similar (3,16). Some of the major features shared by the

TABLE I  
Purification of Cutinase from *F. solani pisi*<sup>a</sup>

Step	Total Units	% Yield
Culture Filtrate (25%)		
+	200,000	
Triton X-100 (0.1%) Wash (75%)		
Acetone (75%) Precipitate	160,000	80
QAE-Sephadex	130,000	56
Octyl-Sepharose	110,000	55
SP-Sephadex		
Isozyme C (95 mg)	72,000	36
Isozyme D (45 mg)	24,000	12

<sup>a</sup>W. Koller, C.L. Soliday and P.E. Kolattukudy, unpublished results.

TABLE II

Host-Pathogen Interactions in Which Cutinase Has Been Detected

Pathogen	Host	Remarks
<u>Fusarium solani pisi</u>	Pea	Cutinase purified; role in infection conclusively proven
<u>Fusarium roseum sambucinum</u>	Squash	Cutinase purified
<u>Fusarium roseum culmorum</u>	Wheat	Cutinase purified
<u>Ulocladium consotiale</u>	Cucumber	Cutinase purified
<u>Helminthosporium sativum</u>	Barley	Cutinase purified
<u>Streptomyces scabies</u>	Potato	Cutinase purified
<u>Colletotrichum gloeosporioides</u>	Papaya	Cutinase purified; role in infection conclusively proven
<u>Colletotrichum capsici</u>	Pepper	Cutinase purified
<u>Colletotrichum graminicola</u>	Corn	Cutinase purified; inhibitors prevent infection
<u>Phytophthora cactorum</u>	Apple	Cutinase purified
<u>Botrytis cineria</u>	Wide Host Range	Cutinase purified
<u>Venturia inequalis</u>	Apple	Cutinase inhibitors prevent infection

various cutinases include the presence of only one tryptophane, one methionine, one or two histidines and two or four cysteines all of which are involved in disulfide bridges. Immunologically, the various cutinases from different organisms appear to be quite different (3,16-19). Only the enzyme from very closely related organisms show cross-reactivity. All fungal cutinases are glycoproteins containing 3 to 6% carbohydrates. The carbohydrates are attached to the protein via O-glycosidic linkages (20). The N-terminus of fungal cutinases appear to be blocked in a novel manner in that glucuronic acid is linked by an amide linkage to the N-terminal amino group (21).

The catalytic properties of fungal cutinases also show similarities. Cutinase shows specificity for primary alcohol esters; secondary alcohol esters are hydrolyzed at much lower rates (3,16). Phospholipids are not hydrolyzed but certain triglycerides, such as tributyrin and triolein but not tripalmitin or tristearin, are hydrolyzed by cutinase. In these cases the enzymes show clear specificity for the primary alcohol esters. All fungal cutinases also catalyze hydrolysis of p-nitrophenyl esters of short chain fatty acids. The relative rates of hydrolysis depend upon on the chain length of the acyl group and the source of the enzyme. In many cases the longer chain esters such as C<sub>16</sub> or C<sub>18</sub> are hydrolyzed extremely poorly when compared to short chain esters such as C<sub>4</sub>. On the other hand cutinase from some genera, such as *Colletotrichum*, catalyzes hydrolysis of longer chain esters at rates comparable to that obtained with the short chain acids (16).

The mechanism of catalysis of ester bonds by fungal cutinases has been studied using protein-modifying reagents that are relatively specific to functional groups. The results of such studies strongly suggest that a catalytic triad involving serine, histidine and a carboxyl group are involved in catalysis by fungal cutinase (22). Active serine directed reagents such as diisopropylfluorophosphate (23) and a variety of other organic phosphates (24,25) as well as transition state analogs such as alkyl and phenyl boronic acids (26), inhibit fungal cutinases. Modification of histidine by diethylpyrocarbonate and carboxyl groups by carbodiimide also inhibit the enzyme activity (22). Correlation of the number of residues modified with the extent of activity loss of the enzyme showed that one serine, one histidine and one carboxy group were essential for catalysis by cutinase (22). The presence of the catalytic triad characteristic of serine hydrolases suggested by these results also raised the possibility of the involvement of an acyl enzyme intermediate. To test this possibility cutinase was first treated with diethylpyrocarbonate to inhibit acyl-enzyme hydrolysis and this enzyme was then treated with p-nitrophenyl[1-<sup>14</sup>C]acetate. Gel filtration of this reaction mixture yielded [1-<sup>14</sup>C]acetyl-cutinase demonstrating the existence of the postulated acyl-enzyme intermediate (22). The mechanism of catalysis by cutinase can be depicted as shown in Figure 1.

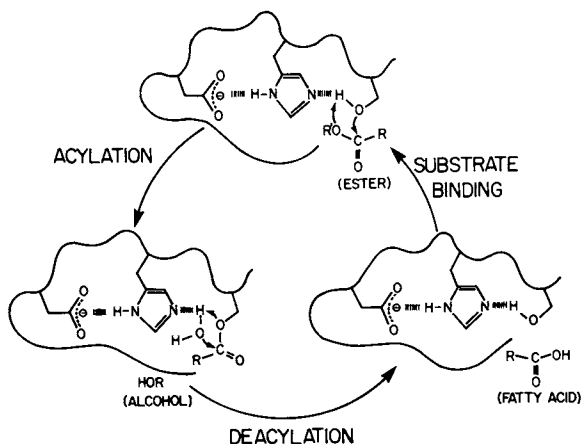


Figure 1. Mechanism of catalysis by cutinase. (Reproduced with permission from Ref. 16. Copyright 1984 Elsevier/North Holland Biomedical Press.)

### Structure of Cutinase

The complete primary structure of the enzyme has been deduced from the nucleotide sequence of the cDNA which was cloned (Figure 2). The cDNA sequence showed an open reading frame which could be translated into a protein with a molecular weight of 23,951 (27). The enzyme isolated from *Fusarium solani pisi* had been shown to have a glucuronic acid attached to a glycine at the N-terminus. The first glycine residue in the primary translation product would be at position 32 according to the nucleotide sequence. Thus the 31 amino acid leader peptide must be removed during the processing of the primary translation product into mature enzyme. In fact, the primary translation product had been shown to be larger than the mature enzyme by *in vitro* translation of isolated mRNA (28). The leader sequence revealed by the nucleotide sequence did show a fairly typical structure except for the fact that the post-hydrophobic core region was longer than that found in most leader sequences of other extracellular proteins (29,30). The biochemistry of the processing of the precursor into the mature enzyme has not been studied.

In order to verify the sequence derived from the nucleotide sequence, proteolytic fragments of the mature enzyme were isolated and the amino acid sequence of these peptides were determined. The peptide containing the only tryptophane of the enzyme was isolated and sequenced. The active serine was located by treatment of the enzyme with radioactive diisopropylfluorophosphate followed by isolation and amino acid sequencing of a tryptic peptide containing the radioactivity. The carboxyl group involved in catalysis was located by labeling this residue by treatment with carbodiimide and [<sup>14</sup>C]glycine ethyl ester followed by isolation and analysis of the labeled tryptic peptide (W.F. Ettinger and P.E. Kolattukudy, unpublished results). These amino acid sequences agreed with the sequences deduced from the nucleotide sequence. The histidine residue involved in catalysis was easily located as the sole histidine residue present in the enzyme. The three members of the catalytic triad are located far apart in the primary structure of the protein. Obviously these three residues are held in juxtaposition so that they can function as a catalytic triad by the secondary and tertiary structure. The disulfide bridges must play a significant role in holding the enzyme in the active conformation because reduction of the disulfide is known to result in inactivation of the enzyme (16). The importance of the disulfide bridges is also reflected in the fact that the positions of the cys residues involved seem to be highly conserved (W.F. Ettinger and P.E. Kolattukudy, unpublished results). In the three cutinases, from which homologous peptides have been sequenced, the conservation of the cys positions is obvious (Figure 3). The high degree of homology shown by the enzymes from *Fusarium* and *Colletotrichum* is striking.

The structure of the cutinase gene has been determined using genomic DNA cloned in  $\lambda$ -phage. This gene contains one 51 bp intron which has the typical junction and splicing signals



AACCACAACCTTCACTTCATCAGCATTCACTTCACTTCTTCCGCTTCTTCCCTTTTCACTCTTTTATCATCCTCACC

10 MET LYS PHE PHE ALA LEU THR THR LEU LEU LEU ALA ALA THR ALA SER ALA LEU PRO THR SER  
 ATG AAA TTC TTC GCT CTC ACC ACA CTT CTC GCC GCC ACC GCT TCG GCT CTG CCT ACT TCT  
 H→C-57

30 ASN PRO ALA GLN GLU LEU GLU ALA ARG GLN LEU LEU GLY ARG THR THR ARG ASP ASP LEU ILE  
 AAC CCT GCC CAG GAG CTT GAG GCG CGC CAG CTT GGT ABA ACA ACT CGC GAC GAT CTG ATC  
 H→C-4

47 ASN GLY ASN SER ALA SER CYS ARG ASP VAL ILE PHE ILE TYR ALA ARG GLY SER THR GLU  
 AAC GGC AAT AGC GCT TCC TGC CGC GAT GTC ATC TTC ATT TAT GCC CGA GGT TCA ACA GAG

70 THR GLY ASN LEU GLY THR LEU GLY PRO SER ILE ALA SER ASN LEU GLU SER ALA PHE GLY  
 ACG GGC AAC TTG GGA ACT CTC GGT CCT AGC ATT GCC TCC AAC CTT GAG TCC GCC TTC GGC

90 LYS ASP GLY VAL TRP ILE GLN GLY VAL GGC GGT GGC TAC CGA GCC ACT CTT GBA GAC AAT  
 AAG GAC GGT GTC TGG ATT CAG GGC GTT GGC GGT GGC TAC CGA GCC ACT CTT GBA GAC AAT  
 99

110 ALA LEU PRO ARG GLY THR SER SER ALA ALA ILE ARG GLU MET LEU GLY LEU PHE GLN GLN  
 GCT CTC CCT CGC GGA ACC TCT AGC GCC GCA ATC AAG GAG ATG CTC GGT CTC TTC CAG CAG

125 ALA ASN THR LYS CYS PRO ASP ALA THR LEU ILE ALA GLY GLY TYR SER GLN GLY ALA ALA  
 GCC AAC ACC AAG TGC CCT GAC GCG ACT TTG ATC GCC GGT GGC TAC AGC CAG GGT GCT GCA  
 130 136

```

150 LEU ALA ALA SER ILE GLU ASP LEU ASP SER ALA ILE ARG ASP LYS ILE ALA GLY THR
    CTT GCA GCC GCC TCC ATC GAG GAC CTC GAC TCG GCC ATT CBT GAC AAG ATC GCC GGA ACT

170 VAL LEU PHE GLY TYR THR LYS ASN LEU GLN ASN ARG GLY ARG ILE PRO ASN TYR PRO ALA
    GTT CTG TTC GGC TAC ACC AAG AAC CTA CAG AAC CGT GGC CBA ATC CCC AAC TAC CCT GCC

187 _____ 190 _____ 194
    ASP ARG THR LYS VAL PHE CYS ASN THR GLY ASP LEU VAL CYS THR GLY SER LEU ILE VAL
    GAC AAG ACC AAG GTC TTC TGC AAT ACA GGG GAT CTC GTT TGT ACT GGT AGC TTG ATC GTT

204 ALA ALA PRO HIS LEU ALA TYR GLY PRO ASP ALA ARG GLY PRO ALA PRO GLU PHE LEU ILE
    GCT GCA CCT CAC TTG GCT TAT GGT CCT GAT GCT CGT GGC CCT GCC CCT GAG TTC CTC ATC

230 GLU LYS VAL ARG ALA VAL ARG GLY SER ALA @@@ GGAGGATGAGAAATTTTAGCAGGCGCCTGTAAAT
    GAG AAG GTT CCG GCT GTC CGT GGT TCT GCT TGA

TATTGGAGBTTTCAAGTTTTCTTTTGGTGAATAGCCATGATAGATTGTTCAACACTCAATGTACTACATGCCCC

```

Figure 2. Nucleotide sequence of the cloned cutinase cDNA and the amino acid sequence deduced from it. C-4 and C-57 indicate the beginning of the nucleotide sequence of two additional cDNA clones. The solid lines represent the regions for which the primary structure was confirmed by amino acid sequencing.



that are homologous to those found in other filamentous fungi and yeast. It showed a typical polyadenylation signal but the CAT box and TATA boxes in the 5'-flanking region were not obvious.

#### Role of Cutinase in Pathogenesis

With the availability of pure cutinase, an immunological approach became possible to test whether cutinase is secreted by fungi during the penetration of their hosts. F. solani pisi spores were placed on pea stem surface in a moist atmosphere and the progress of germination and penetration was followed by scanning electron microscopy (Figure 4a). At the time when the germinating spores were found to be penetrating the surface, the infection area was treated with ferritin-conjugated antibodies prepared against the enzyme and the tissue was processed for transmission electron microscopy. The electron micrographs (Figure 4b,c) clearly showed that the germinating spores secreted cutinase at the infection area (31). Similar experiments were subsequently done with Colletotrichum gloeosporioides and convincing evidence was obtained that this organism also secreted cutinase during penetration (M.B. Dickman, S. Patil, C.L. Soliday and P.E. Kolattukudy, unpublished results). If the secreted enzyme is necessary for penetration, inhibition of the enzyme should stop the penetration process and therefore infection. Bioassays performed by placing Fusarium spores on pea stem surface showed that inclusion of cutinase inhibitors such as antibodies prepared against the enzyme or chemical inhibitors such as organic phosphates prevented the lesion formation (32) (Figure 5). Similar experiments were performed also by placing spores of Colletotrichum gloeosporioides on papaya fruits (18). In this case also, inclusion of cutinase inhibitors such as the antibodies against this enzyme or organic phosphates completely prevented infection. Subsequently, similar experiments were performed by placing spore suspensions of Venturia inequalis on the leaves of apple seedlings, Colletotrichum graminicola on the leaves of corn seedlings and Colletotrichum capsici on pepper fruits (33). In all of these cases, inclusion of inhibitors of cutinase prevented infection. These results clearly showed that at least under the conditions of the bioassay, fungal infection involves cutinase mediated penetration of the cuticular barrier and that specific inhibition of this enzyme can completely prevent infection.

Whether the cutinase-targeted approach to prevent fungal infection of plants can be an effective method in the field is yet to be determined. One field trial that was done suggests that this approach may be feasible. Spraying of an organic phosphate inhibitor of cutinase on papaya in the field protected the fruits from infection by Colletotrichum gloeosporioides (W. Nishijima, M.B. Dickman, S. Patil and P.E. Kolattukudy, unpublished results). This experiment conducted for an entire season was designed only to test the feasibility of the approach under field conditions and therefore parameters such as the optimal amount of inhibitor or the time of application were not

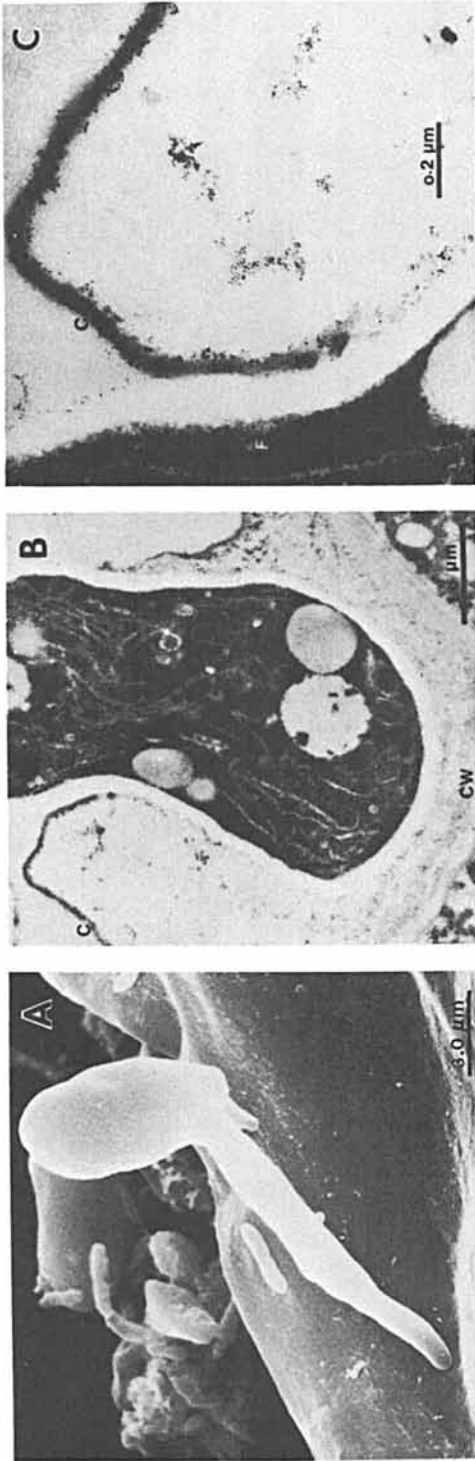


Figure 4. Scanning (a) and transmission (b and c) electron micrographs of the penetration of pea stem cuticle by germinating spores of *F. solani* f. sp. *pisi*. Figures represent the state of events 12 h after placing spores on the stem of the pea seedling. The infection area was treated with ferritin-conjugated, anticutinase IgG. Ferritin granules can be seen at the site of penetration in b and c. C, cuticle; CW, cell wall; F, fungus.

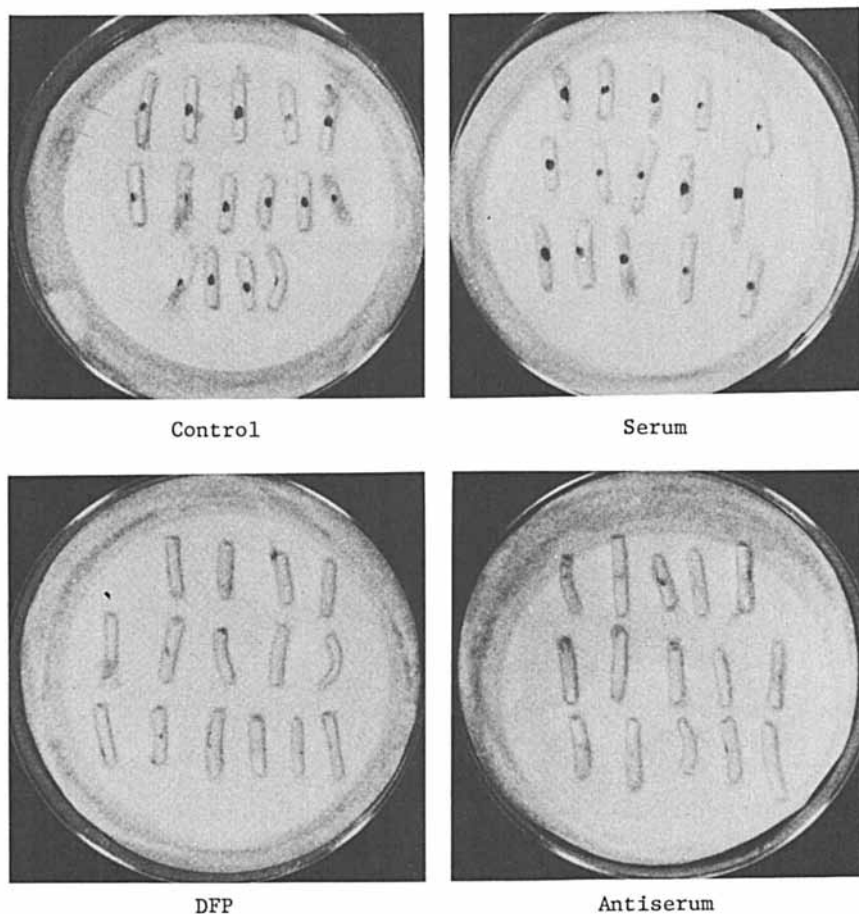


Figure 5. Pea epicotyl segments 72 hours after inoculation with conidial suspension of *F. solani* f. sp. *pisii* in water (control), water containing IgG from preimmune rabbit serum (serum), water containing IgG from antiserum prepared against cutinase (antiserum) and water containing 10  $\mu$ M diisopropylfluorophosphate (DFP).

studied. Instead, the spray program was conducted in alternate weeks for an entire season and results were positive. It is likely that antipenetrant chemicals which inhibit cutinase will work effectively if combined with a low level of a classical fungicide. In this manner, the bulk of the fungal spores will not be able to penetrate and some which might penetrate through some breach in the cuticular barrier can be prevented from setting up infection by the fungicide.

Ability to produce cutinase can determine whether a fungus can infect a plant. An isolate of Fusarium solani pisi (T-30) which lacked the ability to produce cutinase failed to infect pea stem (34) as indicated below. A wound pathogen of papaya, Mycosphaerella, could infect papaya fruits when exogenous cutinase was provided instead of a wound (18). More recently cutinaseless mutants of Colletotrichum gloeosporioides (M.B. Dickman and S. Patil, private communication) and Fusarium solani pisi (Anne Dantzig, private communication) were isolated after mutagenesis. These mutants could not infect their hosts when the cuticular barrier was intact. That the mutation did not affect elements involved in pathogenesis other than penetration was shown by the fact that the mutants were as virulent as the wild type when the cuticular barrier was mechanically breached or when exogenous cutinase was provided in the inoculation medium (M.B. Dickman and S. Patil, private communication). These results clearly demonstrate that cutinase is essential for infection by the two pathogens.

To test whether the ability to produce cutinase might determine the virulence of fungal strains a large number of isolates of Fusarium solani pisi were tested using bioassays with pea stem segments. Spore suspensions were placed on intact stem or, for comparison, on pea stem with mechanically breached cuticle/wall barrier. In this manner, isolates which lacked the ability to penetrate the barrier but had all the other necessary elements for pathogenesis could be detected. Thus Fusarium solani pisi isolate T-8 was found to infect pea stems with or without the cuticular barrier whereas isolate T-30 could infect only if the barrier was mechanically breached (34) (Figure 6). To test whether isolate T-30 lacked the ability to produce cutinase, the spores of isolates T-8 and T-30 were washed with buffer and the cutinase activity was measured using a specially prepared highly radioactive cutin. The results clearly showed that T-30 lacked cutinase (Table III). If this defect were the only reason for the observed inability of T-30 to infect intact pea stem, supplementation with exogenous cutinase might make this isolate as virulent as T-8. Exogenous cutinase did in fact assist T-30 in infecting pea stem segments in the bioassays. However, supplementation with pectinase, cellulase and pectin methylesterase were also required to increase the virulence of T-30 to a level that was equal to that of T-8 (34) (Figure 7). From these observations it was concluded that the infection process involved a set of penetration enzymes to disrupt not only the cuticular barrier but also the carbohydrate polymeric barriers that lie under the cuticle. Although these experiments suggested that T-30 probably lacked the ability to produce all of the penetration enzymes, no direct evidence was obtained.

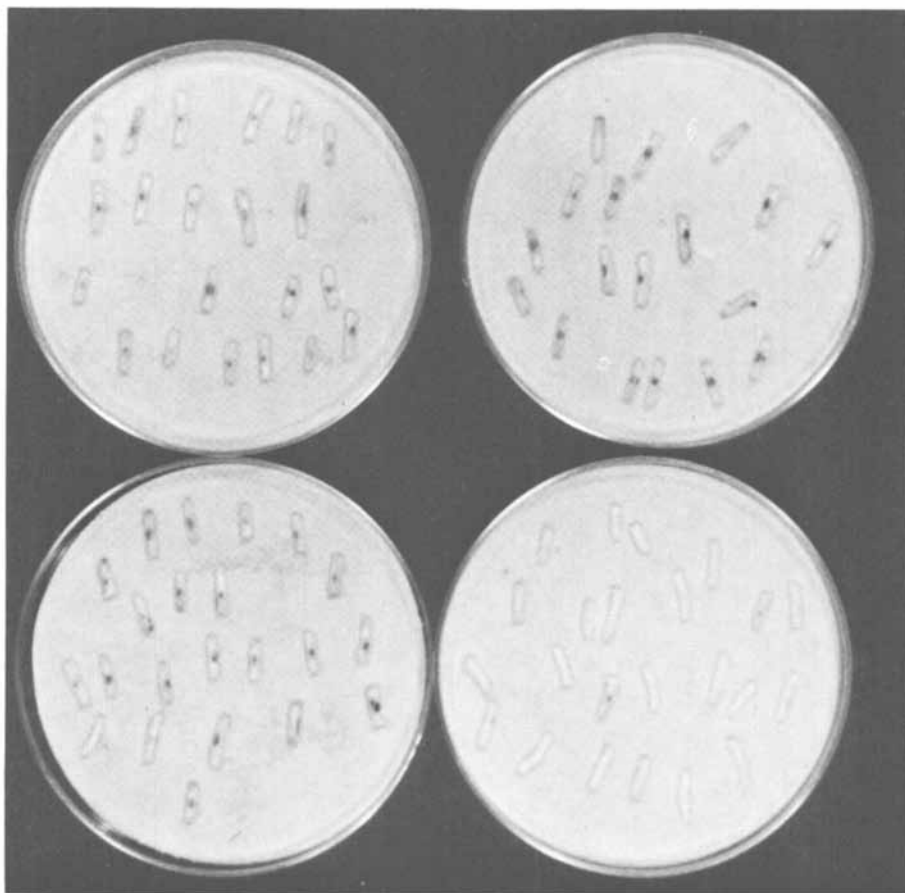


Figure 6. Pea epicotyl segments 72 h after inoculation with conidial suspension of *Fusarium solani pisi* isolate T-8 (left) and isolate T-30 (right). In each case the top and bottom show the results with mechanically breached and intact cuticle/wall barrier, respectively.



TABLE III  
Esterase and Cutinase Activity Released into the Extracellular Fluid During Spore Germination

Isolate	<u>2 h Germination</u>		<u>6 h Germination</u>		<u>24 h Germination</u>				
	Cutinase activity <sup>a</sup> (%)	PNBase activity <sup>b</sup> (%)	Cutinase activity <sup>a</sup> (%)	PNBase activity <sup>b</sup> (%)	Cutinase activity <sup>a</sup> (%)	PNBase activity <sup>b</sup> (%)			
T-8	26.4	50,500	3	132.0	2,610,000	12	153.3	3,020,000	77
T-30	3.0	2800	2	3.0	3100	10	4.6	3900	56

<sup>a</sup>PNB activity is expressed  $\text{nmol min}^{-1}$  per  $10^{10}$  spores using p-nitrophenylbutyrate as substrate.

<sup>b</sup>Cutinase activity is expressed as the amount of  $^3\text{H}$  ct  $\text{min}^{-1}$  released  $\text{h}^{-1}$  per  $10^{10}$  spores.

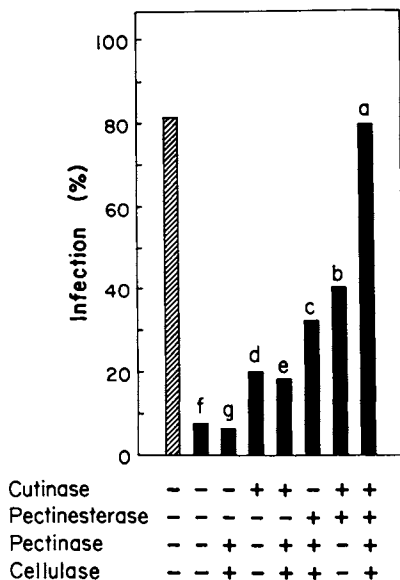


Figure 7. Infection efficiency of isolate T-30 conidia on pea epicotyl with intact cuticle/wall barrier after enzyme supplementation. A conidial suspension was mixed with (+) or without (-) indicated enzymes. The mixtures were used to inoculate intact (■) or wounded (▨) surfaces of pea epicotyl segments. Means followed by the same letter are not significantly different ( $P=0.05$ , Duncan's multiple range test). (Reproduced with permission from Ref. 34. Copyright 1982 Academic Press.)

Since the exogenous enzymes used to supplement the spore medium were from sources other than the pathogen, the particular kind of enzyme mixtures that were required to get effective fungal penetration cannot be taken as firm evidence for the involvement of such enzymes in a natural infection. For example, the requirement for pectin methylesterase might simply imply that the exogenous pectinase used worked more effectively in the presence of this methyl esterase. The nature of the enzymes produced by the pathogen to disrupt the wall barrier can be elucidated only by direct studies on the pathogen itself.

The enzymes used to break the cuticle/wall barriers might either be present in the spore that lands on the plant or these enzymes might be induced as a result of contact of the spore with the plant surface. Conclusive evidence was obtained that cutinase is induced in the spores as a result of contact with cutin (35). Thus, the presence of cutin was required for induction of the extracellular enzyme as measured by hydrolysis of p-nitrophenyl esters, or cutin as substrates, as well as by immunological techniques. The degree of induction of cutinase depended upon the amount of cutin added and the level of the enzyme activity increased with time and reached a plateau in a few hours. Cutin hydrolysate composed of all of the monomers also induced cutinase synthesis in the spores (Figure 8). Isolated monomers were also effective inducers. The most effective inducers were dihydroxy-C<sub>16</sub> acid and trihydroxy-C<sub>18</sub> acid, the most unique components of cutin. These results suggested that the low levels of cutinase present in the spore at the time of its arrival at the plant surface generated small amounts of cutin monomers from the plant surface and these monomers actually induced cutinase production.

To test whether the induction of the enzyme involved transcriptional control, the amount of mRNA produced by the spore was quantitated using <sup>32</sup>P-labeled cDNA as a probe (Figure 9). Such dot blot analysis showed that within 15 minutes after the spores came into contact with cutin or cutin monomers, cutinase gene transcripts were detectable and the level increased for the next few hours (35). These results are in agreement with the observation that the cutinase activity level increased with a slight lag period. The induction observed by the presence of cutin in the medium was highly stimulated by the addition of exogenous cutinase, strongly supporting the hypothesis that the small amount of cutinase originally present in the spore was responsible for producing cutin monomers which then induced the production of the enzyme required for penetration. The fact that the best inducers were the most characteristic cutin components, which are not found anywhere else in nature, shows that the induction mechanism is a highly specific method by which the fungus can sense the presence of the host plant and thus induce cutinase when needed for the penetration process.

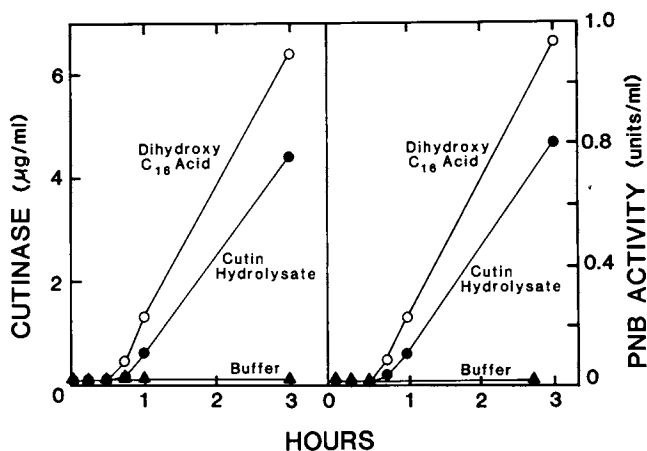


Figure 8. Time course of appearance of cutinase as measured by immunochemical techniques (left) and cutinase activity as measured by *p*-nitrophenyl butyrate hydrolysis (right) in the extracellular fluid of spore suspensions of *F. solani* f. sp. *pisi* induced with either cutin hydrolysate or purified C<sub>16</sub> dihydroxy acid. (Reproduced with permission from Ref. 35. Copyright 1986 The National Academy of Sciences.)

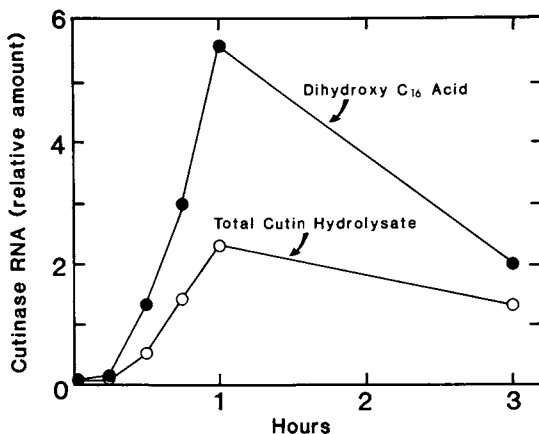


Figure 9. Cutinase mRNA content from spores of *F. solani* f. sp. *pisi* exposed to either cutin hydrolysate or purified dihydroxy-C<sub>16</sub> acid for various periods of time. Cutinase [<sup>32</sup>P]cDNA was used as a probe in the dot blot analysis. (Reproduced with permission from Ref. 35. Copyright 1986 The National Academy of Sciences.)

### Enzymatic Penetration of the Pectinaceous Barrier

The penetrating fungus comes into contact with the carbohydrate polymers when the germinating spore breaches the cutin barrier and these polymers then might trigger the synthesis of pectinases. In fact, when Fusarium solani pisi spores were placed in a pectin-containing medium pectinase production was induced (M.S. Crawford and P.E. Kolattukudy, unpublished results). The pectin hydrolase activity level reached a maximal level in about 8 hours after the spores came into contact with the pectin-containing medium and subsequently decreased (Figure 10). The isolate T-8 which was highly virulent even on intact pea stem showed a much higher level of pectin hydrolase when compared with isolate T-30, which was unable to penetrate intact stems. The maximal level of production of pectin hydrolase coincided with the onset of germination. Only much later a pectate lyase began to appear and in this case also T-8 showed a much higher level of the enzyme when compared to T-30. Indirect evidence suggested that the hydrolase, induced early during germination, produced components which induced the lyase. The pectate lyase from isolate T-8 has been purified to homogeneity and rabbit antibodies have been prepared. These antibodies protected pea stem sections against attack by Fusarium solani pisi under the bioassay conditions indicated above for cutinase. However, the protection in this case was not complete. Presumably the pectin hydrolase also plays an important role in the enzymatic penetration of the carbohydrate barrier that lies under the cuticle. However, this pectinase has not been purified and therefore definitive experiments about the importance of this enzyme have not been performed.

Since cutin is the first barrier that comes into contact with the fungal spore, it is tempting to speculate that the hydrolysis products generated from this polymer might also trigger the production of pectin hydrolase which in turn generates small molecular weight compounds which subsequently induce the synthesis of the lyase. Preliminary experiments have given indications that cutin and cutin hydrolysate do induce pectin hydrolase synthesis in spores. It is possible that penetration enzymes might be coordinately controlled and in the case of T-30 this control is being manifested by the lack of its ability to produce cutinase as well as high levels of the pectin hydrolyzing enzymes. In any case, the enzymes involved in breaching both the cuticular and the carbohydrate barriers might be used as targets to develop methods to prevent fungal penetration of plants and thus to prevent infection.

### Acknowledgments

This is Scientific Paper No. 7226, Project 2001, College of Agriculture Research Center, Washington State University, Pullman, WA 99164. This work was supported a grant PCM-8306835 from the National Science Foundation.

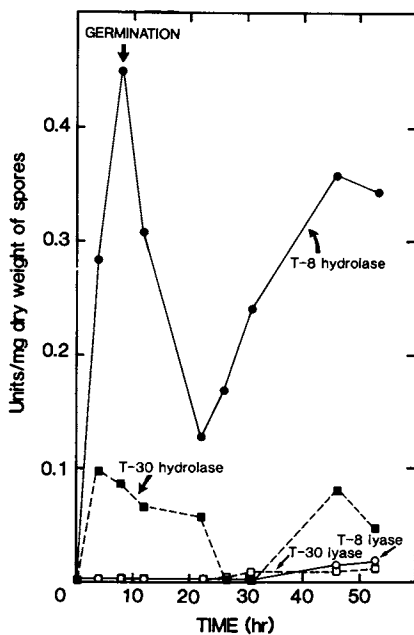


Figure 10. Pectin hydrolase and lyase activities released by spores of *F. solani* f. sp. *pisi* isolates T-8 and T-30, exposed to pectin containing medium. (Reproduced with permission. Copyright 1985 Allan R. Liss, Inc.)

Literature Cited

1. Kolattukudy, P.E. Science, 1980, 208, 990-1000.
2. Kolattukudy, P.E. Ann. Rev. Plant Physiol., 1981, 32, 540-567.
3. Kolattukudy, P.E. "The Biochemistry of Plants, Vol. 4;" Stumpf, P.K.; Ed; Academic Press: New York, 1980, Chapter 18.
4. Kolattukudy, P.E. Biochemistry, 1974, 13, 1354-1363.
5. Espelie, K.E.; Köller, W.; Kolattukudy, P.E. Chem. Phys. Lipids, 1983, 32, 13-26.
6. Kolattukudy, P.E. "The Structure, Biosynthesis and Degradation of Wood;" Loewus, F.A.; Runeckles, V.C.; Eds; Plenum Press, New York, 1977, Chapter 6
7. Agulló, C.; Collar, C.; Seoane, E. Phytochemistry, 1984, 23, 2059-2060.
8. Deas, A.H.B.; Holloway, P.J. "Lipids and Lipid Polymers in Higher Plants;" Tevini, M.; Lichtenthaler, H.K.; Eds; Springer-Verlag: New York, 1977, Chapter 16
9. Riley, R.G.; Kolattukudy, P.E. Plant Physiol., 1975, 56, 650-654.
10. Hunt, G.M.; Baker, E.A. Phytochemistry, 1980, 19, 1415-1419.
11. van den Ende, G.; Linskens, H.F. Ann. Rev. Phytopathol., 1974, 12, 247-258.
12. Kolattukudy, P.E. Ann. Rev. Phytopathol., 1985, 23, 223-250.
13. Aist, J.R. Encycl. Plant Physiol. New. Ser., 1976, 4, 197-221.
14. Dodman, R.L. "Plant Disease - An Advanced Treatise. Vol. 4. How Pathogens Induce Disease;" Horsefall, J.G.; Cowling, E.B.; Eds; Academic Press: New York, 1979, Chapter
15. Verhoeff, K. "The Biology of Botrytis;" Coley-Smith, J.R.; Verhoeff, K.; Eds; Academic Press, New York, 1980, Chapter
16. Kolattukudy, P.E. "Lipases;" Borgström, B.; Brockman, H.; Eds; Elsevier/North Holland Biomedical Press: Amsterdam, 1984, Chapter C.
17. Soliday, C.L.; Kolattukudy, P.E. Arch. Biochem. Biophys., 1976, 176, 334-343.
18. Dickman, M.B.; Patil, S.S.; Kolattukudy, P.E. Physiol. Plant Pathol., 1982, 20, 333-347.
19. Lin, T.S.; Kolattukudy, P.E. Physiol. Plant Pathol., 1980, 17, 1-15.
20. Lin, T.S.; Kolattukudy, P.E. Eur. J. Biochem., 1980, 106, 341-351.
21. Lin, T.S.; Kolattukudy, P.E. Biochem. Biophys. Res. Commun., 1977, 75, 87-93.
22. Köller, W.; Kolattukudy, P.E. Biochemistry, 1982, 21, 3083-3090.
23. Purdy, R.D.; Kolattukudy, P.E. Biochemistry, 1975, 14, 2832-2840.
24. Köller, W.; Allan, C.R.; Kolattukudy, P.E. Phytopathology, 1982, 72, 1425-1430.
25. Dickman, M.B.; Patil, S.S.; Kolattukudy, P.E. Phytopathology, 1983, 73, 1209-1214.

26. Carvalho, S.C.; Allan, C.R.; Kolattukudy, P.E. *Amer. Chem. Soc. 39th NW Regional Meeting*, 1984, Abst. 108.
27. Soliday, C.L.; Flurkey, W.H.; Okita, T.W.; Kolattukudy, P.E. *Proc. Natl. Acad. Sci. USA*, 1984, 81, 3939-3943.
28. Flurkey, H.W.; Kolattukudy, P.E. *Arch. Biochem. Biophys.* 1981, 212, 154-161.
29. Watson, E.E. *Nucl. Acids Res.* 1984, 12, 5145-5164.
30. Perlman, D.; Halvorson, H.O. *J. Mol. Biol.*, 1983, 166, 391-409.
31. Shaykh, M.; Soliday, C.L.; Kolattukudy, P.E. *Plant Physiol.*, 1977, 60, 170-172.
32. Maiti, I.B.; Kolattukudy, P.E. *Science*, 1979, 205, 507-508.
33. Chacko, R.; Kolattukudy, P.E., manuscript in preparation.
34. Köller, W.; Allen, C.R.; Kolattukudy, P.E. *Physiol. Plant Pathol.*, 1982, 20, 47-60.
35. Woloshuk, C.P.; Kolattukudy, P.E., manuscript in preparation.

RECEIVED September 4, 1986



## Chapter 11

# Variability in Steroid Metabolism Among Phytophagous Insects

James A. Svoboda and Malcolm J. Thompson

Insect Physiology Laboratory, Agricultural Research Service, U.S. Department of  
Agriculture, Beltsville, MD 20705

Members of the class Insecta require an exogenous source of sterol to support normal development and reproduction. Cholesterol will satisfy this need in nearly all species studied, but many phytophagous and omnivorous insects thrive on diets containing little or no cholesterol. Most of these species that have been critically examined are able to dealkylate and convert dietary 24-alkyl (C<sub>28</sub> and C<sub>29</sub>) phytosterols to cholesterol. However, significant variations in the utilization and metabolism of dietary sterols between phytophagous species have been discovered in recent years. Thus, it is becoming increasingly difficult to generalize about sterol metabolism even among members of the same Order. These differences in the utilization of neutral sterols can often be correlated with ecdysteroid (molting hormone) production. Certain of the most significant variations in insect sterol utilization and metabolism in phytophagous insects will be discussed with respect to phylogenetic relationships.

Since insects lack the capacity to biosynthesize the steroid nucleus, they generally require a dietary source of sterol for normal development and reproduction (1). This is an important area of biochemical difference, between insects and many other organisms, that might be exploited to develop new pest control strategies. Cholesterol will satisfy this dietary requirement in all but two known cases in which dietary  $\Delta^7$ -sterols are essential (2,3). In addition, some insects may obtain an adequate supply of sterol from symbionts or intestinal microorganisms.

For many years, it was believed that phytophagous insects in general were capable of dealkylating and converting dietary C<sub>28</sub> and C<sub>29</sub> phytosterols to cholesterol to satisfy their need for cholesterol (4). Also, a number of omnivorous species of insects are known to be capable of this conversion (5). Thus, cholesterol

This chapter not subject to U.S. copyright.  
Published 1987, American Chemical Society

can be made available for structural needs in membranes (1) and for essential physiological purposes such as serving as a precursor for the C<sub>27</sub> molting hormones (ecdysteroids, Figure 1), e.g. ecdysone (6).

It has become increasingly evident that considerable variability in steroid utilization and metabolism exists among phytophagous species of insects. In recent years, we have discovered several phytophagous species that are unable to convert C<sub>28</sub> or C<sub>29</sub> phytosterols to cholesterol. This includes one species that dealkylates the C-24 substituent of the side chain but produces mostly saturated sterols and several species that totally lack the ability to dealkylate the sterol side chain. Certain members of this latter group are of particular interest because they have adapted to utilizing a C<sub>28</sub> sterol as an ecdysteroid precursor and makisterone A (C<sub>28</sub>) has been identified as the major ecdysteroid of certain developmental stages of these species.

We will discuss some of our comparative sterol metabolism studies and provide specific examples to illustrate some of these unusual variations in steroid utilization and metabolism in insects, and to show how this information is useful in predicting differences in ecdysteroid biosynthesis in certain species. We will also point out instances in which these variations in neutral sterol metabolism can be related to phylogenetic relationships between species.

#### Phytophagous Insects That Convert C<sub>28</sub> and C<sub>29</sub> Phytosterols to Cholesterol

Lepidoptera. The most extensive studies of the utilization and metabolism of dietary sterols in phytophagous insects have been carried out with two Lepidoptera, the tobacco hornworm, Manduca sexta, in our laboratory (7) and the silkworm, Bombyx mori, by Ikekawa and coworkers in Japan (8). These were pioneering studies utilizing artificial diets, radiolabeled sterols, and state-of-the-art analytical tools. Manduca larvae readily convert C<sub>28</sub> and C<sub>29</sub> phytosterols (e.g. campesterol, sitosterol, and stigmasterol) to cholesterol (Figure 2) and desmosterol is the terminal intermediate in the conversion of each of these sterols to cholesterol (9). This was the first intermediate to be identified in the metabolic conversion of phytosterols to cholesterol in insects. Fucosterol was later determined to be an intermediate between sitosterol and desmosterol and, analogously, 24-methylenecholesterol was found to be the first intermediate between campesterol and cholesterol (4). Stigmasterol is dealkylated and converted to 5,22,24-cholestatrien-3 $\beta$ -ol which is reduced, first to desmosterol, and then to cholesterol (4). The  $\Delta^{24}$ -bond is necessary for enzyme specificity in order to reduce the  $\Delta^{22}$ -bond. Results from research in our laboratory also indicate that other Lepidoptera such as the corn earworm, Heliothis zea, the fall armyworm, Spodoptera frugiperda (10), and the Indian meal moth, Plodia interpunctella, (11) metabolize C<sub>28</sub> and C<sub>29</sub> phytosterols in a manner similar to Manduca. These pathways have been shown to be similar in B. mori and, in addition, fucosterol 24,28-epoxide has been identified as an intermediate between fucosterol and desmosterol in B. mori (8).

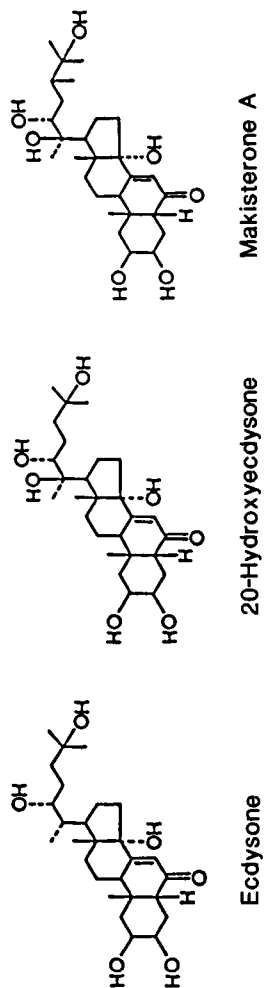


Figure 1. Ecdysteroid structures.

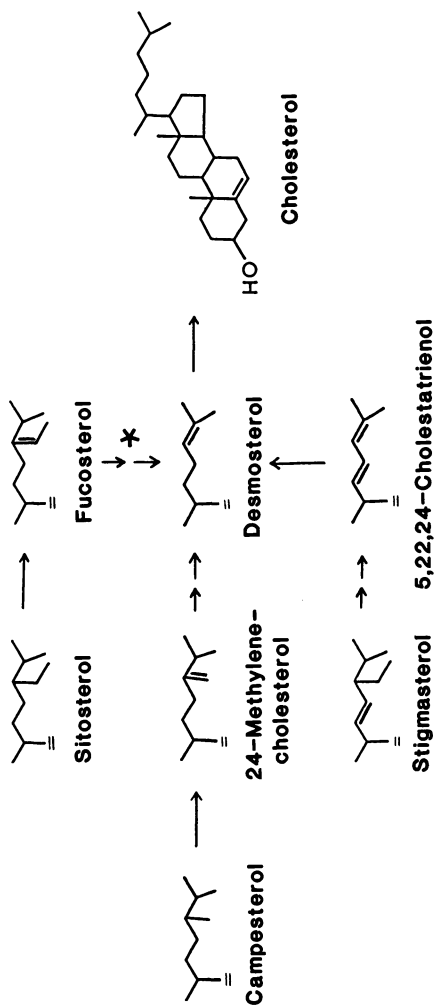


Figure 2. Pathways of conversion of C-24 alkyl sterols to cholesterol in the tobacco hornworm and other phytophagous insects. \*Fucosterol 24,28-epoxide has been shown to be an intermediate between fucosterol and desmosterol in Bombyx mori and Tenebrio molitor.

Coleoptera. The confused flour beetle, Tribolium confusum, was the first phytophagous insect we found that produces an appreciable amount of a sterol other than cholesterol from radiolabeled dietary C<sub>28</sub> and C<sub>29</sub> phytosterols. We found this insect produced large quantities of 7-dehydrocholesterol, equivalent to as much as 70% of the total tissue sterols isolated (12). It was further determined that cholesterol and 7-dehydrocholesterol were in equilibrium in this flour beetle. Another new intermediate, 5,7,24-cholestatrien-3 $\beta$ -ol was identified as an intermediate between desmosterol and 7-dehydrocholesterol (Figure 3). We found very similar pathways of sterol metabolism to exist in the closely related flour beetle, Tribolium castaneum (13). However, another flour beetle, Tenebrio molitor, had only about one-third or less of the levels of 7-dehydrocholesterol as the two Tribolium species, but still much higher levels of this sterol than has been found in most species. Fucosterol 24,28-epoxide was also implicated as an intermediate in the synthesis of cholesterol from sitosterol in T. molitor (14).

A very unique mixture of sterols was found in the Mexican bean beetle, Epilachna varivestis, when sterols from insects fed soybean leaves were analyzed (15). The sterols from bean beetle pupae consisted of >70% saturated sterols and cholesterol was the major sterol isolated from the insect. Metabolic studies with radiolabeled sterols demonstrated that the Mexican bean beetle does dealkylate C<sub>28</sub> and C<sub>29</sub> phytosterols, but reduces the  $\Delta^5$ -bond first (Figure 4)(16). In addition, the  $\Delta^7$ -bond can be incorporated into cholesterol, and thus appreciable amounts (>10% of the total sterols) of lathosterol ( $\Delta^7$ -cholestenol) occur in the sterols of this species. Recent studies (13) have confirmed that in the metabolism of sitosterol and stigmasterol, the side chain dealkylation and conversion to a 24-desalkyl side chain in the Mexican bean beetle parallels the mechanism in Manduca. A  $\Delta^{24}$ -sterol is involved as a terminal intermediate in the metabolism of both sterols, and the side chain of stigmasterol is first dealkylated to form a  $\Delta^{22,24}$ -intermediate, and then the  $\Delta^{22}$ -bond is reduced.

#### Phytophagous Insects Unable to Convert C<sub>28</sub> and C<sub>29</sub> Phytosterols to Cholesterol

Hemiptera. The large milkweed bug, Oncopeltus fasciatus, was the first phytophagous insect discovered to be incapable of converting the major phytosterols (C<sub>28</sub> and C<sub>29</sub>) to cholesterol (17). Dietary sterols of sunflower seeds were incorporated essentially unchanged into the tissues and, when injected, neither radiolabeled campesterol nor sitosterol was metabolized to cholesterol. Apparently, there was some selective uptake of dietary cholesterol, indicated by an enrichment of cholesterol in the insect sterols compared to the cholesterol concentration in the seed sterols. A C<sub>28</sub> ecdysteroid, makisterone A (Figure 1), was identified as the major ecdysteroid of milkweed bug eggs (18) and, subsequently, makisterone A was identified as the major ecdysteroid in hemolymph of last stage milkweed bug nymphs and two other phytophagous species of the Pentatomomorpha group of Hemiptera (19). Makisterone A was

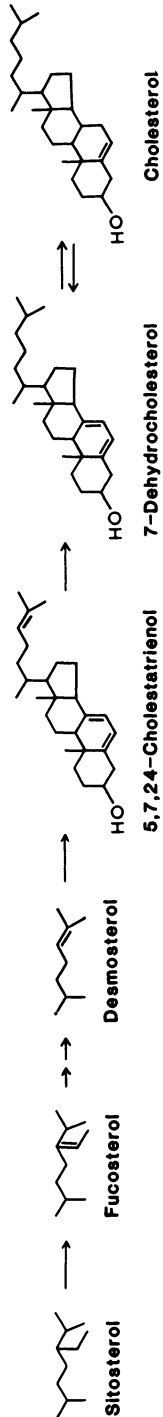


Figure 3. Dealkylation and conversion of sitosterol to 7-dehydrocholesterol and cholesterol in Tribolium confusum.

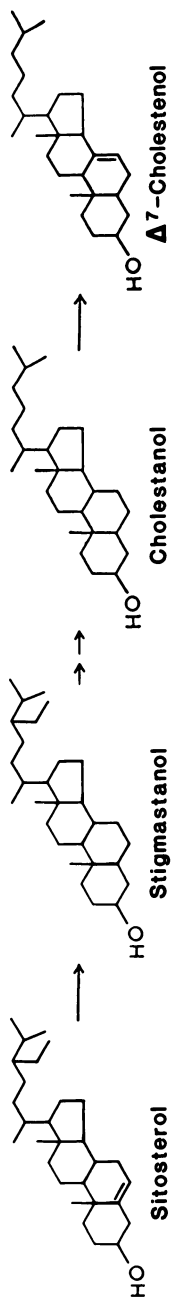


Figure 4. Pathway of conversion of C-24 alky1 sterols to cholestanol and  $\Delta^7$ -cholestenol in the Mexican bean beetle.

also ca. 10 times more active than 20-hydroxyecdysone in stimulating cuticle synthesis and inhibiting vitellogenesis in adult milkweed bugs (20). It was later determined that this adaptation to utilizing a C<sub>28</sub> precursor for ecdysteroid biosynthesis correlated well with neutral sterol metabolism in each of these three species (21). Similar results on sterol utilization and occurrence of makisterone A have been reported for the cotton stainer bug, *Dysdercus fasciatus* (22). These species all have in common the inability to dealkylate the C-24 alkyl substituents of C<sub>28</sub> and C<sub>29</sub> phytosterols.

Coleoptera. Sterol metabolism studies with another important stored products pest, the khapra beetle, *Trogoderma granarium*, revealed another phytophagous insect that is unable to dealkylate and convert C<sub>28</sub> and C<sub>29</sub> phytosterols to cholesterol (23). Similar results were obtained whether a diet consisting of cracked wheat and brewer's yeast or an artificial diet coated with radiolabeled sterols was used (24). There was some selective uptake of cholesterol from the dietary sterols, as indicated by an enrichment of cholesterol in the pupal sterols (1.2% of total), compared to the dietary sterols (0.5% of total). Unlike the previously discussed stored product coleopteran pests, *T. confusum* and *T. castaneum*, both of which had high levels of 7-dehydrocholesterol, no 7-dehydrocholesterol could be identified in the sterols from the khapra beetle.

Hymenoptera. While examining the effects of various dietary sterols on brood production in honey bees, *Apis mellifera*, we discovered that the honey bee utilized dietary C<sub>28</sub> and C<sub>29</sub> phytosterols unchanged (25,26). Regardless of the dietary sterol added to a chemically-defined diet, or even with no sterol added, 24-methylenecholesterol was always the major sterol of the next generation of bees, and sitosterol and isofucosterol were also present in appreciable amounts. Detailed studies with either radiolabeled campesterol, sitosterol, or 24-methylenecholesterol added to the artificial diet provided no evidence for the metabolism of any of these phytosterols to cholesterol or other sterols (27). In fact, <sup>3</sup>H-24-methylenecholesterol has been traced unchanged through two generations of bees (28). Thus, there is a very unusual mechanism that enables the worker bee to selectively transfer certain dietary sterols or sterols cycled from their endogenous pools to the brood food to maintain a constant supply of certain sterols for the brood food. The utilization of neutral sterols by the honey bee and the inability to produce cholesterol from the dealkylation of 24-alkyl C<sub>28</sub> and C<sub>29</sub> phytosterols is reflected in the recent isolation of makisterone A as the major ecdysteroid at peak titer in the honey bee pupa (29).

We have also found another phytophagous hymenopteran, the alfalfa leafcutter bee, *Megachile rotundata*, that utilizes dietary phytosterols similarly to the honey bee (30). As in the honey bee, 24-methylenecholesterol was a major component (34.1% of the total sterols) of the sterols of newly-emerged adults. In addition, there was little cholesterol (<0.5% of the total sterols), indicating that this species also lacks the ability to produce cholesterol from



dietary C<sub>28</sub> and C<sub>29</sub> sterols. The alfalfa leafcutter bee, unlike the honey bee, is a solitary bee but the cells in which the eggs are deposited are provisioned with honey and pollen before sealing. Thus, the larval diet of this species is similar to a major portion of the natural diet of developing honey bee larvae.

### Discussion and Conclusions

We have discovered that a number of interesting phylogenetic relationships can be correlated with steroid metabolism in several insect species. Certain phytophagous Hemiptera apparently successfully adapted to the disappearance of C<sub>27</sub> dietary sterols in tracheophytes by developing the capacity to utilize a C<sub>28</sub> precursor (e.g. campesterol) in the synthesis of ecdysteroids. It is of interest to note that a predacious hemipteran, the spined soldier bug, *Podisus maculiventris*, which belongs to the same group (Pentatomomorpha) as the phytophagous Hemiptera mentioned above, has retained the ecdysteroid synthesizing capabilities of its phytophagous ancestors. Makisterone A is the major hemolymph ecdysteroid of last stage soldier bug nymphs (19) even though their diet consists of other insects and contains high levels of cholesterol (21).

The khapra beetle, *T. granarium*, inhabits an environment more similar to that of the confused flour beetle than to the usual environment of other members of the family Dermestidae to which the khapra beetle belongs. However, with respect to utilization of dietary sterols, the khapra beetle is more similar to other dermestids such as the hide beetle, *Dermestes maculatus*, (31) which usually feeds on animals or animal products rather than plant derived material. Apparently, the khapra beetle has not had to alter its sterol metabolism to adapt to living in its stored product environment. It will be of interest to examine the ecdysteroids of the khapra beetle to see if the synthesis of molting hormones in this species has been modified to utilize other than a C<sub>27</sub> sterol as an ecdysteroid precursor.

The significance of the relatively high concentrations of 7-dehydrocholesterol in the *Tribolium* species we have studied remains a matter of conjecture. It may be due to some physiological adaptation to its normal environment, but the Indian meal moth inhabits a similar environment and still metabolizes dietary sterols more similarly to green leaf-feeding species of Lepidoptera than to these flour beetles. The only known physiological function of 7-dehydrocholesterol is as a precursor for ecdysone (32), but it may have some specific structural role as well in these insects which produce high levels of this sterol.

The Mexican bean beetle is another unusual coleopteran with respect to sterol metabolism. It is a member of the family Coccinellidae which includes mostly predacious species, that usually obtain adequate cholesterol from their diet of other insects. However, the subfamily Epilachninae to which the Mexican bean beetle belongs is made up of phytophagous species. Members of this subfamily are apparently secondarily phytophagous and have adapted to utilizing a diet containing high levels of the usual C<sub>28</sub> and C<sub>29</sub> phytosterols, but produce mostly saturated sterols and

$\Delta^7$ -sterols rather than cholesterol. We know of no other leaf-feeding insect with similar sterol metabolizing capabilities.

The honey bee has provided some most interesting data on steroid utilization and metabolism. Finding a hymenopteran that is unable to dealkylate C-24 alkyl sterols indicated that at least three orders of insects include phytophagous members that lack this capability (Coleoptera, Hemiptera, and Hymenoptera). Since the honey bee evolved from predacious ancestors, it apparently has modified its ecdysteroid biosynthetic capability but not its ability to modify the side chain of neutral sterols. The efficient selective transfer mechanism ensures that an adequate supply of C<sub>28</sub> sterol is available for ecdysteroid production. It will be of interest to determine whether the normal precursor of makisterone A is campesterol or some other 28-carbon sterol. The recent studies with the alfalfa leafcutter bee suggest that there may be a number of phytophagous Hymenoptera that utilize a C<sub>28</sub> molting hormone rather than the usual C<sub>27</sub> ecdysteroids such as ecdysone and 20-hydroxyecdysone. Certain of these species will be subjects of a future study. Also, the report of a hymenopteran, Atta cephalotes isthmicola, in which no cholesterol could be identified indicates that unusual ecdysteroids may be important to yet another insect (33).

Although this discussion has dealt only with some unusual variations in sterol utilization and metabolism among phytophagous insects, similar reviews of this area of insect biochemistry in zoophagous and omnivorous species could include equally interesting information. Considering the small fraction of the more than one million identified species of the class Insecta that have been investigated, there will undoubtedly be many more discoveries of unique aspects of steroid biochemistry in insects.

#### Literature Cited

1. Clayton, R. B. J. Lipid Res. 1964, 5, 3-19.
2. Heed, W. B.; Kircher, H. W. Science 1965, 149, 758-761.
3. Chu, H. M.; Norris, D. M.; Kok, L. T. J. Insect Physiol. 1970, 16, 1379-1387.
4. Svoboda, J. A.; Kaplanis, J. N.; Robbins, W. E.; Thompson, M. J. Annu. Rev. Entomol. 1975, 20, 205-220.
5. Robbins, W. E.; Kaplanis, J. N.; Svoboda, J. A.; Thompson, M. J. Annu. Rev. Entomol. 1971, 16, 53-72.
6. Karlson, P.; Hoffmeister, H. Z. Physiol. Chem. 1963, 331, 298-300.
7. Svoboda, J. A.; Thompson, M. J.; Robbins, W. E.; Kaplanis, J. N. Lipids 1978, 13, 742-753.
8. Ikekawa, N. Experientia 1983, 39, 466-472.
9. Svoboda, J. A.; Thompson, M. J.; Robbins, W. E. Life Sci. 1967, 6, 395-404.
10. Svoboda, J. A.; Robbins, W. E. Lipids 1971, 6, 113-9.
11. Cohen, C. F.; Svoboda, J. A.; Thompson, M. J. Southwest. Entomol. 1983, 8, 57-60.
12. Svoboda, J. A.; Robbins, W. E.; Cohen, C. F.; Shortino, T. J. In "Insect and Mite Nutrition"; Rodriguez, J. G., Ed.; Amsterdam: North-Holland, 1972; pp. 505-516.

13. Svoboda, J. A. and Cohen, C. F., unpublished data.
14. Nicotra, F.; Pizzi, P.; Ronchetti, F.; Russo, G.; Toma, L. J. Chem. Soc. Perkin Trans. 1981, 480-3.
15. Svoboda, J. A.; Thompson, M. J.; Elden, T. C.; Robbins, W. E. Lipids 1974, 9, 752-5.
16. Svoboda, J. A.; Thompson, M. J.; Robbins, W. E.; Elden, T. C. Lipids 1975, 10, 524-7.
17. Svoboda, J. A.; Dutky, S. R.; Robbins, W. E.; Kaplanis, J. N. Lipids 1977, 12, 318-321.
18. Kaplanis, J. N.; Dutky, S. R.; Robbins, W. E.; Thompson, M. J.; Lindquist, E. L.; Horn, D. H. S.; Galbraith, M. N. Science 1975, 190, 681-2.
19. Aldrich, J. R.; Kelly, T. J.; Woods, C. W. J. Insect Physiol. 1982, 28, 857-861.
20. Aldrich, J. R.; Svoboda, J. A.; Thompson, M. J. J. Exp. Zool. 1981, 218, 133-7.
21. Svoboda, J. A.; Lusby, W. R.; Aldrich, J. R. Arch. Insect Biochem. Physiol. 1984, 1, 139-145.
22. Gibson, J. M.; Majumder, M. S. I.; Mendis, A. H. W.; Rees, H. H. Arch. Insect Biochem. Physiol. 1983, 1, 105-120.
23. Svoboda, J. A.; Nair, A. M. G.; Agarwal, N.; Agarwal, H. E.; Robbins, W. E. Experientia 1979, 35, 1454-5.
24. Svoboda, J. A.; Nair, A. M. G.; Agarwal, N.; Robbins, W. E. Experientia 1980, 36, 1029-1030.
25. Herbert, E. W. Jr; Svoboda, J. A.; Thompson, M. J.; Shimanuki, H. J. Insect Physiol. 1980, 26, 287-9.
26. Svoboda, J. A.; Thompson, M. J.; Herbert, E. W. Jr.; Shimanuki, H. J. Insect Physiol. 1980, 26, 291-4.
27. Svoboda, J. A.; Herbert, E. W. Jr.; Thompson, M. J.; Shimanuki, H. J. Insect Physiol. 1981, 27, 183-8.
28. Svoboda, J. A.; Thompson, M. J.; Herbert, E. W. Jr.; Shortino, T. J.; Szczepanik-VanLeeuwen, P. A. Lipids 1982, 17, 220-5.
29. Feldlaufer, M. F.; Herbert, E. W. Jr.; Svoboda, J. A.; Thompson, M. J.; Lusby, W. R. Insect Biochem. 1985, In press.
30. Svoboda, J. A.; Lusby, W. R. Arch. Insect Biochem. Physiol. 1985, In press.
31. Clark, A. J.; Block, K. J. Biol. Chem. 1959, 234, 2583-8.
32. Galbraith, M. N.; Horn, D. H. S. Chem. Commun. 1970, 3, 179-180.
33. Ritter, K. S.; Weiss, B. A.; Norrbom, A. L.; Nes, W. R. Comp. Biochem. Physiol. 1982, 71B, 345-9.

RECEIVED January 10, 1986

## Chapter 12

# Free and Conjugated Ecdysteroids in the Tobacco Hornworm, *Manduca sexta*, at Various Developmental Stages

Malcolm J. Thompson, Ruben Lozano, James A. Svoboda, Mark F. Feldlaufer, and  
Gunter F. Weirich

Insect Physiology Laboratory, Agricultural Research Service, U.S. Department of  
Agriculture, Beltsville, MD 20705

The molting hormones (MHs) and ecdysteroids of the tobacco hornworm (*Manduca sexta* L.) at different developmental stages are discussed. From this insect nine ecdysteroids have been identified and each has varying degrees of MH activity. There are also qualitative and quantitative differences at the various stages. 20-Hydroxyecdysone is the major ecdysteroid of *Manduca* during pupal-adult development at peak titer of MH activity. Five days later, 20,26-dihydroxyecdysone is the major ecdysteroid. Meconium of *Manduca* contains 3-epi-20-hydroxyecdysone as the major ecdysteroid. In *Manduca* ovaries and eggs, 26-hydroxyecdysone 26-phosphate is the major ecdysteroid occurring at levels of 31 µg/g in ovaries, 25 µg/g and 17 µg/g in 48- to 64- and 72- to 88-hour-old eggs, respectively. Female pupae at peak titer have a complex mixture of conjugates which account for nearly 18% of the total ecdysteroids. Ecdysteroids can now be identified and their fate followed in *Manduca* at every developmental stage.

The oligophagous tobacco hornworm, *Manduca sexta* (L.), has been our primary insect in studies concerned with molting hormones (MH) and ecdysteroids (compounds with a common structure and inclusive of roles other than molting) (1). *Manduca*, because it has about a six week life cycle and can be laboratory-reared on an artificial diet, has permitted us to study different developmental stages within a reasonable period of time. As a result, nine ecdysteroids including an ecdysteroid conjugate (Fig. 1) have been isolated and identified from various developmental stages of the hornworm, and seven of them were first characterized in our laboratory. It also has allowed us to isolate sufficient quantities of the ecdysteroids so that others could be provided with standard samples.

With the development of new and improved instrumentation, techniques, the availability of ecdysteroid standards, together with knowledge acquired on the biosynthesis and metabolism of

This chapter not subject to U.S. copyright.  
Published 1987, American Chemical Society

ecdysteroids of *Manduca* and insects in general, we are now able to identify and follow the fate of ecdysteroids and their conjugates at every developmental stage of the tobacco hornworm. Thus, current methodology combined with the efficient conversion of radiolabeled cholesterol to labeled ecdysteroids or ecdysteroid conjugates in *Manduca* at different developmental stages has permitted us to work with far less biological material and derive more useful information. In this symposium paper we will discuss the ecdysteroids of the tobacco hornworm at different developmental stages, and will report on some of our most recent studies concerning the fate of labeled cholesterol, ecdysteroids, and ecdysteroid conjugates at two different developmental stages of *Manduca*.

Since our initial and most recent studies of MHs of *Manduca* focused on the pupal-adult development period, the discussion will begin with this period and then proceed in reverse sequence to the immature stages of *Manduca* development.

### Pupal-adult

To insure maximum yield of molting hormone(s) from a minimum quantity of biological material, the MH titer was determined prior to isolation of the MHs (2). The peak titer of MH activity occurred between 6 and 8 days after the larval-pupal ecdysis or 14 days prior to adult emergence. Thus, 7-day-old hornworm pupae were selected for the isolation of the MHs (2). The average titer was 425 house fly units (HFU; for ecdysone 5.0 ng = 1 HFU) or 2 µg of ecdysone per gram of tissue, the highest titer reported up to that time for any species. From kilogram quantities of pupae milligram quantities of crystalline ecdysone and 20-hydroxyecdysone were obtained, and a small but significant amount of a more polar ecdysteroid was detected. 20-Hydroxyecdysone accounted for 60 to 80% of the total ecdysteroids in 7-day-old pupae (2).

After processing a larger group of 7-day-old pupae the more polar ecdysteroid was isolated and identified as 20,26-dihydroxyecdysone (3). This ecdysteroid was only 1/10 to 1/15 as biologically active as ecdysone or 20-hydroxyecdysone in the house fly assay. The structures and biological activity of the ecdysteroids indicated the sequence of conversion shown in [1]



The fact that 20,26-dihydroxyecdysone was less active than ecdysone suggests that it was an inactivation product and that we could expect an increase in the quantity of this compound at a later period of pupal-adult development. Indeed, 20,26-dihydroxyecdysone was the major ecdysteroid during pupal-adult development five days after peak titer of MH activity, followed by lesser amounts of 3-epi-20-hydroxyecdysone, 20-hydroxyecdysone, 3-epi-20,26-dihydroxyecdysone, 3-epiecdysone and ecdysone (4). While the increased concentration of 20,26-dihydroxyecdysone was anticipated as a result of 20-hydroxyecdysone metabolism, it cannot be ruled out that 20,26-dihydroxyecdysone has a physiological role of its own in insect development (3). This assumption was supported



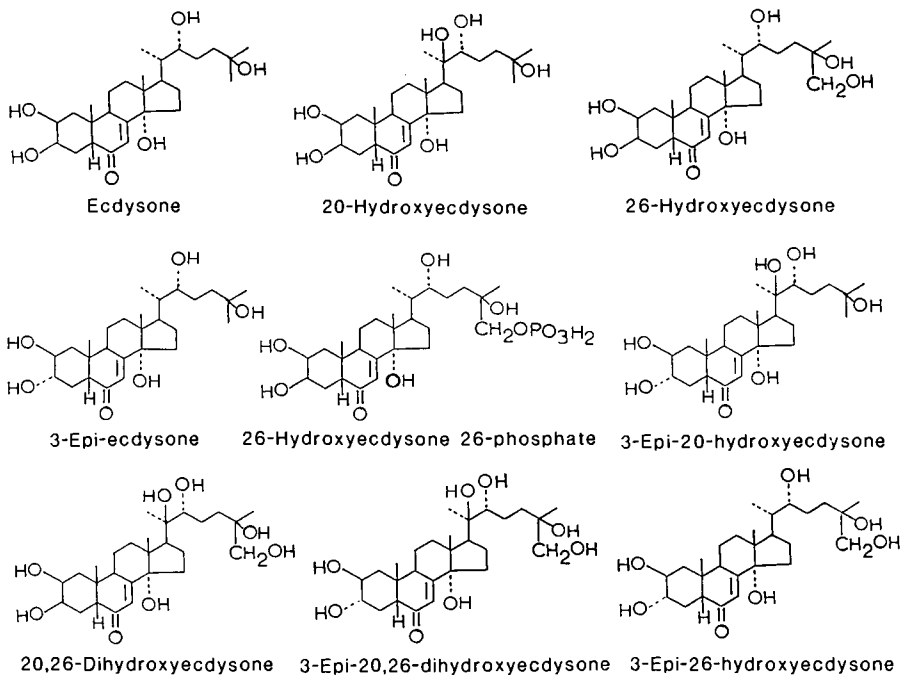


Figure 1. Ecdysteroids isolated from Manduca.

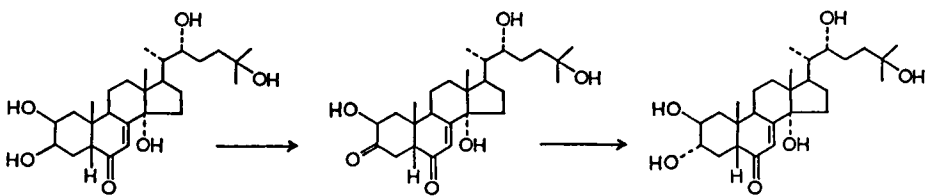


Figure 2. Metabolic scheme for the production of 3-epiecdysone.

the middle and immediately placed in and stored in methanol at  $-20^{\circ}\text{C}$  until work-up.

The analyses thus far have only been conducted with female pupae and are incomplete and the scheme of isolation is shown in Figure 3. The butanol phase which contained the free ecdysteroids was further fractionated by counter-current distribution (CCD) [60 transfers in a system of cyclohexane-butanol-water (4:6:10) with 10 ml of each phase]. The material from CCD tubes 23-42 was subjected to three C<sub>18</sub> SEP-PAK fractionations (13). Quantitative RP-HPLC and radioassay analyses of fractions 4 and 5 resulting from using a SEP-PAK elution system as in Figure 3 yielded 200  $\mu\text{g}$  of labeled 20-hydroxyecdysone. The combined residues from CCD tubes 43-57 when fractionated over a Florisil SEP-PAK cartridge and chromatographed over 4 g of Silica gel yielded 22  $\mu\text{g}$  of labeled ecdysone.

After applying the 3.74 g of residue from the aqueous phase to the XAD-16 column better than 92% of the impurities were removed from the column with water (Fig. 3). The SEP-PAK fractionation removed additional impurities. A refractionation via SEP-PAK of combined SEP-PAK fractions 3-5 (Fig. 3) followed by ion suppression RP-HPLC and radioassay analyses of 2% of the most radioactive fraction having the least amount of mass gave the chromatogram shown in Figure 4. The peaks eluting between 5 to 7 min and the shoulder at 11.0 min as well as the peak at 13.3 min were highly radioactive. Thus, female THW pupae at peak titer have a complex mixture of conjugates which accounts for nearly 18% of the total radioactive ecdysteroids. HP-TLC and RP-HPLC analyses indicate that the conjugates are phosphates. Also in our Laboratory, recent incubation studies with high-speed supernatant of *Manduca* midgut homogenates showed that [<sup>3</sup>H]ecdysone was converted to 3-[<sup>3</sup>H]epiecdysone and tritium-labeled highly polar metabolite (14). From the highly polar metabolite fraction four ecdysone conjugates and two epiecdysone conjugates were resolved by ion-pair high-performance liquid chromatography. These ecdysteroid conjugates were demonstrated to be phosphates. The studies further demonstrated that midgut cytosol contains several ATP:ecdysteroid phosphotransferases (14).

Before we can begin to understand the fate and the physiological significances of free and conjugated ecdysteroids and their interrelationship in the tobacco hornworm during pupal-adult development, we need to continue to isolate and identify the labeled free and conjugated ecdysteroids of both male and female pupae before and after peak titer of MH activity. Even though the task appears to be a tedious and difficult one, the progress will be rapid.

### Meconium Fluid

The meconium, which serves as a depository of metabolic waste products, was collected from tobacco hornworms of mixed sexes shortly before eclosion and analyzed for its ecdysteroid content. 3-Epi-20-hydroxyecdysone was the major ecdysteroid isolated in crystalline form, followed by 20-hydroxyecdysone and what appeared to be 3-epiecdysone (15). 3-Epiecdysone was also identified as the product of an *in vitro* enzyme system from *Manduca* midgut. These two



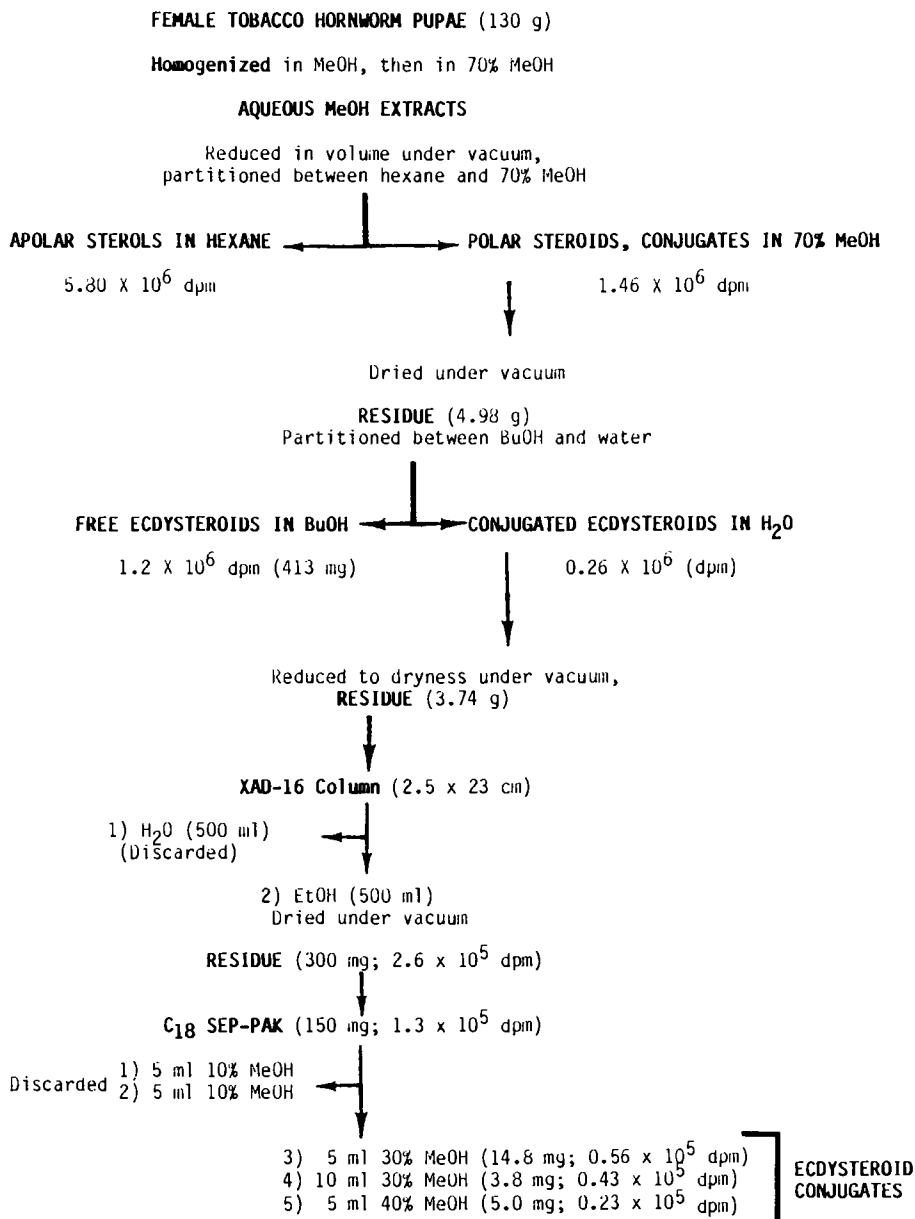


Figure 3. Procedure used for the isolation of ecdysteroid conjugates from Manduca pupae.

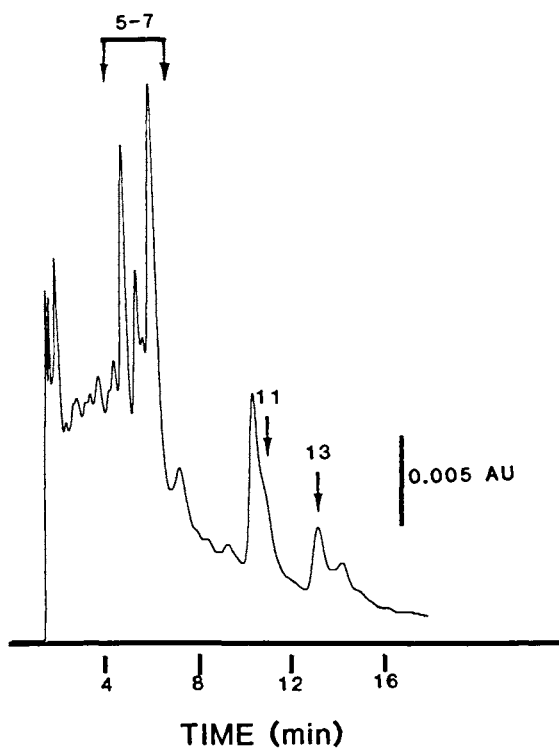


Figure 4. Ion suppression reversed-phase HPLC trace indicating radioactive peaks of partially purified ecdysteroid conjugates from 8-day-old *Manduca* pupae, on IBM C<sub>8</sub> column (4.6 mm x 15 cm) by isocratic elution with 30% methanol in 0.03 M aqueous NaH<sub>2</sub>PO<sub>4</sub> solution (pH 5) at flow rate of 0.8 ml/min.

lines of research led to the first isolation and identification of  $3\alpha$ -ecdysteroids. Since the first sterol conjugate from an insect source was isolated from the meconium of the tobacco hornworm (16), we are currently conducting qualitative and quantitative comparative studies of free and conjugated ecdysteroids of the meconium of male and female pupae injected with [ $^{14}\text{C}$ ]cholesterol on day 13.

#### Embryonated Eggs of the Hornworm

Young eggs, 1- to 4-hour-old contain negligible MH activity (17), whereas older embryonated eggs, 24- to 44-hour-old (18) or 48- to 64-hour-old (17), have relatively high molting hormone titer.

In both the 24- to 44-hour-group and 48- to 64-hour-group, 26-hydroxyecdysone was the major ecdysteroid accounting for nearly 80% of the total free ecdysteroids, whereas the three MH's isolated during pupal-adult development, ecdysone, 20-hydroxyecdysone, and 20,26-dihydroxyecdysone, were included in the remaining 20% of the ecdysteroid of eggs. 26-Hydroxyecdysone was also the major ecdysteroid in younger embryonated eggs (4- to 18-hour-old) accounting for about 90% of the total ecdysteroids (19).

In addition, ecdysone, 20-hydroxyecdysone, and 20,26-dihydroxyecdysone were also present in 4- to 18-hour old eggs. These accounted for all of the MH activity though comprising less than 2% of the total recovered ecdysteroids. Interestingly, 26-hydroxyecdysone was shown to be devoid of MH activity in the house fly assay and ecdysone was the major MH-active ecdysteroid in hornworm eggs (19).

In addition to the  $3\beta$ -ecdysteroids, two  $3\alpha$ -ecdysteroids, 3-epi-26-hydroxyecdysone, and 3-epi-20,26-dihydroxyecdysone were isolated and identified from 4- to 18-hour-old eggs. 3-Epi-26-hydroxyecdysone comprised 3% of the total recovered ecdysteroids and was the second major component isolated from this groups of eggs. Thus, from these eggs, six ecdysteroids were conclusively or tentatively identified and six other unidentified ecdysteroids were isolated.

In our recent studies concerned with ecdysteroids in developing ovaries and eggs of the THW (20), and the fate of radiolabeled steroids in ovaries and eggs of the THW (12), we encountered some interesting and conflicting results. In Table I we show that in 1- to 18-hour-old eggs more than 63% of the ecdysteroid existed in the free form. The proportion was similar in 48- to 64-hour-old eggs, though the sum of the free and conjugated ecdysteroid was far less in 48- to 64-hour-old eggs. In our latest study, we showed that following injection into *Manduca* female pupae (day 16), [ $^{14}\text{C}$ ]cholesterol was converted to labeled ecdysteroid conjugates (12), of which the latter is mainly 26-hydroxyecdysone 26-phosphate (11). In this study, however, 48- to 64-hour-old or 72- to 88-hour-old eggs were found to contain little if any free ecdysteroids (Table II). The absence of free ecdysteroids in these egg groups was unexpected and completely different from results of our earlier studies (20). In our search for an explanation for the differences between the results of this study and those of our earlier studies (20), we determined that, in this study, we had

Table I. Titer of Free and Conjugated Ecdysteroids of Ovaries and Eggs at Various Developmental Stages of the Tobacco Hornworm (20)

	Free Ecdysteroids µg/g fresh weight*	Conjugated Ecdysteroids µg/g fresh weight**
<u>Ovaries** from 93-hour-old</u>		
<u>Adult Females</u>		
Ecdysone	not detected	0.58
26-Hydroxyecdysone	not detected	20.20
<u>0- to 1-hour-old Eggs**</u>		
Ecdysone	not detected	0.73
26-Hydroxyecdysone	0.96	21.00
<u>1- to 18-hour-old Eggs**</u>		
Ecdysone	0.38	0.22
26-Hydroxyecdysone	13.40	7.80
<u>48- to 64-hour-old Eggs**</u>		
Ecdysone	0.02	not detected
26-Hydroxyecdysone	6.90	6.0

\*Determined by RP-HPLC

†Expressed as Free Ecdysteroids Recovered After Enzymatic Hydrolysis of Conjugates

\*\*Tissues Stored in MeOH AT -20°C

\*\*Tissues Stored at -20°C

immediately placed and stored all biological material in methanol at -20°C (Table II). Previously, this was done only with ovaries and 0- to 1-hour-old eggs. Older eggs were routinely placed in glass bottles and kept at -20°C until work-up. Perhaps certain phosphatases of these eggs were activated by the lowering of the temperature and subsequent hydrolysis of the ecdysteroid conjugates caused an accumulation of free ecdysteroids. There could also have been a sudden burst of enzyme activity at a critical temperature during cooling of the eggs to -20°C. Undoubtedly, the low temperature destroyed the normal embryonic developmental processes, but did not eliminate this hydrolytic activity. Efforts are in progress to determine the exact conditions that cause the hydrolysis. We are also accumulating large quantities of eggs (1- to 18-hour-old), stored in methanol, from which the free (if any) and conjugated ecdysteroids will be isolated. The conjugates will then be enzymatically hydrolyzed to determine if the six ecdysteroids previously identified in 4- to 18-hour-old eggs (19) are indeed the natural ecdysteroids of this age group.

Our results of analyses of ecdysteroids of ovaries in both studies (12,20) agree in that no free ecdysteroids were detected although improvements in the method of analyses of ecdysteroid conjugates could account for the greater amount of the 26-hydroxyecdysone conjugate (31 µg/g of ovaries) (Table II) being found in the radiolabeled study compared to 20 µg/g found previously (20)(Table I). We would advise that every effort be made to destroy or eliminate any potential for initiation or continuation of enzyme

Table II. Titer of Free and Conjugated Ecdysteroids of Ovaries \* and Eggs at Various Developmental Stages of the Tobacco Hornworm \*

	Free Ecdysteroids µg/g fresh weights <sup>†</sup>	Conjugated Ecdysteroids µg/g fresh weights <sup>†</sup>
<u>Ovaries** from 93-hour-old</u>		
<u>Adult Females</u>		
ecdysone	not detected	trace
26-Hydroxyecdysone	not detected	31
<u>48- to 64-hour-old Eggs**</u>		
Ecdysone	not detected	trace
26-Hydroxyecdysone	not detected	25
<u>72- to 88-hour-old-Eggs**</u>		
Ecdysone	not detected	trace
26-Hydroxyecdysone	not detected	17

\*<sup>14</sup>C-Cholesterol Injected Into Pupae (Day 16)

<sup>†</sup>Determined by RP-HPLC

\*\*Tissues Stored in MeOH at -20°

action before storing any biological material. This is very important if analytical results or final conclusions derived from such material are to be considered valid. On the other hand, if eggs initially had been stored in methanol at -20°C, the discovery of 26-hydroxyecdysone in M. sexta eggs (17) would have at least been delayed.

Unlike conjugates in eggs of other insect species, the conjugates in eggs of the THW contain, primarily, 26-hydroxyecdysone (Tables I and II). This enhanced the isolation and identification of the major conjugate of eggs of the THW. For this work we used 48- to 64-hour-old eggs that had been stored frozen at -20°C although this age group of eggs contained only about 6 µg of the 26-hydroxyecdysone conjugate per gram of eggs (Table I). Approximately 750 µg of a chromatographically pure conjugate was isolated from 120 g of 48- to 64-hour-old eggs (11). Enzymatic hydrolysis of the conjugate with acid phosphatase from human seminal fluid gave 26-hydroxyecdysone. The conjugate was identified as 26-hydroxyecdysone 26-phosphate (Fig. 1) by NMR and fast atom bombardment mass spectrometry. The compound is also the major conjugate of newly-laid eggs (0- to 1-hour-old). Thus, large quantities of this conjugate are readily available from a relatively small quantity of 1- to 18-hour-old THW eggs (25 to 31 µg/g of eggs) providing that when they are collected they are immediately stored in methanol at -20°C until work-up (Table II). This age group of eggs is easily collected and accumulated.

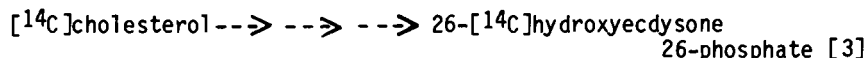
Though the ratio of 26-hydroxyecdysone to other ecdysteroids in eggs and ovaries of the THW can be as high as 20:1 (20), it was not anticipated that the C-26 hydroxyl would be the only position involved in conjugate formation. In a study in which the conjugate was labeled, RP-HPLC and radioassay analyses showed a radioactive peak preceding the peak of 26-hydroxyecdysone 26-phosphate. This

peak was also present in the chromatogram of eggs from which we isolated 26-hydroxyecdysone 26-phosphate (11). We have now accumulated enough of this material to determine whether it is a phosphate conjugate of ecdysone or 26-hydroxyecdysone and the position of conjugation.

It has been surmised that formation of conjugates allows for storage of large quantities of ecdysteroids in insect eggs to be subsequently released during a developmental stage (i.e. embryogenesis) incapable of steroid uptake and/or conversion (21-24). Our results with eggs stored in methanol at -20°C (Table II) suggest that controlled release of free ecdysteroids in *M. sexta* eggs is more limited than previously thought (20). Certainly no significant amounts of free ecdysteroids could be detected. In fact, RP-HPLC and radioassay analyses of the ecdysteroid conjugate fraction of 72- to 88-hour-old eggs show the presence of additional and substantial quantities of more polar conjugates (12). Presently, the fate of 26-hydroxyecdysone 26-phosphate in THW eggs will have to await the identification of these more polar conjugates.

Our isolation of ecdysone, 20-hydroxyecdysone, 20,26-dihydroxyecdysone, 3-epi-20,26-dihydroxyecdysone, and 26-hydroxyecdysone from THW eggs led us to suggest that there were at least two biosynthetic pathways for ecdysteroids during embryonic development of the hornworm: the pathway through 26-hydroxyecdysone as the principal route and the formation of 20-hydroxyecdysone as a minor pathway, with ecdysone serving as an intermediate in both pathways (17). There still is no direct evidence that ecdysone serves as a precursor for 26-hydroxyecdysone. In fact, following injection of [<sup>3</sup>H]ecdysone into female pupae (day 16), there was no incorporation of radioactivity into ovaries or eggs (12). On the other hand [<sup>14</sup>C]cholesterol was readily incorporated and metabolized to 26-[<sup>14</sup>C]hydroxyecdysone 26-phosphate (12). Perhaps, [<sup>3</sup>H]ecdysone should be injected in tobacco hornworms at an earlier stage of the life cycle (5th instar) to determine whether it is incorporated and metabolized to 26-hydroxyecdysone, the major ecdysteroid of ovaries and eggs of *Manduca*.

The ecdysteroid composition of eggs indicates that there are at least two ecdysteroid conjugates present in ovaries and 0- to 1-hour-old eggs and several in older eggs (72- to 88-hour-old) and very little if any free ecdysteroids (12). 26-Hydroxyecdysone 26-phosphate is the major conjugate of ovaries and is by far the predominant conjugate throughout embryogenesis. Thus, our present knowledge of no known intermediates in the incorporation of [<sup>14</sup>C]cholesterol into the ovarian ecdysteroids in *Manduca* can only be expressed as shown in [3]:



Though 26-hydroxyecdysone is without molting hormone activity in the house fly assay, the exceptionally high concentration of 26-hydroxyecdysone conjugate in ovaries and eggs, together with the assumption that it is unlikely that ovaries and eggs only serve as a depository of waste for inactivated ecdysteroids, certainly indicate some physiological role for 26-hydroxyecdysone.

## Conclusions

The isolation and identification of free ecdysteroids from Manduca and insects in general has not been too difficult, because we had partition systems that effectively separated free ecdysteroids from their impurities. For example, from processing 130 g of pupae, the ecdysteroids which partition into the butanol phase are now present in only 413 mg of residue (Fig. 3). This material could be further purified by column and thin-layer chromatography and countercurrent distribution. On the other hand, the ecdysteroid conjugates are present in 3.74 g of residue that is water-soluble which presents additional obstacles to further purification. The nature of the conjugation or the impurities present quite often prevented successful column or thin-layer chromatography of the conjugates. More recently, however, a method was described for the separation of free and conjugated ecdysteroids and the isolation of ecdysteroid conjugates (21).

We have now successfully employed Amberlite XAD-2 (11) and XAD-16 resin in the purification of our conjugates. After applying the 3.74 g of residue from the aqueous phase to the XAD-16 column better than 92% of the impurities were removed from the column with water (Fig. 3) and the partially purified conjugates were eluted with ethanol. The C<sub>18</sub> SEP-PAK fractionation removed additional impurities. Final purification can now be achieved by ion suppression RP-HPLC followed by desalting on SEP-PAK.

Thus, with the present-day instrumentation and techniques we are now routinely able to isolate and identify both free and conjugated ecdysteroids. The conversion of [<sup>14</sup>C]cholesterol to labeled free and conjugated ecdysteroids in Manduca during various developmental stages further enhances our capabilities for determining the fate of ecdysteroids in Manduca at any developmental stage. Tremendous progress is being made in following the fate of ecdysteroids in Manduca eggs during embryogenesis. The mixture of ecdysteroid conjugates of Manduca pupae at peak titer (Fig. 4) also awaits identification. We are now in a position to investigate the interrelationship between free and conjugated ecdysteroids.

Certainly, the continued identification of free and conjugated ecdysteroids at various developmental stages will enhance our understanding of the physiological control of ecdysteroids in Manduca and insects in general as well as our knowledge and understanding of the various enzymes involved. With each stage of insect development representing a possible target for selective disruption of steroid metabolizing pathways, inhibitors of these pathways (molting hormone metabolism) offer potential means of insect control. It is also possible that one could develop insect resistant crops by introducing the capability of producing certain of the insect inhibitors in the plant.

Literature Cited

1. Goodwin, T. W.; Horn, D. H. S.; Karlson, P.; Koolman, J.; Nakanishi, K.; Robbins, W. E.; Siddall, J. B.; Takemoto, T. Nature 1978, 272, 122.
2. Kaplanis, J. N.; Thompson, M. J.; Yamamoto, R. T.; Robbins, W. E.; Louloudes, S. J. Steroids 1966, 8, 605-623.
3. Thompson, M. J.; Kaplanis, J. N.; Robbins, W. E.; Yamamoto, R. T. Chem. Commun. 1967, 650-3.
4. Kaplanis, J. N.; Thompson, M. J.; Dutky, S. R.; Robbins, W. E. Steroids 1979, 34, 333-345.
5. Kaplanis, J. N.; Tabor, L. A.; Thompson, M. J.; Robbins, W. E.; Shortino, T. J. Steroids 1966, 8, 625-631.
6. Nigg, H. N.; Svoboda, J. A.; Thompson, M. J.; Kaplanis, J. N.; Dutky, S. R.; Robbins, W. E. Lipids 1974, 9, 971-4.
7. Thompson, M. J.; Kaplanis, J. N.; Robbins, W. E.; Dutky, S. R.; Nigg, H. N. Steroids 1974, 24, 359-366.
8. Karlson, P.; Koolman, J. Insect Biochem. 1973, 3, 409-417.
9. Hoffmann, J. A.; Koolman, J.; Karlson, P.; Joly, P. Gen. Comp. Endocrinol. 1974, 22, 90-7.
10. Blais, C.; LaFont, R. Hoppe-Seyler's Z. Physiol. Chem. 1984, 365, 809-817.
11. Thompson, M. J.; Weirich, G. F.; Rees, H. H.; Svoboda, J. A.; Feldlaufer, M. F.; Wilzer, K. R. Arch. Insect Biochem. Physiol. 1985, 2, 227-236.
12. Thompson, M. J.; Svoboda, J. A.; Feldlaufer, M. F.; Lozano, R. Lipids 1985, In press.
13. Weirich, G. F. In "Methods in Enzymology"; Law, J. H.; Rilling, H. C., Eds.; Academic Press: New York, 1985; Vol. 111, pp. 454-458.
14. Weirich, G. F.; Thompson, M. J.; Svoboda, J. A. Arch. Insect Biochem. Physiol., In press.
15. Thompson, M. J.; Kaplanis, J. N.; Robbins, W. E.; Dutky, S. R.; Nigg, H. N. Steroids 1974, 24, 359-366.
16. Hutchins, R. F. N.; Kaplanis, J. N. Steroids 1969, 13, 605-614.
17. Kaplanis, J. N.; Robbins, W. E.; Thompson, M. J.; Dutky, S. R. Science 1973, 180, 307-308.
18. Kaplanis, J. N.; Robbins, W. E.; Thompson, M. J.; Dutky, S. R. Steroids 1976, 27, 675-9.
19. Kaplanis, J. N.; Thompson, M. J.; Dutky, S. R.; Robbins, W. E. Steroids 1980, 36, 321-335.
20. Thompson, M. J.; Svoboda, J. A.; Weirich, G. F. Steroids 1984, 43, 333-341.
21. Dinan, L. N.; Rees, H. H. J. Insect Physiol. 1981, 27, 51-8.
22. Lagueux, M.; Sall, C.; Hoffmann, J. A. Am. Zool. 1981, 21, 715-726.
23. Sall, C.; Tsoupras, G.; Kappler, C.; Lagueux, M.; Zachary, D.; Luu, B.; Hoffmann, J. A. J. Insect Physiol. 1983, 29, 491-507.
24. Isaac, R. E.; Sweeney, F. P.; Rees, H. H. Biochem. Soc. Trans. 1983, 11, 379-380.

RECEIVED May 1, 1986



## Chapter 13

# Metabolism and Function of Sterols in Nematodes

David J. Chitwood, Ruben Lozano, William R. Lusby, Malcolm J. Thompson, and  
James A. Svoboda

Insect Physiology Laboratory, Agricultural Research Service, U.S. Department of  
Agriculture, Beltsville, MD 20705

Current knowledge of sterol biochemistry and physiology in nematodes is reviewed. Nematodes possess a nutritional requirement for sterol because they lack the capacity for *de novo* sterol biosynthesis. The free-living nematode *Caenorhabditis elegans* has recently been used as a model organism for investigation of nematode sterol metabolism. *C. elegans* is capable of removal of the C-24 alkyl substituent of plant sterols such as sitosterol and also possesses the remarkable ability to attach a methyl group at C-4 on the sterol nucleus. An azasteroid and several long-chain alkyl amines disrupt the phytosterol dealkylation pathway in *C. elegans* by inhibiting its  $\Delta^{24}$ -sterol reductase. These compounds inhibit growth and reproduction in certain parasitic nematodes and provide model compounds for development of novel nematode control agents.

Nematodes are nonsegmented roundworms which include several diverse groups. Free-living nematodes include microscopic soil-dwelling or aquatic species that feed on microorganisms and dead organic matter. Of greater economic importance in the soil are plant-parasitic nematodes, which cause an estimated annual loss of six billion dollars to American agriculture (1). Because of their frequently larger size as well as their threat to human health, animal-parasitic nematodes are more familiar to the general public and include ascarids, hookworms, pinworms, the dog heartworm, and the causal agents of trichinosis, elephantiasis, and river blindness. Readers with further curiosity about the life history or biology of nematodes are referred to recent monographs by Poinar (2) and Maggenti (3).

The present difficulty in routine culture of parasitic nematodes through their entire life cycles away from their plant or animal hosts has severely hindered investigations of their biochemistry and physiology. Consequently, most studies of sterol nutrition and metabolism in nematodes have necessarily involved the

This chapter not subject to U.S. copyright.  
Published 1987, American Chemical Society

use of free-living genera such as Caenorhabditis, Turbatrix and Panagrellus. These can be easily propagated upon bacteria or in sterile liquid media containing semidefined components such as yeast extract and soy peptone or, with greater difficulty, in chemically defined liquid media.

Historically, parasitic nematodes have been difficult to control for several reasons, including the resistance of the nematode cuticle to penetration by potential nematicides, the resistance of the soil to the migration of nematicides applied to it, the high mammalian toxicity of many anthelmintics, and the general similarity of the metabolic pathways found in these parasites and their hosts. However, the existence of key differences in sterol metabolism between nematodes and their animal or plant hosts have presented the possibility that nematode sterol metabolism could be selectively inhibited. Moreover, the likely function of nematode steroids in the hormonal regulation of important life processes such as molting and reproduction together with the potential benefit of selectively disrupting these life processes have provided further impetus for the recent intensification of research efforts in the area of nematode steroid biochemistry.

#### Nutritional Requirement for Sterol in Nematodes

Interest in nematode sterol metabolism was stimulated by the discovery that the DD-136 strain of Steinernema feltiae, an insect associate, would not grow and reproduce upon bacterial cultures unless a sterol was present (4). Several compounds were capable of satisfying this nutritional requirement, including cholesterol, cholestanol, sitosterol, stigmastanol, 22-dihydrobrassicasterol, cholest-4-en-3-one, 7-dehydrocholesterol, and lathosterol. Growth and reproduction did not occur in cultures supplemented with stigmasterol or ergosterol, two sterols containing  $\Delta^{22}$ -bonds. Hieb and Rothstein (5) demonstrated a similar requirement in Caenorhabditis propagated upon the bacterium Escherichia coli; addition of cholesterol, 7-dehydrocholesterol, ergosterol, stigmasterol or sitosterol resulted in excellent growth and reproduction. Turbatrix aceti reproduced successfully upon Bacillus subtilis supplemented with cholesterol, cholestanol, desmosterol, lathosterol, 7-dehydrocholesterol, 25-norcholesterol, cholest-4-en-3-one, cholest-5-en-3-one, campesterol, 24-methylenecholesterol, stigmasterol, sitosterol, or fucosterol; two sterols with a cis-A/B ring configuration, coprostanol and coprost-7-enol, were not utilized (6). In other experiments, squalene or lanosterol increased T. aceti and Caenorhabditis populations in chemically defined media supplemented with casamino acids and myoglobin or cytochrome c (7). Similarly, some population increase occurred in cultures of the snail associate Rhabditis maupasi in chemically defined media containing lanosterol, although the reproductive rate was greater in cholesterol or ergosterol-supplemented cultures (8).

Difficulties in interpretation of the results of some of these nutritional experiments are a consequence of possible sterol contaminants in media ingredients, possible impurities in the added

sterols, possible bacterial metabolism of the supplemented sterols, or the potential ability of many sterols to substitute for cholesterol in a structural role and free small quantities of endogenous cholesterol in such cultural systems for use as a specific precursor of steroid hormones or other metabolites by a "sparing" process, as can occur in many insects (9). In order to minimize these effects, we have propagated Caenorhabditis elegans in a chemically defined axenic culture (CbMM, 10) supplemented with chloroform/methanol (2:1, v/v)-extracted bovine hemoglobin and various highly purified steroids at 6 µg/ml (unpublished). Nematodes were initially transferred to a sterol-deficient medium, incubated two weeks, and then transferred to media supplemented with the appropriate steroid. Two subcultures were performed at two-week intervals to greatly minimize the amount of residual sterol. At the conclusion of these experiments, thriving populations were present in cultures supplemented with cholesterol, lathosterol, desmosterol, 7-dehydrocholesterol, campesterol, ergosterol, sitosterol, stigmasterol, stigmastanol, isofucosterol and cholesteryl acetate. Interestingly, 29-fluorostigmasterol, a compound which is toxic to the insect Manduca sexta by virtue of the generation of fluoroacetate during C-24 dealkylation (11) satisfied the sterol nutritional requirement in these experiments. (Toxicity did appear at concentrations of 50 µg/ml.) In the same experiments, reproduction and movement eventually ceased in media containing coprost-7-enol, 4 $\alpha$ -methylcholest-8(14)-enol, lanosterol, 22,23-dihydroxysitosterol, progesterone, ecdysone, or 20-hydroxyecdysone. Although additional sterols should be investigated in this sterile, highly defined system before conclusions can be drawn, apparently, 4 $\alpha$ -methyl-, 4,4-dimethyl-, heavily hydroxylated, or cis-A/B sterols cannot satisfy the sterol nutritional requirement in C. elegans in continuous culture.

Among parasitic nematodes, a sterol requirement has been investigated in only Nippostrongylus brasiliensis, a rat parasite whose eggs will develop into third-stage, infective larvae in a culture medium containing formalin-killed E. coli and cholesterol, 7-dehydrocholesterol, ergosterol, sitosterol, stigmasterol, lanosterol, or cholestane, but not coprostanol or coprostanone (12). In addition, in vitro development of third-stage juveniles of Ascaris to fourth-stage juveniles and the size of the resultant juveniles are markedly increased by the addition of cholesterol (13).

Lack of De Novo Sterol Biosynthesis in Nematodes. The dietary requirement for sterol results from the lack of de novo sterol biosynthesis in nematodes. Species in which radiolabeled acetate or mevalonate are not converted to radiolabeled sterol include T. aceti (14, 15), Caenorhabditis (15) and the animal parasites Ascaris (16), Dirofilaria immitis (17), and Brugia pahangi (17). On occasion, radiolabeled sterols identified by thin-layer chromatography have been detected from nematodes incubated with radiolabeled acetate; in the lone case in which such compounds were further characterized by gas-liquid chromatography, the radiolabeled components possessed retention times much earlier than cholesterol (16).

It is not known which enzymes in the typical de novo

biosynthetic pathway are absent in nematodes. Nutritional investigation revealed that in *T. aceti* and *Caenorhabditis* the block occurs subsequent to farnesol (7). *Panagrellus redivivus* was reported to possess the interesting ability to convert tritiated 2,3-oxidosqualene to lanosterol; tritiated C<sub>27</sub> sterols were not detected (18). Experiments with additional radiolabeled precursors are necessary to further investigate this interesting subject.

#### Sterol Composition of Parasitic Nematodes

Because of the previously described problems in culture of parasitic nematodes, investigation of sterol metabolism in these organisms has been largely limited to comparison of the sterol compositions of host and parasite. For example, lathosterol and cholesterol were the major sterols of *N. carpocapsae* DD-136 propagated in wax moth larvae, organisms that contained cholesterol as their principal sterol. Radiolabeled cholesterol injected into host larvae was recovered as radiolabeled lathosterol and cholesterol in the nematode (19).

Not unexpectedly, cholesterol is the major sterol of the few vertebrate-parasitic nematodes examined thus far (16, 20, 21). Fleming and Fetterer (22) have demonstrated recently via occlusion of the digestive tract, continuous perfusion of the pseudocoelom and collection of perienteric fluid from *Ascaris* incubated in the presence of tritiated cholesterol that transcuticular and transmuscular transport is the primary means of short-term cholesterol absorption. The intestine of this animal selectively absorbs cholesterol at about twice the rate of sitosterol (23). Absorption of phytosterols is undoubtedly responsible for the frequent occurrence of substantial quantities of these 24-methyl or 24-ethyl substituted compounds in many parasitic nematodes, including digestive-tract parasites whose host diets can include substantial quantities of phytosterols. Because all of the phytoparasitic nematodes examined thus far contain substantially greater relative proportions of cholesterol and/or lathosterol than their hosts (24-27) and because most (but not all) phytophagous insects (28) as well as certain free-living nematodes (subsequently described) are capable of converting phytosterols to cholesterol via a C-24 dealkylation process, it has been speculated that plant-parasitic nematodes are capable of a similar dealkylation (24, 25, 27). Obviously, experiments with radiolabeled 24-alkylsterols are needed to conclusively establish whether the existence of cholesterol or other 24-desalkylsterols in phytoparasitic nematodes is due to dealkylation rather than some selective uptake mechanism, as in some insects (29, 30). *Ascaris* adults did not convert injected [<sup>14</sup>C]sitosterol to any other sterol (16); it is unknown if the C-24 dealkylation pathway is similarly not functional in other stages of this intestinal parasite or in other animal-parasitic nematodes.

#### Metabolism of Sterols in Free-living Nematodes

Definitive evidence for existence of dealkylation and other metabolic pathways in nematodes has been obtained through extensive

experimentation with axenically propagated free-living nematodes. Cole and Krusberg (14) conclusively demonstrated C-24 dealkylation in *T. aceti* by its conversion of [ $^3\text{H}$ ]sitosterol to tritiated cholesterol and 7-dehydrocholesterol. More recently, our laboratory has undertaken a comprehensive investigation of the metabolism of eight different sterols by *C. elegans* in sterile, semidefined liquid media containing chloroform/methanol-extracted ingredients (31-35). Dietary desmosterol, sitosterol and stigmasterol were radiolabeled; when possible to measure, all sterols recovered from *C. elegans* in such experiments contained approximately the same specific activity as the original supplemented sterol and hence were true metabolites of the dietary sterol and not trace media contaminants or the products of *de novo* synthesis. These experiments have demonstrated that *C. elegans* performs several different sterol metabolic processes, including C-7 dehydrogenation,  $\Delta^5$ -reduction, 4 $\alpha$ -methylation,  $\Delta^7$ -sterol to  $\Delta^{8(14)}$ -sterol isomerization, C-24 dealkylation, and  $\Delta^{22}$ -reduction.

Initial experiments with supplemented cholesterol demonstrated that *C. elegans* can introduce a double bond at C-7 and, to a lesser extent, reduce the  $\Delta^5$ -bond of the resulting  $\Delta^{5,7}$ -diene, as 7-dehydrocholesterol and lathosterol were major and minor metabolites (Table I). Unexpectedly, we detected substantial quantities of two different 4 $\alpha$ -methylsterols in *C. elegans* fed cholesterol, and the steryl ester fraction was especially rich in these compounds. Because 4 $\alpha$ -methylsterols are generally regarded as intermediates between lanosterol (or cycloartenol) and cholesterol in organisms with *de novo* sterol biosynthetic capability, our initial reaction was that these compounds were endogenous media contaminants. However, attempts to isolate them from incubated, nematode-free media failed. Subsequent experiments with [ $^{14}\text{C}$ ]desmosterol and [ $^{14}\text{C}$ ]sitosterol revealed that the 4 $\alpha$ -methylcholest-8(14)-enol and 4 $\alpha$ -methylcholest-7-enol contained approximately the same specific activity as the original dietary sterol and were produced by a direct nuclear methylation pathway. The biosynthesis of 4-methylsterols from methylation of a 4-desmethylsterol precursor has not been suggested to occur in any other organism.

The nuclear methylation pathway is not unique to *C. elegans*. We have recently discovered similar but not identical pathways in *T. aceti* and *P. redivivus* (Chitwood et al., unpublished). Cysts of *Heterodera zeae* did not contain 4-methylsterols (27), but possibly other life stages of *H. zeae* or other parasitic nematodes may contain 4-methylsterols.

Experiments with sitosterol-supplemented media demonstrated the C-24 dealkylation of a 24 $\alpha$ -ethylsterol by *C. elegans* (Table I). The ability of this nematode to produce 24-desalkylsterol metabolites from campesterol, 22-dihydrobrassicasterol, 24-methylenecholesterol, stigmasterol and stigmastanol (Table I) indicates that 24 $\alpha$ -methyl, 24 $\beta$ -methyl, and 24-methylene substituents are effectively removed and that C-24  $\alpha$ -ethyl group removal is not dependent upon lack of a  $\Delta^{22}$ -bond or presence of a  $\Delta^5$ -bond. However, the fact that substantially larger quantities of campesterol remained unmetabolized in *C. elegans*, as compared to the other five phytosterols, indicates either that substrate specificity for the

C-24 dealkylation enzyme system occurs or that different enzymes are utilized for different substrates (34).

Several other interesting observations were noted during our comparative investigations (Table I). For example, stigmastanol-fed *C. elegans* did not contain any  $\Delta^5$ - or  $\Delta^{5,7}$ -sterols; therefore, it is likely that this nematode lacks a  $\Delta^5$ -dehydrogenase. Although nuclear modification of dietary 24-ethylsterols did not occur prior to dealkylation, the nucleus of dietary 24-methylsterols was directly metabolized to a surprisingly large degree, resulting in production of significant quantities of 24-methylcholesta-5,7-dienol, 24-methylcholest-7-enol, and 4 $\alpha$ ,24-dimethylcholest-8(14)-enol. Apparently, the nuclear modification enzymes have little affinity for 24-ethylsterols but might indeed bind to sterols with a less bulky 24-methyl group. Alternatively, separate enzymes for nuclear metabolism of 24-methylsterols and 24-desmethylsterols could exist concurrently.

Rothstein (15) originally demonstrated the esterification of [ $^{14}$ C]cholesterol by *Caenorhabditis* and *T. aceti*. In our investigations, esterified sterols comprised from 7.3% to 21.3% of the total sterol from *C. elegans* and were radiolabeled when radiolabeled dietary sterols were employed (32, 34). Steryl ester fractions consistently contained larger proportions of 4 $\alpha$ -methylsterols than free sterol fractions. Speculative explanations for the abundance of 4 $\alpha$ -methylsteryl esters include an esterification requirement for 4 $\alpha$ -methylsterol synthesis or transport or a specific hormonal, pheromonal or other physiologic role for a 4 $\alpha$ -methylsteryl ester or metabolite.

### Inhibition of Sterol Metabolism in Nematodes

Like nematodes, insects nutritionally require sterol because they lack the capacity for de novo sterol biosynthesis (9). Many azasteroids and nonsteroidal alkylamines and amides interfere with steroid metabolism, growth and development in insects and have provided model compounds for development of novel agents for insect control (36). Consequently, several investigators have evaluated these as well as related compounds for toxicity or growth-inhibitory activity towards various nematodes. Cole and Krusberg (14) demonstrated the accumulation of [ $^3$ H]-desmosterol in *T. aceti* sterily propagated in media containing [ $^3$ H]-sitosterol and triparanol succinate, a vertebrate hypocholesterolemic agent by virtue of its inhibition of  $\Delta^{24}$ -sterol reductase, an enzyme that converts desmosterol to cholesterol. Feldmesser et al. (37) demonstrated the toxicity of many different N-substituted long-chain (C<sub>11</sub> to C<sub>15</sub>) alkyl amines and amides to *P. redivivus* and the root-knot nematode *Meloidogyne incognita*; 100% lethality occurred at concentrations of 5-40  $\mu$ g/ml. Other related amines possessed *in vitro* toxicity against the pinewood nematode, *Bursaphelenchus xylophilus* (38). Douvres et al. (39) found toxicity of many of these alkylamines and amides to be as low as 1.0 to 2.5  $\mu$ g/ml *in vitro* against the cattle stomach worm *Ostertagia ostertagi*.

Less is known about the effects of azasteroids upon nematodes because of their undesirable resemblance to human sterols as well

Table I. Relative percentages of sterols in free sterol (FS) and steryl ester sterols

Recovered sterol	Cholesterol		Desmosterol		Campesterol	
	FS	SE	FS	SE	FS	SE
Cholesterol	52.3	41.2	26.9	18.7	3.9	3.7
7-Dehydrocholesterol	40.5	26.7	31.2	39.0	29.4	10.9
Lathosterol	3.6	5.7	1.7	1.4	3.7	1.8
Cholesta-5,7,9(11)-trienol	1.4	1.5	1.4	1.4	1.9	-
Cholest-8(14)-enol	-	-	-	-	-	-
Cholestanol	-	-	-	-	-	-
Desmosterol	-	-	32.3	22.8	-	-
Cholesta-5,7,24-trienol	-	-	2.4	4.5	-	-
Campesterol	-	-	-	-	35.8	53.3
Dihydrobrassicasterol	-	-	-	-	-	-
24-Methylenecholesterol	-	-	-	-	3.1	3.7
24-Methylcholesta-5,7-dienol	-	-	-	-	14.1	12.9
24-Methylcholest-7-enol	-	-	-	-	0.6	1.1
24-Methylenecholesta-5,7-dienol	-	-	-	-	1.3	-
24-Methylcholesta-5,7,9(11)-trienol	-	-	-	-	1.0	0.3
Sitosterol	-	-	-	-	-	-
Stigmasterol	-	-	-	-	-	-
Stigmastanol	-	-	-	-	-	-
Fucosterol	-	-	-	-	-	-
4 $\alpha$ -Methylcholest-8(14)-enol	2.1	23.4	3.8	10.6	3.6	9.7
4 $\alpha$ -Methylcholest-7-enol	0.1	1.3	0.3	1.6	0.2	0.2
4 $\alpha$ ,24-Dimethylcholest-8(14)-enol	-	-	-	-	0.7	1.4
4 $\alpha$ ,24-Dimethylcholestanol	-	-	-	-	0.4	0.8

<sup>a</sup>Contained 1.5% campesterol.

(SE) fractions from Caenorhabditis elegans propagated with different dietary

Supplemented sterol									
Dihydro-brassicasterol		24-Methylene-cholesterol		Sitosterol <sup>a</sup>		Stigmasterol		Stigmastanol	
FS	SE	FS	SE	FS	SE	FS	SE	FS	SE
5.1	8.5	8.6	12.2	6.7	9.3	8.6	11.3	-	-
45.0	21.5	49.6	43.8	66.5	30.5	55.6	26.5	-	-
3.5	1.6	5.0	2.5	4.4	3.6	3.9	9.4	68.3	28.6
6.5	3.4	2.1	2.5	0.8	0.3	5.8	2.4	-	-
-	-	-	-	-	-	-	-	3.7	6.4
-	-	-	-	-	-	-	-	3.8	5.6
-	-	-	-	-	-	-	-	-	-
-	-	-	0.6	-	-	-	-	-	-
-	-	-	-	0.7	1.3	-	-	-	-
24.2	31.7	-	-	-	-	-	-	-	-
1.3	8.8	31.0	16.5	-	-	-	-	-	-
5.4	7.1	-	-	-	-	-	-	-	-
0.2	-	-	-	-	-	-	-	-	-
0.3	-	2.5	6.3	-	-	-	-	-	-
1.1	1.3	-	-	-	-	-	-	-	-
-	-	-	-	16.0	30.3	-	-	-	-
-	-	-	-	-	-	20.5	21.9	-	-
-	-	-	-	-	-	-	-	14.2	30.1
-	-	-	-	0.1	0.1	-	-	-	-
6.7	14.7	1.1	14.9	4.2	23.3	5.4	27.5	9.3	27.0
0.3	0.6	0.1	0.7	0.7	1.4	0.2	1.0	0.7	2.3
0.3	0.4	-	-	-	-	-	-	-	-
0.1	0.4	-	-	-	-	-	-	-	-



as their complex structure and resultant expensive chemical synthesis. The azasteroid 25-azacoprostanone strongly inhibited reproduction in *C. elegans* (32) and *in vitro* development of first-stage to third-stage juveniles of *N. brasiliensis* and the mouse parasite *Nematospiroides dubius* (40). Specific biological effects in nematodes of many alkylamines and alkylamides were described in detail in *O. ostertagi*, where effects included reduced survival, decreased motility or induced paralysis, delayed development, lowered yields of advanced stages, delayed or blocked or incompletely third or fourth molt, and decreased or nonexistent egg production (39). A similar paralysis and inhibition of motility and reproduction occurred in *C. elegans* treated with various alkylamines (33) or 25-azacoprostanone (32). The latter compound or 25-azacholestanone induced *N. brasiliensis* juveniles to develop morphological abnormalities observed in juveniles cultured in sterol-deficient media, including degeneration of intestinal cells, abnormal dispersion of lipid globules, and small length (40).

Effects of inhibitors on sterol metabolic pathways in *C. elegans*. The most specific effects on nematodes of the azasteroids, amines and amides have been obtained through our investigations of *C. elegans* propagated in media supplemented with one of several different inhibitors (31, 32, 33, 35). Our results have demonstrated that these inhibitors can act at several different metabolic sites; moreover, the accumulation of many previously undetected sterols has led to the discovery of several key intermediates in the sterol metabolic pathways of this organism (Figure 1).

Initially, *C. elegans* was propagated in media supplemented with 5.0  $\mu\text{g/ml}$  25-azacoprostanone hydrochloride, a concentration previously shown to decrease reproductive rate in *C. elegans* by about 50%. Excluding dietary sitosterol, nearly 96% of the sterols from such organisms were  $\Delta^{24}$ -sterols normally present in no more than trace quantities: cholesta-5,7,24-trienol, desmosterol, cholesta-7,24-dienol, and fucosterol (Table II). The abundance of these compounds indicated that the azasteroid significantly inhibited  $\Delta^{24}$ -sterol reductase in *C. elegans* and that  $\Delta^{24}$ -sterols are major intermediates in the *C. elegans* pathway for sitosterol dealkylation. In addition, the predominance of  $\Delta^7$ -4 $\alpha$ -methylsterols revealed that the azasteroid inhibits the isomerase that converts  $\Delta^7$ - to  $\Delta^8(14)$ -4 $\alpha$ -methylsterols.

Four nonsteroidal dimethylamines similarly inhibited the  $\Delta^{24}$ -sterol reductase in sitosterol-fed *C. elegans*, but to a lesser extent (Table II). Among these compounds, maximal inhibition occurred upon addition of N,N,3,7,11-pentamethyldodecanamine, followed by N,N-dimethyldodecanamine, N,N-dimethyltetradecanamine, and N,N-dimethylhexadecanamine. Although inhibitory to growth and reproduction in *C. elegans*, the corresponding  $\text{C}_{12}$  dimethylamide possessed little effect on sitosterol (Table II) or stigmasterol (Table III) metabolism; perhaps substitution of an amide group for the amine group destroyed the ability of the inhibitor to competitively bind to the *C. elegans*  $\Delta^{24}$ -sterol reductase. The branched-chain alkylamine resulted in an accumulation of unmetabolized dietary sitosterol, possibly because its greater

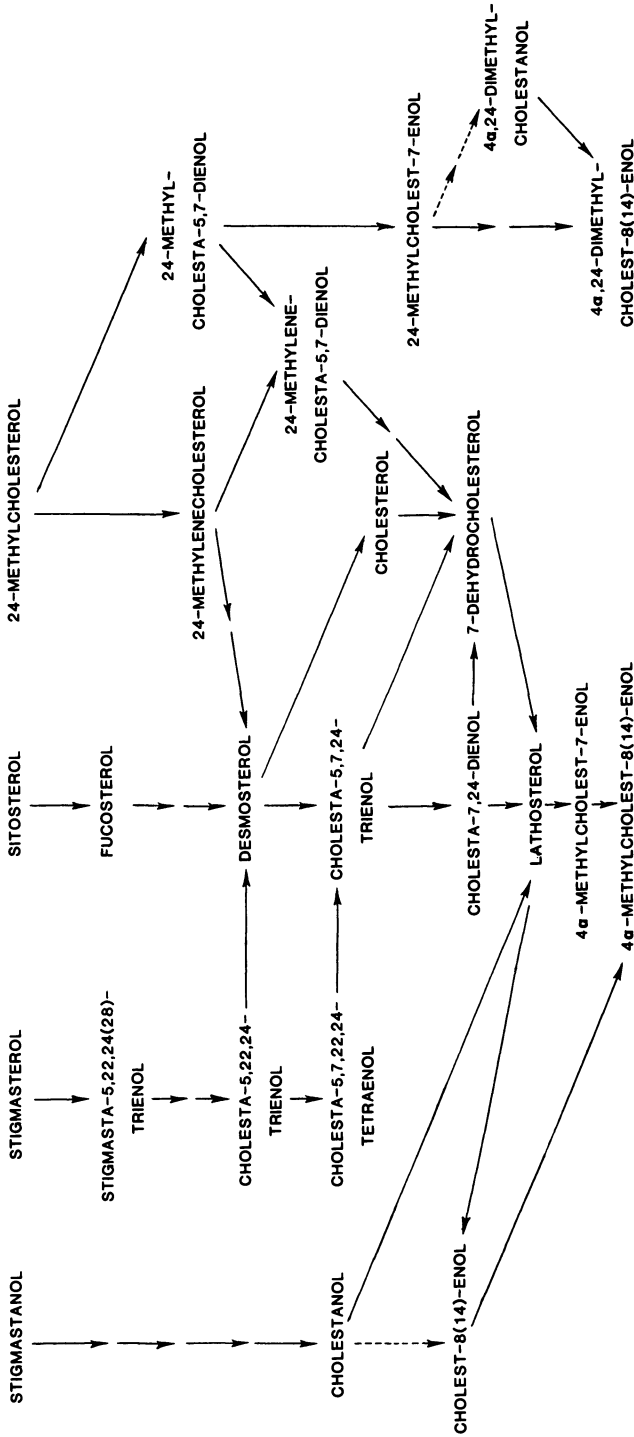


Figure 1. Major pathways of sterol metabolism in *Caenorhabditis elegans*. Dotted lines represent possible but unproven steps.

Table II. Relative percentages of total sterols from *Caenorhabditis* metabolic inhibitors. Dietary sitosterol contained 1.5% campesterol.

Recovered sterol	None	25-azaco- propane • HCl (5 µg/ml)	C <sub>12</sub> amine <sup>a</sup> (25 µg/ml)
Cholesterol	8.1	0.4	9.0
7-Dehydrocholesterol	56.4	1.2	23.1
Lathosterol	5.5	0.2	7.7
Cholesta-5,7,9(11)-trienol	2.0	0.1	0.7
Desmosterol	-	9.0	4.7
Cholesta-5,7,24-trienol	-	44.5	14.8
Cholesta-7,24-dienol	-	2.1	1.2
Cholesta-5,7,9(11),24-tetraenol	-	1.2	1.4
Cholesta-8,24-dienol	-	0.1	0.1
Campesterol	0.6	0.9	1.5
Fucosterol	0.1	2.3	0.4
Sitosterol	18.2	29.8	19.6
4 $\alpha$ -Methylcholest-8(14)-enol	8.6	0.2	4.1
4 $\alpha$ -Methylcholest-7-enol	0.5	0.5	2.9
4 $\alpha$ -Methylcholesta-8(14),24-dienol	-	2.5	2.2
4 $\alpha$ -Methylcholesta-7,24-dienol	-	3.1	1.7
Others	-	1.9	4.9
Total $\Delta^{24}$ -sterols	0.1	64.8	26.5
Total 24-alkylsterols	18.9	33.0	21.5

<sup>a</sup>N,N-Dimethyldodecanamine

<sup>b</sup>N,N-Dimethyltetradecanamine

<sup>c</sup>N,N-Dimethylhexadecanamine

<sup>d</sup>N,N-Dimethyldodecanamide

<sup>e</sup>N,N-Dimethyl-3,7,11-trimethyldodecanamine

<sup>f</sup>Trans-1,4-bis(2-chlorobenzylaminomethyl)cyclohexane dihydrochloride

elegans propagated with 25 µg/ml sitosterol and various

C14 amine <sup>b</sup> (25 µg/ml)	C16 amine <sup>c</sup> (25 mg/ml)	branched		AY-9944 <sup>f</sup> (50 µg/ml)
		C12 amide <sup>d</sup> (35 µg/ml)	C12 amine <sup>e</sup> (25 µg/ml)	
9.3	8.5	9.0	2.6	6.8
33.7	29.0	47.0	3.7	71.1
6.1	7.2	6.0	2.0	4.6
3.8	11.0	6.4	0.5	0.6
-	0.8	-	11.8	-
9.7	2.4	-	7.4	-
1.2	1.1	-	6.9	-
1.6	-	-	-	-
-	-	-	3.3	-
1.3	2.2	1.0	3.6	0.6
2.7	1.7	1.1	3.8	0.2
16.8	20.0	20.1	35.7	10.2
5.3	11.4	6.7	2.8	1.9
2.6	2.9	0.7	2.1	3.1
1.0	1.4	-	9.1	-
0.9	0.4	-	4.5	-
4.0	-	2.0	0.2	0.9
17.1	7.8	1.1	46.8	0.2
20.8	23.9	22.2	43.1	11.0

resemblance to the side chain of sitosterol than the straight-chain amines results in competition with sitosterol for binding to the C-24(28) dehydrogenase.

Table III. Relative percentages of total sterols from *Caenorhabditis elegans* propagated with 25  $\mu\text{g/ml}$  stigmaterol either alone or supplemented with 25  $\mu\text{g/ml}$  N,N-dimethyldodecanamine or 35  $\mu\text{g/ml}$  N,N-dimethyldodecanamide

Recovered sterol	Supplemented Inhibitor		
	None	C <sub>12</sub> amine	C <sub>12</sub> amide
Cholesterol	9.1	5.7	10.9
7-Dehydrocholesterol	50.6	23.3	47.2
Lathosterol	4.8	4.4	7.3
Cholesta-5,7,9(11)-trienol	5.2	5.6	5.3
Desmosterol	-	4.2	-
Cholesta-5,7,24-trienol	-	14.8	-
Cholesta-5,7,9(11),24-tetraenol	-	3.2	-
Cholesta-5,22E,24-trienol	-	3.0	-
Cholesta-5,7,22E,24-tetraenol	-	4.1	-
Cholesta-5,7,9(11),22E,24-pentaenol	-	0.6	-
Stigmasta-5,22E,24(28)-trienol	-	0.9	0.4
Stigmaterol	20.8	17.8	18.9
4 $\alpha$ -Methylcholest-8(14)-enol	9.2	5.0	8.7
4 $\alpha$ -Methylcholest-7-enol	0.3	1.7	0.5
4 $\alpha$ -Methylcholesta-8(14),24-dienol	-	1.8	-
4 $\alpha$ -Methylcholesta-7,24-dienol	-	0.9	-
Other sterols (unidentified)	-	3.0	0.8

In stigmaterol-supplemented cultures (Table III), N,N-dimethyldodecanamine caused the accumulation of a number of  $\Delta^{24}$ - and  $\Delta^{22,24}$ -sterols, including the  $\Delta^{22,24}$ -analogue of fucosterol: stigmasta-5,22E,24(28)-trienol. Presence of this compound and lack of detection of fucosterol and  $\Delta^{5,22}$ -,  $\Delta^{5,7,22}$ -, or  $\Delta^{7,22}$ -dealkylated sterols indicates that  $\Delta^{22}$ -reduction (Figure 1) probably occurs prior to  $\Delta^{24}$ -reduction, as it does in insects (41-43).

In campesterol-supplemented cultures (Table IV), N,N-dimethyldodecanamine similarly caused an accumulation of  $\Delta^{24}$ -sterols; however,  $\Delta^{24}$ -sterol reductase inhibition was much less than in stigmaterol- or sitosterol-supplemented media. Production of the analogous  $\Delta^{24}$ - and  $\Delta^{24(28)}$ -sterol intermediates indicates that campesterol dealkylation proceeds via the pathway depicted in Figure 1. In addition, high concentrations of the amine caused a curious accumulation of unmetabolized dietary campesterol that was reflected by a greater total sterol content in such nematodes (35). It is unknown if this increase results from a direct stimulation of campesterol uptake as opposed to inhibition of the C-24(28) dehydrogenase.

Table IV. Relative percentages of total sterols from *Caenorhabditis elegans* propagated with 25  $\mu\text{g/ml}$  campesterol and different concentrations of N,N-dimethyldodecanamine

Recovered sterol	N,N-dimethyldodecanamine concentration		
	0 $\mu\text{g/ml}$	10 $\mu\text{g/ml}$	25 $\mu\text{g/ml}$
Campesterol	38.9	37.0	60.0
Cholesterol	4.0	2.6	0.8
7-Dehydrocholesterol	26.3	26.8	12.5
Lathosterol	3.4	2.1	0.7
Cholesta-5,7,9(11)-trienol	1.5	1.6	0.8
Cholesta-5,7,24-trienol	-	1.7	5.4
Campesta-5,7-dienol	13.9	14.2	12.5
Campesta-7-enol	0.7	0.7	-
Campesta-5,7,9(11)-trienol	0.9	1.3	0.6
24-Methylenecholesterol	3.2	3.8	1.9
Campesta-5,7,24(28)-trienol	1.1	1.0	0.2
4 $\alpha$ -Methylcholest-8(14)-enol	4.6	4.3	1.6
4 $\alpha$ -Methylcholest-7-enol	0.2	0.8	0.9
4 $\alpha$ ,24-Dimethylcholest-8(14)-enol	0.8	0.9	0.4
4 $\alpha$ ,24-Dimethylcholest-7-enol	-	0.2	0.3
4 $\alpha$ ,24-Dimethylcholestanol	0.5	0.6	0.3
4 $\alpha$ -Methylcholesta-8(14),24-dienol	-	0.3	0.4
4 $\alpha$ -Methylcholesta-7,24-dienol	-	0.1	0.3
Other sterols (unidentified)	-	-	0.4

Our findings demonstrate that the azasteroids, alkylamines and alkylamides can block one or more of several different sterol metabolic sites in *C. elegans*, including its  $\Delta^{24}$ -reductase, C-24(28) dehydrogenase, and  $\Delta^{8(14)}$ -isomerase. Because these and related compounds can alter ecdysteroid metabolism in insects (28), one should expect future discovery of additional sites of action of these compounds.

Table II also contains previously unpublished results of experiments in which medium was supplemented with AY-9944, a compound originally developed as a hypocholesterolemic agent due to its inhibition of the  $\Delta^7$ -sterol reductase involved in the conversion of 7-dehydrocholesterol to cholesterol in de novo sterol biosynthesis in mammals (44). In other biological systems (bramble suspension cells, algae and yeast), AY-9944 inhibits  $\Delta^8$ - to  $\Delta^7$ -isomerases and, in one case, a  $\Delta^{14}$ -sterol reductase (45-47). When sitosterol-containing media was supplemented with AY-9944, the 4-desmethylsterol composition of *C. elegans* was not substantially altered; however, the  $\Delta^7$ -4 $\alpha$ -methylsterol was in greater abundance than its  $\Delta^{8(14)}$ -analog. Thus, AY-9944 apparently inhibits the  $\Delta^7$ - to  $\Delta^{8(14)}$ -sterol isomerase in *C. elegans*. Nematode growth and reproduction was not inhibited by AY-9944 at 50  $\mu\text{g/ml}$ .

### Function of Sterols in Nematodes

One should expect sterols to function similarly in nematodes and other animals; consequently, sterols should be integral structural

components of nematode membranes as well as metabolic precursors to steroid hormones. In the only investigation of the structural role of sterols in nematodes, freeze-fracture electron microscopic examination of *T. aceti* propagated in a medium supplemented with cholesterol and filipin revealed the occurrence of filipin/sterol complexes in intracellular membranes (48).

With respect to a possible hormonal function of steroids, the small size of free-living and plant-parasitic nematodes has confined investigation of nematode endocrinology to the larger animal parasites. Endocrine control of nematode development has been associated with exsheathment of third-stage, infective trichostrongylid juveniles (49, 50) and in ecdysis of a cod and seal parasite, *Phocanema decipiens* (51, 52). The identity of the hormones involved in these systems is unknown; greater attention has been focused on possible involvement of analogs of insect juvenile hormones (epoxyfarnesoic acid methyl ester derivatives) rather than steroids.

The probability that nematode endocrine control involves some compounds similar in structure to vertebrate or insect steroid hormones has prompted several investigations of the effects upon nematodes of exogenous application of various concentrations of several of these compounds (53-62). When observed, effects have consisted primarily of inhibition or stimulation of growth, reproduction or molting. Although the high concentrations of added steroid or the nature of the assay system in some of these experiments might lead to speculation about the *in vivo* extrapolation of such results, the body of work is compatible with the hypothesis that steroids are hormonally active within nematodes.

Several investigators have taken an alternative approach and have attempted to isolate from nematodes steroids with hormonal function in other animals. No typical vertebrate steroids have yet been isolated from nematodes, except for a recent preliminary report of isolation from *Trichostrongylus colubriformis* of testosterone in worms from male (but not female) goats and progesterone in worms from female (but not male) goats (63). Efforts to isolate ecdysteroids from nematodes have been more successful. Initially, extracts from nematodes were discovered to have molting hormone activity in various insect bioassays (64-66). Subsequently, Horn et al. (66) isolated from 15.5 kg of *Ascaris* adults about 4.5 µg of 20-hydroxyecdysone, identified by reversed phase partition chromatography and UV spectroscopy. Material that reacted positively in a radioimmunoassay (RIA) for ecdysteroids was found in homogenates of *P. redivivus*, *Haemonchus contortus*, and the fungal-feeding *Aphelenchus avenae* (67). In a major contribution towards nematode steroid biochemistry, Mendis et al. (68) identified ecdysone, 20-hydroxyecdysone, and possibly 20,26-dihydroxyecdysone and ponasterone A from highly purified extracts from male and female *Dirofilaria immitis* by gas-liquid chromatography-mass spectrometry and by RIA of fractions purified by high-performance liquid chromatography (HPLC). Each sex contained both free and conjugated ecdysteroids. The same researchers have similarly identified free and conjugated ecdysteroids from *A. avenae*, *C. elegans* and *Ascaris suum* (69), a species in which Fleming (70) has recently identified ecdysteroids by HPLC and RIA.

The presence of ecdysteroids in nematodes and the previously described molt-promoting or growth-stimulating activity of some of these compounds at low concentrations indicate that these compounds are strong bioactive substances within nematodes, if not hormones in the classic sense. Given the widespread occurrence of ecdysteroids in nature, it would be of further interest to determine that ecdysteroids are indeed synthesized by nematodes from a sterol precursor rather than merely assimilated from the diet or environment as well as to identify the anatomical location of such a synthesis (69, 71). A combination of an organ-specific bioassay system with classical analytical chemistry would result in true endocrinological investigation of nematodes and thereby directly establish that some nematode hormone is in fact a steroid.

#### Acknowledgments

We thank Dr. Glenn Prestwich for his gift of 29-fluorostigmasterol, Ayerst Laboratories for the gift of AY-9944, and Sherry Cohen and Martin Weinrich for assistance in preparation of this and related manuscripts. We also thank Dr. Glenn Patterson for arrangement of a cooperative agreement with the Department of Botany, University of Maryland, that supported Dr. Lozano and Patricia Oakley, who has provided excellent technical assistance. Mention of a company name or a proprietary product does not imply endorsement by the U.S. Department of Agriculture.

#### Literature Cited

1. Feldmesser, J.; Kochansky, J.; Jaffe, H.; Chitwood, D. In "Agricultural Chemicals of the Future"; Hilton, J. L., Ed., Rowman and Allanheld: Totowa, New Jersey, 1985; pp. 327-344.
2. Poinar, G. O. "The Natural History of Nematodes"; Prentice-Hall: Englewood Cliffs, New Jersey, 1983.
3. Maggenti, A. "General Nematology"; Springer-Verlag: New York, 1981.
4. Dutky, S. R.; Robbins, W. E.; Thompson, J. V. Nematologica 1967, 13, 140.
5. Hieb, W. R.; Rothstein, M. Science 1968, 160, 778.
6. Cole, R. J.; Dutky, S. R. J. Nematol. 1969, 1, 72.
7. Lu, N. C.; Newton, C.; Stokstad, E. L. R. Nematologica 1977, 23, 57.
8. Brockelman, C. R.; Jackson, G. J. J. Parasitol. 1978, 64, 803.
9. Kircher, H. W. In "Cholesterol Systems in Insects and Animals"; Dupont, J., Ed., CRC Press: Boca Raton, Florida, 1982; pp. 1-50.
10. Vanfleteren, J. R. Ann. Rev. Phytopathol. 1978, 16, 131.
11. Prestwich, G. D.; Gayen, A. K.; Phirwa, S.; Kline, T. B. Bio/Technology 1983, 1, 62.
12. Bolla, R. I.; Weinstein, P. P.; Lou, C. Comp. Biochem. Physiol. 1972, 43B, 487.
13. Urban, J. F.; Douvres, F. W.; Xu, S. Vet. Parasitol. 1984, 14, 33.
14. Cole, R. J.; Krusberg, L. R. Life Sci. 1968, 7, 713.



15. Rothstein, M. Comp. Biochem. Physiol. 1968, 27, 309.
16. Barrett, J.; Cain, G. D.; Fairbairn, D. J. Parasitol. 1970, 56, 1004.
17. Comley, J. C. W.; Jaffe, J. J. J. Parasitol. 1981, 67, 609.
18. Willett, J. D.; Downey, W. L. Biochem. J. 1974, 138, 233.
19. Dutky, S. R.; Kaplanis, J. N.; Thompson, M. J.; Robbins, W. E. Nematologica 1967, 13, 139.
20. Cole, R. J.; Krusberg, L. R. Comp. Biochem. Physiol. 1967, 21, 109.
21. Fulk, W. K.; Shorb, M. S. J. Parasitol. 1971, 57, 840.
22. Fleming, M. W.; Fetterer, R. H. Exp. Parasitol. 1984, 57, 142.
23. Beames, C. G.; Bailey, H. H.; Rock, C. O.; Schanbacher, L. M. Comp. Biochem. Physiol. 1974, 47A, 881.
24. Cole, R. J.; Krusberg, L. R. Exp. Parasitol. 1967, 21, 232.
25. Svoboda, J. A.; Rebois, R. V. J. Nematol. 1977, 9, 286.
26. Orcutt, D. M.; Fox, J. A.; Jake, C. A. J. Nematol. 1978, 10, 264.
27. Chitwood, D. J.; Hutzell, P. A.; Lusby, W. R. J. Nematol. 1985, 17, 64.
28. Svoboda, J. A. In "Isopentenoids in Plants"; Nes, W. D.; Fuller, G.; Tsai, L.-S., Eds.; Marcel Dekker: New York, 1984, pp. 367-388.
29. Thompson, M. J.; Louloudes, S. J.; Robbins, W. E.; Waters, J. A.; Steele, J. A.; Mosettig, E. J. Insect Physiol. 1963, 9, 615.
30. Svoboda, J. A.; Nair, A. M. G.; Agarwal, N.; Robbins, W. E.; Experientia 1980, 36, 1029.
31. Chitwood, D. J.; Lusby, W. R.; Lozano, R.; Thompson, M. J.; Svoboda, J. A. Steroids 1983, 42, 311.
32. Chitwood, D. J.; Lusby, W. R.; Lozano, R.; Thompson, M. J.; Svoboda, J. A. Lipids 1984, 19, 500.
33. Lozano, R.; Chitwood, D. J.; Lusby, W. R.; Thompson, M. J.; Svoboda, J. A.; Patterson, G. W. Comp. Biochem. Physiol. 1984, 79C, 21.
34. Lozano, R.; Lusby, W. R.; Chitwood, D. J.; Svoboda, J. A. Lipids 1985, 20, 102.
35. Lozano, R.; Lusby, W. R.; Chitwood, D. J.; Thompson, M. J.; Svoboda, J. A. Lipids 1985, 20, 158.
36. Svoboda, J. A.; Thompson, M. J.; Robbins, W. E.; Kaplanis, J. N. Lipids 1978, 13, 742.
37. Feldmesser, J.; Thompson, M. J.; Robbins, W. E.; Sponaugle, R. P. Experientia 1976, 32, 466.
38. Nagase, A.; Kuwahara, Y.; Tominaga, Y.; Sugawara, R. Agric. Biol. Chem. 1983, 47, 53.
39. Douvres, F. W.; Thompson, M. J.; Robbins, W. E. Vet. Parasitol. 1980, 7, 195.
40. Bottjer, K. P.; Weinstein, P. P.; Thompson, M. J. Comp. Biochem. Physiol. 1984, 78B, 805.
41. Svoboda, J. A.; Hutchins, R. F. N.; Thompson, M. J.; Robbins, W. E. Steroids 1969, 14, 469.
42. Svoboda, J. A.; Robbins, W. E.; Cohen, C. F.; Shortino, T. J. In "Insect and Mite Nutrition"; Rodriguez, J. G., Ed.; North Holland: Amsterdam, 1972, pp. 505-516.

43. Fujimoto, Y.; Kimura, M.; Takasu, A.; Khalifa, F.; Morisaki, M.; Ikekawa, N. Tetrahedron Lett. 1984, 25, 1501.
44. Kraml, M.; Bagli, J. F.; Dvornik, D. Biochem. Biophys. Res. Commun. 1964, 15, 455.
45. Patterson, G. W.; Doyle, P. J.; Dickson, L. G.; Chan, J. T. Lipids 1974, 9, 567.
46. Schmitt, P.; Benveniste, P. Phytochemistry 1979, 18, 445.
47. Pereira, R.; Holmlund, C. E.; Whittaker, N. Lipids 1983, 18, 545.
48. Silberkang, M.; Havel, C. M.; Friend, D. S.; McCarthy, B. J.; Watson, J. A. J. Biol. Chem. 1983, 258, 8503.
49. Rogers, W. P. Proc. R. Soc. Lond. Ser B. 1960, 152, 367.
50. Rogers, W. P.; Brooks, F. Int. J. Parasitol. 1976, 6, 315.
51. Davey, K. G. In "The Organization of Nematodes"; Croll, N. A., Ed.; Academic Press: London, 1976, pp. 273-291.
52. Davey, K. G.; Goh, S. L. Can. J. Zool. 1984, 62, 2293.
53. Webster, J. M.; Craig, S. J. Nematol. 1969, 1, 308.
54. Dennis, R. D. Comp. Biochem. Physiol. 1976, 53A, 53.
55. Fleming, M. W. J. Exp. Zool. 1985, 233, 229.
56. Hitcho, P. J.; Thorson, R. E. J. Parasitol. 1971, 57, 787.
57. Dropkin, V. H.; Lower, W. R.; Acedo, J. J. Nematol. 1971, 3, 349.
58. Thong, C. H. S.; Webster, J. M. Can. J. Zool. 1971, 49, 1059.
59. Johnson, R. N.; Vigliierchio, D. R. Exp. Parasitol. 1970, 27, 301.
60. Hansen, E. L.; Buecher, E. J. Experientia 1971, 27, 859.
61. Gwadz, R. W.; Spielman, A. J. Parasitol. 1974, 60, 134.
62. Ogura, N.; Kobayashi, M.; Yamamoto, H. Dokkyo J. Med. Sci. 1981, 8, 74.
63. Chung, W.-L.; Kiser, C. A. S.; Parish, E. J.; Bone, L. W. Fed. Proc. 1985, 44, 658 (Abstract).
64. Rajulu, G. S.; Kulasekarapandian, S.; Krishnan, N. Curr. Sci. 1972, 41, 67.
65. Rogers, W. P. Parasitology 1973, 67, 105.
66. Horn, D. H. S.; Wilkie, J. S.; Thomson, J. A. Experientia 1974, 30, 1109.
67. Dennis, R. D. W. Int. J. Parasitol. 1977, 7, 181.
68. Mendis, A. H. W.; Rose, M. R.; Rees, H. H.; Goodwin, T. W. Mol. Biochem. Parasitol. 1983, 9, 209.
69. Rees, H. H.; Mendis, A. H. W. In "Biosynthesis, Metabolism and Mode of Action of Invertebrate Hormones"; Hoffman, J.; Porchet, M., Eds.; Springer-Verlag: Berlin, 1984, pp. 338-345.
70. Fleming, M. W. Exp. Parasitol. 1985, 59, in press.
71. Koolman, J.; Walter, J.; Azhner, H. In "Biosynthesis, Metabolism and Mode of Action of Invertebrate Hormones"; Hoffmann, J.; Porchet, M., Eds.; Springer-Verlag: Berlin, 1984, pp. 324-330.

RECEIVED January 10, 1986

## Chapter 14

# Fatty Acids and Esters as Antimicrobial/Insecticidal Agents

Jon J. Kabara

Department of Biomechanics, Michigan State University, East Lansing, MI 48824

Structure-function relationships of fatty acids and monoesters are reviewed. Many of the generalistics found concerning chain length, unsaturation, esterification and geometrical isomerism hold whether germicidal or insecticidal properties are being considered.

Saturated fatty acids reach their optimal effects with chains 12 carbons long. Monounsaturations increase the activity of long ( $C_{14}$  or greater) but not short ( $<C_{12}$ ) chain fatty acids. Cis unsaturated fatty acids are more active than trans acids. Adding a second double bond further increases activity while the addition of a third double bond does not substantially improve activity. Esterification to monohydric alcohol causes deactivation of the fatty acid while esterification to polyhydric compounds (glycerol, polyglycerol, sucrose, etc.) generally leads to a more active derivative.

While the potency of these lipid derivatives are not as strong as many current germicides/insecticides, they offer greater safety in their application. For the most part, these chemicals are approved in food applications. Because of this safety factor, these lipids need to be further exploited.

Soap (oils heated with alkali) was first mentioned on a 4000 year old clay tablet unearthed at Tello in Mesopotamia. Even before the Christian era, soap was used by the "barbarous" people of Gaul and Germany. Roman legions brought the cleaning product to "civilized" people prior to 79 A.D, when Vesuvius overwhelmed Pompeii. From Rome the value of soap spread to the Greeks. The pharmacological application of soaps followed its detergent uses. The germicidal value of soaps was probably recognized much earlier than meager records show. In more recent history, the earliest (1899) mention of soap as an antifungal agent can be found in a paper by J. R. Clark (1).

0097-6156/87/0325-0220\$06.00/0  
© 1987 American Chemical Society

Much of the modern history of fatty acids as germicidal agents can be found in a few excellent reviews (2-4). Until more modern times, the literature between 1920 and 1940 covers the period of greatest research with fatty acids as germicides (5).

A considerable number of chemicals used as insecticides were early recommended by Pliny the Elder (23-79 A.D.) in his Natural History. These were largely collected from folklore and Greek literature of the previous three or four centuries. Again, while soap must have been used for insecticidal purposes by the so-called barbaric peoples of northern Europe, the first early (1787) mention of soap as an insecticide was made by Goeze (6). In 1822, fish oil by itself was advocated for the control of certain caterpillars. A money prize was offered in 1842 by the Massachusetts Horticulture Society for the cheapest and most effective insecticide to destroy the rose chafer. This prize was awarded for the recommendation of whale-oil soap.

From the above it is obvious that fatty acids and saponified products of soap production have played an important role in the history of germicidal and insecticidal agents. Through the modern study of structure-function relationships, it has been possible to better identify those lipid products which are most active against microorganisms and insects.

Interestingly, although bacteria and insects have few characteristics in common, a review of the literature and our own efforts in this field suggest certain universal traits to lipid activity and structure. The following review attempts to collate the known facts and to emphasize structure-function relationships of lipids which maximize their biological effects.

#### Lipophilic Antimicrobial Agents

A complete review of the subject has recently been published (7). Sufficient for our discussion are the variables in fatty acid structures that influence antimicrobial activity: chain length, unsaturation and esterification.

Saturated Fatty Acids. Fatty acids represented by low members of the series  $C_2-C_8$  have little or no germicidal effects. Slightly longer ( $C_8-C_{14}$ ) fatty acids endow much higher antimicrobial activity while saturated fatty acids greater than  $C_{14}$  are usually inactive (Table I). This effect of saturated fatty acids is most pronounced on gram-positive bacteria; Streptococci being more effected than Staphylococci. Fungi, yeast and molds are also inhibited whereas gram negative strains (*Ps aeruginosa* and *E. coli*) are generally not affected. Where reports are given for the antibacterial effect of fatty acids on gram-negative organisms, the effects are weak and usually only static. By contrast, the action of lauric acid ( $C_{12}$ ), the most active of the saturated fatty acids on gram-positive bacteria, is both static and cidal. The optimum chain length ( $C_{12}$ ) found against bacteria is longer by one or two carbons than the optimal size for fungi, i.e.  $C_{10}-C_{11}$ . Branching of the carbon chain decreases the activity of the acyl

chain particularly if one considers the total number of carbon atoms in the acid. The effect of branching is less when only carbon atoms of the largest chain are considered.

TABLE I. Minimal Inhibitory Concentration (mM) of Saturated and Unsaturated Fatty Acids

Fatty Acid	Gram(-)	Gram(+)		Yeast
	Pseudo- monas aeruginosa	Strepto- coccus Group A	Staphylo- coccus aureus	Candida albicans
Caproic	NI	NI	NI	NI
Caprylic	NI	NI	NI	NI
Capric	NI	1.45	2.9	0.9
Lauric	NI	0.12	2.5	2.5
Myristic	NI	0.55	4.4	4.4
Myristoleic	NI	0.11	0.44	0.55
Palmitic	NI	3.9	NI	NI
Palmitoleic	NI	0.1	1.0	0.5
Stearic	NI	NI	NI	NI
Oleic	NI	1.77	NI	NI
Elaidic	NI	NI	NI	NI
Linoleic	NI	0.35	1.79	NI
Linoelaidic	NI	NI	NI	NI
Arachidonic	NI	NI	NI	NI

Source: Reproduced with permission from Ref. 5. Copyright 1972 American Society for Microbiology.

Keeny et al. (8) showed that the optimum chain length was apparently determined by the resistance of the organisms and the solubility of the fatty acids in question. Thus for Aspergillus niger the optimum chain length was 11-carbon atoms, but for the more sensitive Trichophyton interdigitale the 13-carbon acids were most active. Trichophyton purpureum was even less resistant and was inhibited by the 14-carbon acids. Longer-chain compounds fail to show antimycotic effects because, owing to lack of solubility, a static concentration cannot be obtained. The data showed that the fatty acids increase in activity with decreasing pH, provided that the low pH values do not make the compound so insoluble that a static concentration for the organism under test cannot be obtained. The change in activity with hydrogen ion concentration was much greater for the short-chain acids, suggesting that the ion of the long-chain compound - or some aggregate or micellar form of it which may exist in solution - exerts additional action.

It should be emphasized that while fatty acids can be compounded into effective topical agents, their systemic action is nil. This is due to their being readily metabolized by the host through the usual fatty acid pathways. In general, they may be esterified to form glycerides and/or may be degraded to small fragments by beta-oxidation. Asami et al. (9) have demonstrated that 11-iodo-10-undecenoic acid was esterified, in part, in the rat to a glyceride. Although the specific effect of fluorine in the 2-position of fatty acids on esterification has not yet been reported, these fatty acids are believed not to undergo beta-oxidation (10). Thus the 2-fluoro fatty acids

possess at least one potential advantage over the nonfluorinated analogs which would be useful for systemic antifungal activity (11). Taking advantage of this fluorine structure's inability to undergo metabolism, the 15 (2-fluoro) fatty acids were synthesized. Parallel study with the corresponding nonfluorinated analogs against four fungi (11): Aspergillus niger, Trichoderma viride, Myrothecium verrucaria, and Trichophyton mentagrophytes were carried out. Both series of compounds were about equally active, except that the nonfluorinated fatty acids showed maximal activity at chain lengths of 4-10 carbons, whereas the 2-fluoro fatty acids were most active at chain lengths of 8-14 carbons. Eggerth (12-15) found that the  $\alpha$ - bromo fatty acids were usually more germicidal than the unsubstituted soaps.

The effect of a hydroxyl in the  $\alpha$ -position was to increase the selective germicidal action of saturated soaps and to diminish that of unsaturated species. Larson (16) found that Pneumococci and Streptococci would not grow in the presence of even small amounts (<0.1%) of sodium ricinoleate. Larson and Nelson (17) reported that Pneumococci instantly lost their pathogenicity on treatment with castor oil soap at a final dilution of 0.1%. Scarlet fever Streptococci was inhibited after 5 min in 0.5 % sodium ricinoleate. Miller and Castles (18) found the same fatty acid to inhibit the growth of Gonococci on artificial media in dilutions of 1:20,000. Violle (19) studied the effect of a 1:1000 solution of hydroxy fatty acids on many kinds of bacteria. The common and pathogenic bacteria of the intestinal tract were unaffected. Streptococci were killed, but Staphylococci were not. Barnes and Clarke (20) determined that 0.004% sodium ricinoleate and 0.0004% sodium oleate were approximately the minimal Pneumococidal concentrations of the soaps against three types of Pneumococci. In other words, the oleate was much more effective by a factor of 10. Kolmer et al. (21) reported that a 20% solution of sodium ricinoleate was completely bactericidal for S. aureus in an exposure of 5 min, yet a 10% solution was not completely bactericidal in exposures as long as 1 hr when tested according to the Reddish method. The hydroxylated salts, gluconate and trihydroxy stearate, seemed to lack any ability to kill the Pneumococci under the conditions employed (2). The stearate was peculiar in that the addition of a fourth hydroxyl group restored the power to destroy this organism in fairly low concentrations. The hydroxyl group, in the case of the ricinoleate, enhanced the bactericidal activity against the Streptococcus, whereas there was a decrease in activity against the Pneumococcus.

Since early times sulfur has been considered efficacious in the treatment of fungus diseases. Since a sulfur atom is considered structurally equivalent to a  $-\text{CH}=\text{CH}-$  moiety, n-heptylmercaptoacetic acid may be regarded as the isostere of an unsaturated 11-carbon fatty acid. However neither this compound nor a variety of other substituted mercaptoacetic acids showed activity of the same magnitude as the fatty acids (4).

With the exception of 11-thiohendecanoic acid, none of the

other thiohendecanoic acids approached undecylenic acid in activity. The latter fatty acid was found by Rothman et al. (22) to be responsible for the spontaneous cure of ringworm of the scalp during puberty.

Unsaturated Fatty Acids: Whereas lauric acid is the most active of the saturated fatty acids, palmitoleic ( $C_{16:1}$ ) is the most active of the monounsaturated series. Both the type of unsaturation, geometrical configuration, bond position and chain length are important to conferring antimicrobial activity.

Unsaturated fatty acids with acetylenic linkages were reported to be less active against some types of bacteria as compared to ethylenic fatty acids (2). In a more detailed comparative study of the problem, we found acetylenic derivatives to be slightly more active (23). It is emphasized that cis-unsaturated isomers were active while the trans isomers were always less active or inactive when compared to the more "common" unsaturation form.

Esterification of Fatty Acids. It was earlier discovered that while the esterification of a fatty acid leads to an inactive species, esterification of a hydroxy fatty acid still yielded an active ester (Table II). From these and other experiments, (24-26), a free single or multiple hydroxy group was necessary for effective biological activity. One of the more common polyhydric alcohols, glycerol, was esterified and found to be more active than the corresponding fatty acids (see Table III). Details of these findings have been confirmed by others (27-30). There seems to be some controversy or confusion as to which fatty acid ester is the most biocidal. As a general statement, the fatty acid used to esterify the polyol determines the potency of the ester. The structure-function relationship for saturated and unsaturated esters follows the activity of their respective fatty acids as reviewed in an earlier section. This means that lauric acid ( $C_{12}$ ) and palmitoleic acid ( $C_{16:1}$ ) form the most active saturated and unsaturated derivatives, respectively (25, 28).

TABLE II. Minimal Inhibitory Concentrations

Dodecyl derivative	Microorganism (gram-positive)	
	Pneumococci (mM)	Group A Streptococcus (mM)
Lauric acid	0.06	0.12
Lauryl alcohol	0.07	0.07
Lauryl aldehyde	0.14	0.14
Methyl laurate	>4.6	>4.6
Cholesteryl laurate	NI	NI
Dodecanedioic acid	NI	NI
Hydroxy Lauric acid	-	0.23
Hydroxy Methyl laurate	-	0.54

Source: Reproduced with permission from Ref. 5. Copyright 1972 American Society for Microbiology.

TABLE III. Minimum Inhibitory Concentrations ( $\mu\text{g/ml}$ ) for Fatty Acids and Their Corresponding Monoglycerides

Organism <sup>a</sup>	Unde-	10-Unde-	10-Unde-	10-Unde-	11-Dode-	Dode-	Dode-	12-Tride-	Tride-
	canoic acid	canoic acid	canoic acid	canoic acid	canoic acid	canoic acid	canoic acid	canoic acid	canoic acid
	Ni <sup>b</sup>	500	500	500	Ni	500	Ni	1000	Ni
Streptococcus faecalis	125	1000	125	125	250	62	8	125	1000
Streptococcus pyogenes	1000	1000	500	500	Ni	500	250	1000	Ni
Staphylococcus aureus	31	31	62	62	125	31	16	31	Ni
Corynebacterium sp.	62	125	125	62	62	62	16	125	1000
Nocardia asteroides	1000	1000	250	100	1000	1000	500	1000	Ni
Candida albicans	500	500	250	100	500	1000	250	500	1000
Saccharomyces cerevisiae									

<sup>a</sup> Escherichia coli and Pseudomonas aeruginosa were not affected.

<sup>b</sup> Ni - MIC 1000  $\mu\text{g/ml}$

Source: Reproduced with permission from Ref. 24. Copyright 1977 American Oil Chemists' Society.



Beuchat (30) and Shibasaki (31) have supported the finding that the lauric acid derivative is the most active monoglyceride even in the presence of potential inhibiting materials. Beuchat (30) compared the effects of glycerides, sucrose esters, benzoate, sorbic acid, and potassium sorbate against *Vibrio parahaemolyticus*. His remarks indicated that the C<sub>12</sub> monoglyceride was more active than lower (C<sub>8</sub>, C<sub>10</sub>) or higher (C<sub>14</sub>) chain length derivatives. Also, the low Minimum Inhibitor Concentration (MIC) value for Monolaurin (5 G/ml or less) indicated it to be more effective than sodium benzoate (300 G/ml) or sorbic acid (70 MG/ml).

Antimicrobial activity was also found for esters of more complex polyhydric alcohols (25, 32). Kato and Arima (32) reported that the sucrose mono ester of lauric acid was active against a gram-negative organism, whereas Conley and Kabara (25) and Shibasaki and Kato (29) indicated that these and other esters are primarily active against gram-positive bacteria and fungal organisms. In contrast to data generated for glycerides, the diester of sucrose rather than the monoester was more active. Sucrose dicaprylate possessed the highest activity of the sucrose esters tested, but was still less active than monolaurin (30,33,34).

The monoesters of glycerol and the diesters of sucrose not only have higher antimicrobial activity than their corresponding free fatty acids, but also compared favorably in activity with commonly used antiseptics such as parabens, sorbic acid, and dehydroacetic acid (see Tables IV and V).

TABLE IV. Comparison of the Antifungal Activities of Fatty Acid Esters and Some Commonly Used Preservatives

Food additive	Minimum inhibitory concentration ( $\mu\text{g/ml}$ ) <sup>a</sup>		
	<i>Aspergillus niger</i>	<i>Candida utilis</i>	<i>Saccharomyces cerevisiae</i>
Monocaprin	123	123	123
Monolaurin	137	69	137
Butyl-p-hydroxybenzoate	200	200	200
Sodium lauryl sulfate	100	400	100
Sorbic acid	1000	1000	1000
Dehydroacetic acid	100	200	200

<sup>a</sup>By the agar dilution method

Source: Reproduced with permission from Ref. 33. Copyright 1975 Nippon Bokin Bobai.

TABLE V. Comparison of the Antibacterial Activities of Fatty Acid Esters and Some Commonly Used Preservatives

Food additive	Minimum inhibitory concentration ( $\mu\text{g/ml}$ ) <sup>a</sup>		
	<i>Bacillus subtilis</i>	<i>Bacillus cereus</i>	<i>Staphylococcus aureus</i>
Sucrose	74	74	148
dicaprylin			
Monocaprin	123	123	123
Monolaurin	17	17	17
Butyl-p-hydroxybenzoate	400	200	200
Sodium lauryl sulfate	100	100	50
Sorbic acid	4000	4000	4000

<sup>a</sup>By the agar dilution method

Source: Reproduced with permission from Ref. 33. Copyright 1975 Nippon Bokin Bobai.

Another series of food-grade esters was derived from a group of compounds called polyglycerol esters. These linear polymers of glycerol, discovered and advocated for food use by Babayan et al. (35), were supplied to us by Dr. Babayan for biocidal testing. Similar to results with sucrose esters, the medium-chain fatty acid esters appeared to be the most active (25). It was of interest that, regardless of whether the polyol was tri-, hexa-, or decapolyglycerol, the fatty acid moiety seemed to determine overall biocidal activity. Generally, as the polyol became bulkier, the spectrum of biocidal activity became narrower. Other polyol esters have been tested, and except for special applications, none have proven more useful than monolaurin (see Table VI).

TABLE VI. MIC Values of Various Glycerol and Polyglycerol Esters (mM) Against *Streptococcus pyogenes*

Fatty Acid Moiety	Glycerol	Triglycerol	Hexaglycerol	Decaglycerol
Acetate C <sub>2</sub>	>7.46	>3.55	>1.98	>1.25
Butyrate C <sub>4</sub>	>6.17	>3.23	>1.98	0.12
Caproate C <sub>6</sub>	2.63	1.47	0.89	>1.17
Caprylate C <sub>8</sub>	2.29	0.27	0.17	0.11
Caprate C <sub>10</sub>	0.20	0.15	0.19	0.11
Laurate C <sub>12</sub>	0.05	0.29	0.19	0.26

Source: Reproduced with permission from Ref. 25. Copyright 1973 American Society for Microbiology.

#### FATTY ACIDS AS INSECTICIDES

With the use of chemicals the housewife gave up the broom and swatter as mechanical control of pests. Chemical control has obvious advantages in that it brings prompt relief. On the other hand, "chemical warfare" has effects more widespread than

simply eliminating the pests. These side effects often present dangerous fallout to household pets and family members.

Close relations between the entomologist and the plant physiologist are important in the study of insecticides. When insecticides are applied to plants to kill insects thereon, there is often only a slight margin of safety in favor of the plant. A plant, like a pest, is a living organism susceptible to poisoning. Therefore, with any insecticide, the relative susceptibility of different plant species to fatty acids needs to be considered. This includes information not only of the plant in question but also the rate of application, time of application, etc.

Insecticides that we applied for the control of insects on plants are generally divided into two types: Systemic poisons and Contact poisons. The latter are divided into irritants killing by contact and substances that close spiracles and kill by suffocation. Fatty acids probably work by interfering with exchange of respiratory gases, i.e. killing by suffocation. This contact poison joins pyrethrum and petroleum products as a contact insecticide.

It became an early practice to combine kerosene and soaps to form emulsions that had varying degrees of effectiveness. Such mixtures were reported in Gardener's Monthly as early as 1868. A formula published in the American Agriculturist (vol. 35, p. 207, June 1876) involved whale oil soap, kerosene, and water. From 1880 to 1900 it was considered the ideal contact insecticide (6).

William Forsyth is said to be the first (1800) to advise the use of whale oil against scale insects on plants (6). By the 1850s, whale oil soap was found to be the cheapest and most effective insecticide to destroy rose chafer. By 1866 vegetable oils replaced kerosene by being much safer. Free vegetable oil found little use in the U.S. and other countries where petroleum and coal tar preparations were developed.

The saponified oils rather than the oils themselves were found to be most effective. Fleming and Baker (1931) reported on the effectiveness of potassium soaps of certain vegetable oils against adult Japanese beetles (Table VII(36)). No relationship exists between the saponification number of the oil and order of effectiveness. However, Van der Molen and VanLeeuwen (37) did show a correlation between film characteristics and per cent mortality (Table VIII).

One of the better early reviews on structure-function relationships of fatty acids as insecticides was written by F. Tattersfield (41). The author points out that as the series of fatty acids is ascended, toxicity from acetic to undecanoic increases, declines somewhat at dodecanoic and tridecanoic and disappears in tetradecanoic acid and higher.

In the unsaturated series, the undecenoic is no more active than the saturated fatty acid. However, oleic acid is more toxic than stearic acid.

Methylation of the carboxyl group results in a loss or a diminution in insecticidal activity. The correlation between partition coefficient (hydrophobic/hydrophilic balance) and insecticidal value of fatty acids may be explained by the

TABLE VII. The Effectiveness of Potassium Soaps of Certain Vegetable Oils Against Adult Japanese Beetles

Vegetable Oil	Approximate soap analysis	Saponification number	Descending order of Effectiveness
Cottonseed oil	(Palmitate, 23 percent)		
	( )		
	(Oleate, 21 percent)	195	1
	( )		
	(Linoleate, 46 percent)		
Soybean oil	(Oleate, 40 percent)		
	( )	188	2
	(Linolenate, 60 percent)		
Raw linseed oil	(Linolenate, 28 percent)		
	( )	189	3
	(Linoleate, 72 percent)		
Boiled linseed oil	Same	190	4
(Standard oleate)	----	---	5
Blown castor oil	Ricinoleate, 100 percent	288	6
Peanut oil	Oleate, 100 percent	197	7
Coconut oil	----	---	8

Source: Reproduced with permission from Ref. 36. Copyright 1934 Entomological Society of America.

TABLE VIII. Correlation of Soap Film Qualities With Soap Toxicity

Potassium soap	Film characteristics	Japanese beetle mortality percent
Palm oil )	Very tough	70
)		
Beef tallow )	A little less tough	55 to 68
)		
Mutton tallow )		
)		
Coconut oil )		
)		
Cocoa butter )	Little or not at all adherent	50 to 54
)		
Lard )		
)		
Cottonseed oil )		
)		
Soybean oil )		
)		
Corn oil )		
)		
Neatsfoot oil )	Not continuous	25 to 48
)		
Menhaden oil )	Weak or none	2 to 8
)		
Linseed oil )		
)		
Olive oil )		
)		
Oleic acid )		
)		
Japan wax )		
)		
Castor oil )	Weak or none	2 to 8
)		
Rape oil )		
)		
Whale oil )		
)		
Codliver oil )		

Source: Reproduced with permission from Ref. 36. Copyright 1934 Entomological Society of America.

relationship between membrane solubility, penetration and toxic action.

Although Siegler and Popence, (1925) are responsible for the early lively interest in the toxicity of the fatty acid derivatives containing a 12-carbon chain, the common use of coconut oil is only in part due to their suggestions (38). It should be noted that coconut oil is the most important and widely used oil in the soap industry. The principal pests treated were aphids: rosy and green apple aphids; bean aphids; black chrysanthemum, etc.

Dills and Menusan (1935) found that the fatty acids most toxic to insects caused the greatest plant injury (Table IX(39)). In the case of the potassium soaps, toxicity to plants and insects was not correlated. Plant injury decreases as the size of the soap molecule increases. Ginsburg and Kent (1937) pointed out that not all plants can tolerate the concentrations of soap necessary to kill some insects (4). They determined that 0.25 per cent potassium coconut oil soap produced no injury of any plants tested, 0.5 per cent injured some delicate flowers, 1.0 percent produced injury; a considerable proportion of cases, and 2.0 percent injured most of the plants, including such orchard foliage as peach, cherry and grape.

TABLE IX. Toxicity of Emulsified Fatty Acids and Their Potassium Soaps to *Aphis rumicis*

Fatty Acid	No. carbon atoms	Mortality with soap, percent
Caproic	6	6.9
Caprylic	8	36.9
Capric	10	55.2
Lauric	12	67.1
Myristic	14	30.4
Palmitic	16	24.9
Stearic	18	12.8
Oleic	18	85.1
Emulsifier 1/4%	--	--

Source: Reproduced with permission from Ref. 39. Copyright 1939 Boyce Thompson Institute for Plant Research, Inc.

Salzberg and Dietz combined the lauryl chain with the rhodanate as lauryl rhodanate (United States patents 1,963,100 (1934) and 1,992,040 (1935)). They found the lauryl derivative to be of uniformly high toxicity to several insects. Against the red spider the lauryl derivative was more effective than the capric or myristic rhodanates. On the other hand, the green chrysanthemum aphid was killed almost as well by the 8-, 10- and 14- carbon rhodanates as by the 12-carbon one.

One of the more important and useful papers in this field of fatty acid insecticides was written by Ginsburg and Kent (42). While it had been found that concentrations of 1.0% percent or stronger soap is sufficient to kill many soft bodied insects and the larvae (43-44), these concentrations are injurious to certain plants. Using a potassium coconut oil as an example, Ginsburg and Kent reported on injury of greenhouse (Table X), orchard (Table XI), and flowering plants (Table XII).

TABLE X. Tests With Various Concentrations of Coconut Oil Soap on Greenhouse Plants

Name of Plant	Injury from 0.25% & 0.5% Soap	Injury from 1% Soap	Injury from 2% Soap
Begonia	None	None	Slight injury to leaves
Buddleia	None	Injury to leaves	Severe injury
Crassula	None	None	None
Carnation	None	None	Injury to stems & leaves
Chrysanthemum	None	None	None
Dracaena	None	None	None
Euphorbia	None	Injury to young leaves	Severe injury
English Ivy	None	Injury to young leaves	Injury to leaves
Fern (Boston)	None	Injury to leaves	Severe injury to leaves
Fern (Whitmanii)	None	Injury to leaves	Severe injury to leaves
Hydrangea	None	None	Slight injury
Poinsettia	None	None	Severe injury
Snapdragon	None	Injury to leaf tips	Severe injury
Stevia	None	None	None

Source: Reproduced with permission from Ref. 42. Copyright 1937  
New York Entomological Society.

TABLE XI. Tests With Various Concentrations of Coconut Oil Soap on Orchard and Garden Plants

Name of Plant	Injury from 0.25% & 0.5% Soap	Injury from 1% Soap	Injury from 2% Soap
Apple	None	None	None
Peach	None	None	Slight injury to foliage.
Cherry	None	None	Slight injury to foliage.
Grape	None	None	Appreciable injury to shoots & foliage
Beets	None	None	None
Cabbage (red)	None	None	None
Cabbage (green)	None	None	None
Kohlrabi	None	None	None
Corn	None	None	None
Cucumber	None	None	None
Cantaloupe	None	None	None
Egg Plant	None	No injury	Injury to blossoms, none to leaves
Lettuce	None	Injury to leaves	Severe injury
Lima Beans	None	None	None
Pumpkin	None	Injury to foliage	Injury to foliage
Blackberries	None	Injury to foliage & young shoots	Injury to foliage & young shoots
Sweet Potatoes	None	No injury	Slight injury to foliage
Squash	None	None	None
Rose	None	None	Injury to young leaves & flowers
String Beans	None	Slight injury to young leaves	
Sweet Peas	None	Slight injury to young leaves	
Tomato (young plants)	None	None	Slight injury
Tomato (plant in blossom)	None	None	Injury to blossoms
Peppers	None	Injury to leaves	Injury to leaves

Source: Reproduced with permission from Ref. 42. Copyright 1937  
New York Entomological Society.



TABLE XII. Tests With Coconut Oil Soap on Flowering Plants in Bloom

Name of Plant	Injury from 0.25% Soap	Injury from 0.5% Soap
Canna	None	None
Chrysanthemum, Hardy	"	"
Dahlia, Dwarf	"	"
Daisy	"	"
Delphinium	"	Injury to flowers
Geranium	"	None
Geranium, Sweet	"	"
Heliotrope	"	"
Hollyhock	"	Injury to flowers
Ice Plant	"	Injury to flowers
Marigold	"	None
Petunia	"	Injury to flowers
Phlox	"	None
Ragged Sailor	"	"
Roses	"	"
Gladiolus	"	Injury to flowers

Source: Reproduced with permission from Ref. 42. Copyright 1937 New York Entomological Society.

From these results it appears that coconut oil soap in concentrations of 0.5 per cent or lower is safe to apply on all kinds of plants with the exception of delicate blossoms.

Although most studies on structure-function relationships involving germicidal/insecticidal activity are in agreement, esters and potassium salts of fatty acids are active insecticides while remaining inactive germicides (45). Rather than postulate different mechanisms of action, one reasonable but not proven explanation is that the ester undergoes hydrolysis to the free acid in insects.

The picture of acid versus potassium salt soaps (K-soaps) is not clear. While K-soaps significantly enhanced the toxicity of capric, stearic and linoleic, it reduced the effects of caproic and palmitic; the other fatty acids were unchanged. These observations by Puritch (46) need to be confirmed before any mechanism of action can be postulated since it is accepted that the unionized fatty acid is the active specie.

Fatty acids have lethal effects on a variety of organisms (Table XIII). This wide spectrum of activity against so many pests and its safety to humans highly recommend the further application of natural and synthetic lipids as potential insecticides.

TABLE XIII. Control of Various Household and Garden Pests by Coconut Fatty Acid Soap

Adelgids	Lace Bugs	Scales
Aphids	Leaf Hoppers	Spittlebugs
Cricket	Mealybugs	Springtails
Earwigs	Mites	Tent Caterpillars
Fruitfly Adults	Plant Bugs	Thrips
Fungus Gnats	Psyllids	Whitefly
Grasshoppers	Sawflies	Woolly Aphids

#### Future Role of Fatty Acids as Germicides/Insecticides

To paraphrase Mark Anthony in Shakespeare's Julius Caesar. "The evil that insecticides/germicides do lives after them." The Love Canal and other monuments to man's chemical follies may indeed be "interred with our bones". In a report by the World Health Organization (WHO,1971) it was noted that of 1500 consecutive compounds entering the WHO screening procedure, only five emerged as of potential value (47). All of the above point out the risk and hazard of looking for new compounds as potential germicide/insecticide agents. The tremendous investment of time and money makes industry very cautious. Not only are development expenses astronomical, but also the liability costs must be factored.

Figures are hard to generate for individual products because of the proprietary nature of such information. A review of the problem (48) showed that in 1970 the pesticide industry claimed an average cost of \$5.5 million for the development of a

new pesticide. The time required for commercialization was 77 months. A similar survey of 14 companies reported an average cost of \$4 million in 60 months in 1969. These figures are too low since they do not include the cost of pilot plants, process development, or studies of waste control and other environmental factors. Their more realistic estimate leads to a figure of \$11 million over 10 years, which includes not only the cost of developing unsuccessful compounds, which must be borne by successful ones, but also the additional cost involved in the failure to invest \$11 million at 8 percent interest if the company had instead chosen merely to deposit the money in the bank. In 1985, the cost is probably closer to \$50 million and the time required for commercialization is 10-12 years. Little wonder that only a few major companies can afford the money, much less the gamble on a "new" chemical.

The above scenario makes the timing of looking for applications of old, safe chemicals more attractive. There is little doubt in my mind that these old chemicals (fatty acids and mono-esters) will be the agents of choice in the future. What is currently needed is how we can best formulate these lipids into effective, safe and cheap products. With these chemicals it is comforting to know that they can become part of the solution and not part of the problem.

#### Literature Cited

1. Clark, J.R. Bot. Gaz. 1899, 28, 289-327.
2. Bayliss, M. J. Bacteriol. 1936, 31, 489-504.
3. Kodicek, E. Soc. Exp. Biol. Symp. 1949, 3, 217-32.
4. Nieman, C. Bacteriol. Rev. 1954, 18, 147-63.
5. Kabara, J.J.; Swieczkowski, D.M.; Conley, A.J.; Truant, J.P. Antimicrob. Agents Chemother. 1972, 2, 23-8.
6. Shepard, H.H. "The Chemistry and Toxicology of Insecticides"; Burgess: Minneapolis, 1939; p. 11.
7. Kabara, J.J. "Cosmetic & Drug Preservation", Marcel Dekker: New York, 1984, p. 275-304.
8. Keeny, E.L. Clin. Invest. 1944, 23, 929-34.
9. Asami, Y.; Kusakabe, A.; Eriguchi, K.; Amemiga, M.; Itabe, A.; Ueno, G.; Saito, S.; Sakai, Y.; Tanaka, Y.G.; Rikagaku Kenkyusho Hokoku 1965, 41, 259-65; Chem. Abstr., 1966, 64, 18271.
10. Pattison, F.L.M.; Buchanan, R.L.; Dean, F.H. Can. J. Chem. 1965, 43, 1700-9.
11. Gershon, H.; Parmegiani, R. J. Med. Chem. 1967, 10, 186-90.
12. Eggerth, A.H. J. Gen. Physiol. 1926, 10, 147-60.
13. Eggerth, A.H. J. Exp. Med. 1927, 46, 671-88.
14. Eggerth, A.H. J. Exp. Med. 1929, 49, 53-62.
15. Eggerth, A.H. J. Exp. Med. 1931, 53, 27-36.
16. Larson, W.P. Proc. Soc. Exp. Biol. Med. 1921, 19, 62-3.
17. Larson, W.P.; Nelson, E. Proc. Soc. Exp. Biol. Med. 1931, 22, 339-42.
18. Miller, C.P.; Castles, R. J. Bacteriol. 1931, 22, 339-50.

19. Violle, H. Rend. Acc. Sci. 1933, 197-204, 714.
20. Barnes, L.A.; Clarke, C.M. J. Bacteriol. 1934, 27, 107-17.
21. Kolmer, J.A.; Rule, A.M.; Madden, B. J. Lb. Clin. Med. 1934, 19, 972-85.
22. Rothman, S.; Smiljanic, M.; Shapiro, A.L.K. Proc. Soc. Exp. Biol. 1945, 60, 394-95.
23. Kabara, J.J.; Conley, A.J.; Swieczkowski, D.J.; Ismail, I.A.; Lie Ken Jie, M.; Gunstone, F.D. J. Med. Chem. 1973, 16, 1060-63.
24. Kabara, J.J.; Vrable, R.; Lie Ken Jie, M. Lipids 1977, 9, 753-59.
25. Conley, A.J.; Kabara, J.J. Antimicrob. Agents Chemother. 1973, 4, 501-6.
26. Kabara, J.J. J. Food Prot. 1981, 44, 633-47.
27. Kato, N.; Shibasaki, I. J. Ferment. Technol. 1975, 53, 793-801.
28. Sands, J.A.; Auperin, D.A.; Landin, P.D.; Reinhardt, A.; Cadden, S.P. In "Pharmacological Effect of Lipids"; Kabara, J.J., Ed.; American Oil Chemists' Society: Champaign, Ill., 1979; pp. 75-95.
29. Shibasaki, I.; Kato, N. In "Pharmacological Effect of Lipids"; Kabara, J.J., Ed.; American Oil Chemists' Society: Champaign, Ill., 1979; pp. 15-24.
30. Beuchat, L.R. Appl. Environ. Microbiol. 1980, 39, 1178-84.
31. Shibasaki, I. J. Food Safety 1982, 4, 35-58.
32. Kato, A.; Arima, K. Biochim. Biophys. Res. Commun. 1971, 42, 596-601.
33. Kato, A.; Shibasaki, I. J. Antibacterial Antifung. Agents 1975, 8, 355-61.
34. Kato, N.; Shibasaki, I. J. Anti-bacterial Antifung. Agents 1976, 4, 254-61.
35. Babayan, V.K.; Kaufman, T.G.; Lehjman, H.; Tkaczuk, R.J. J. Soc. Cosmet. Chem. 1964, 15, 473-79.
36. Fleming, W.E.; Baker, F.E. Jour. Econ. Ent. 1934, 23, 625-30.
37. Van der Moulén, P.A.; VanLeeuwen, E.R. Jour. Econ. Ent. 1929, 23, 812-14.
38. Siegler, E.H.; Popence, C.H. Jour. Econ. Ent. 1925, 18, 292-99.
39. Dills, L.E.; Menusan, H., Jr. Contrib. Boyce Thompson Inst. 1935, 7, 63-82.
40. Ginsburg, J.M.; Kent, C. Jour. New York Ent. Soc. 1937, 45, 109-13.
41. Tattersfield, F. J. Agric. Sci. (Cambridge) 1927, 17, 181-208.
42. Ginsberg, J.M.; Kent, C. Jour. New York Ent. Soc. 1937, 45, 109-13.
43. Bourcart, E. "Insecticides, Fungicides & Weed Killers"; D. Vostrand Co.: New York, 1925.
44. Metcalf, C.L.; Flint, W.P. "Fundamentals of Insect Life"; McGraw-Hill Co.: New York, 1932.

45. McFarlane, J.E. Comp. Biochem. Physiol. 1968, 24, 377-84.
46. Puritch, G.S. In "The Pharmacological Effects of Lipids"; Kabara, J.J., Ed.; American Oil Chem. Soc.: Champaign, IL., 1978, pp. 105-12.
47. Wright, J.W. Wld. Hlth. Org. tech. Rep. Ser. 1971, 513, 8-9.
48. Djerassi, C.; Shih-Coleman, C.; Diekman, J. Operational & Policy Aspects Science 1974, 186, 596-607.

RECEIVED August 19, 1986

## Chapter 15

# Hopanoids: Sterol Equivalents in Bacteria

Karl Poralla and Elmar Kannenberg

Institute for Biology II, Microbiology I, University of Tübingen, Auf der Morgenstelle 1,  
D-7400, Tübingen, Federal Republic of Germany

Sterols (e.g., cholesterol and ergosterol) are known to occur nearly exclusively in higher organisms (eukaryotes). Only a few bacteria synthesize or utilize sterols. Hopanoids, a class of pentacyclic triterpenoids, are found in about 50% of the bacterial strains investigated. These hopanoids mostly contain an extended side chain with polar groups, therefore they resemble the geohopanoids found in crude oil and geological sediments. In model membrane systems hopanoids condense phospholipids, enhance viscosity, and diminish permeability. In certain bacteria, subsequent to an increase in growth temperature or in ethanol concentration, the hopanoid content in the cellular lipid fraction is enhanced. In cholesterol-dependent bacteria, hopanoids can substitute for the sterol. From these experiments one can conclude that hopanoids possess a membrane-stabilizing function very similar to sterols.

If one specifies the differences between bacteria (prokaryotes) and higher organisms (eukaryotes), one point is always mentioned. Higher organisms contain sterols whereas bacteria do not. Only a few exceptions are known for the occurrence of sterols in bacteria. Do bacteria dispense with such an important group of compounds? It will be shown that bacteria contain similar compounds - namely the hopanoids. These are structural and functional equivalents of sterols.

### Occurrence of Sterols

Sterols comprise a subgroup of steroids normally containing one hydroxy group. The side chain is not shortened and  $4\alpha$ -,  $4\beta$ - and  $14\alpha$  methyl groups can be cleaved off. Sterols occur in all higher organisms, e.g., protozoa, photosynthetic plants, fungi, and animals [1]. If they cannot synthesize sterols by themselves the organisms take them up from the environment. Thus the sterols

0097-6156/87/0325-0239\$06.00/0  
© 1987 American Chemical Society

play the role of a growth factor. The best known examples for such a role are in insects [2].

Sterols occur in significant amounts in a few bacterial species. The presence of significant amounts must be stressed, otherwise they could not fulfill a function as a membrane strengthener. Detection in low amounts is not always significant because the possibility of an impurity from outside the bacterium exists. However an impurity can be excluded by incorporation of a labelled precursor, e.g. mevalonate, squalene or even glucose.

The bacterial genus Mycoplasma is well known for the occurrence of and dependence on sterols [3], but these bacteria without cell walls do not synthesize their sterols by themselves [4]. Bacterial species which contain and synthesize sterols by themselves are Methylococcus capsulatus [5,6], Nannocystis exedens [7] and an L-form of Staphylococcus aureus [8]. It is interesting to note that Methylococcus capsulatus simultaneously contains hopanoids [6].

### Biosynthesis of Sterols

Biosynthesis starts from mevalonic acid to produce squalene in 6 enzymatic steps. This intermediate is epoxidized in a reaction in which one atom is incorporated from molecular oxygen [9]. Sterols are cyclized from epoxysqualene to form cycloartenol in photosynthetic plants and lanosterol in fungi and animals [10,11]. This circumstance presents a very strong argument for biosynthesis of sterols being invented at least twice in the course of evolution. The invention probably occurred after the separation of photosynthetic plants from fungi and animals [12]. The connection between biosynthesis in the large taxonomic groups bacteria, protozoa, plants, fungi and animals is shown in Fig. 1.

The cyclization products of epoxysqualene are further aerobically processed to obtain sterols devoid of the methyl groups at C-4 and C-14. The side chain is conserved in its length but not always in its bulkiness. Furthermore, the degree of unsaturation in ring B can differ. The configuration of the rings A - D is all transatic; thus the sterol molecules are flat. All sterols contain a  $\beta$ -3-hydroxy group [1].

### Functions of Sterols in Membranes

The main occurrence of free sterols is in the cytoplasmic membrane, where they interact with other lipids and proteins. Two modes of action for sterols in membranes are proposed. One is the so called bulk membrane function, i.e., the interaction with phospholipids and the spatial separation of these charged molecules [13]. The other role is a cofactor function for the incorporation of unsaturated fatty acids into lipids [14].

The tetracyclic, amphipathic sterols incorporate into a bilayer membrane and through van der Waals forces they interact with the acyl chains of phospholipids. When the temperature is below the transition temperature of the phospholipids, sterols introduce a sort of disturbance into the ordered lipids. Thereby the transition temperature is lowered and, depending on the sterol concentration, the phase transition is diminished or even abolished. Above the phase transition temperature, sterols reduce acyl chain mobility, a

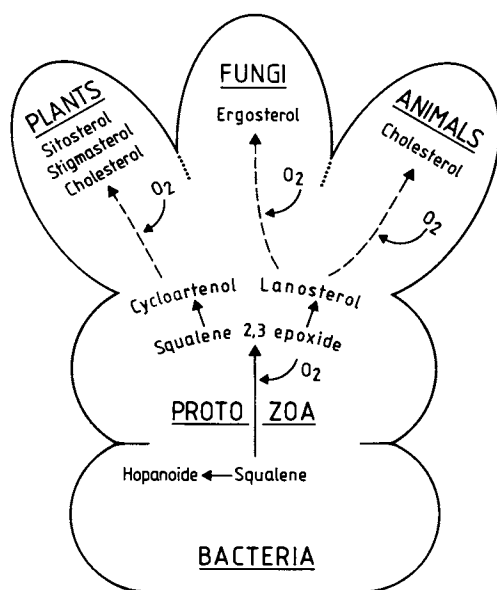


Fig. 1: The distribution of biosynthetic pathways for polycyclic triterpenoids with membrane function in the major taxonomic groups.



phenomenon called condensation. In other types of experiments one can observe an increase in microviscosity and a lower permeability of the membrane for small molecules [13,15]. These effects can be observed with different types of sterols; to a low degree with an unprocessed sterol containing all methyl groups (e.g. lanosterol) and to a high degree with cholesterol. It should also be mentioned that sterols possess the potential to stabilize membranes of negatively charged lipids merely by separating the charged molecules [14]. By this separation, repulsion of the molecules is diminished.

Furthermore (processed) sterols can have a totally different function beside their influence on bulk membrane function. First indications for a metabolic function were obtained by experiments showing a synergism between two sterols. Low amounts of a processed sterol and high amounts of precursor sterol produce as much growth as high amounts of the processed sterol alone [17]. In Mycoplasma it could be shown that cholesterol promotes unsaturated fatty uptake from culture medium into lipids [17]. This growth promoting effect is not accompanied by an enhancement of the microviscosity in the membrane. Such synergism was also shown in yeast [18,19].

### Structures and Biosynthesis of Hopanoids

William R. Nes was the first to propose that bacteria could have sterol-like pentacyclic molecules [20]. Some years later these were found to be hopanoids by Ourisson, Rohmer and Albrecht [21,22]. Hopanoids comprise a group of pentacyclic triterpenoids for which some structures are shown in Fig. 2. Hopanoids are rigid, flat, amphipathic molecules with geometric dimensions similar to sterols. Their main structural differences are: 1. The cyclic system contains 5 rings. As a consequence, the side chain is shorter. 2. The hydrophilic part is the side chain, not the nucleus. 3. The side chain can be extended and further hydroxyl functions or other groups introduced [23]. By mainly structural arguments, it was surmised by Ourisson and Coworkers that hopanoids have a sterol-like function [21].

Hopanoids (diplopterol and/or hopene) are cyclized directly from squalene. Thus the biosynthesis is anaerobic and all methyl groups are conserved [24,25]. The hydroxy group of diplopterol probably originates from water. This mode of biosynthesis was proven for another pentacyclic triterpenoid, tetrahymanol [26].

Besides not being removed, methyl groups are not shifted during the enzymatic cyclization. Thus, one perceives the hopanoids as a sort of crude sterol. In contrast to the lack of alteration of the nucleus the variations of the extended side chain are numerous (Fig. 2).

### Occurrence of Hopanoids

The biological significance of hopanoids was detected indirectly. First, they were known to occur exclusively as secondary metabolites of higher organisms; trees, grasses, ferns, and lichens. These hopanoids contain 30 C-atoms and are, therefore, devoid of an extended side chain [27]. Examples for such hopanoids are diplopterol and hopene (Fig. 2).

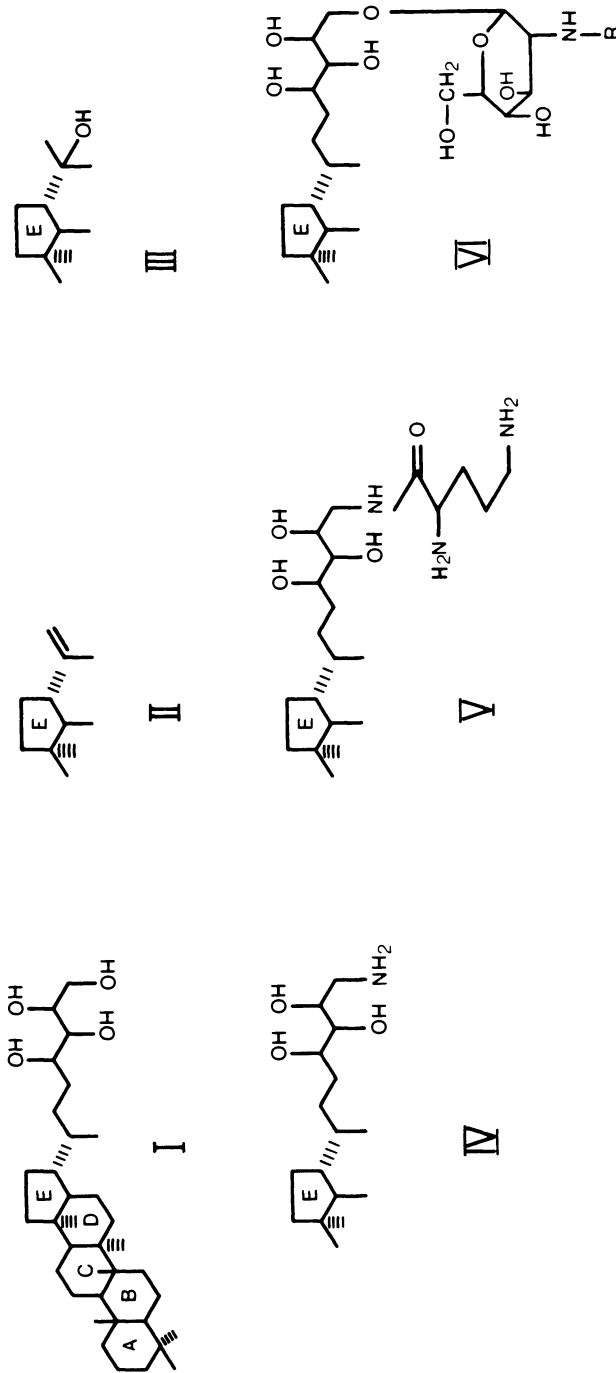


Figure 2. Bacterial hopanoids. Compounds II and III occur also in plants. I, 32,33,34,35-tetrahydroxybacteriohopane (= THBH) II, hopene; III, diplopterol (= hopanol); IV, 35-amino-32, 33, 34-trihydroxybacteriohopane; V, 35-ornithyl-32,33,34-trihydroxybacteriohopane; VI, 35-(O- $\beta$ -N-acetylglucosaminyl)-32,33,34-trihydroxybacteriohopane.

Hopanoids and their derivatives were detected in crude oils and in the organic part of geological sediments by the groups of Ourisson, Albrecht and Eglinton [22,28]. The global stock of hopanoids and their derivatives in soluble organic matter of geological sediments is estimated by Ourisson to be more than 5%. Therefore, hopanoids comprise more material than all living matter on earth. The hopanoid derivatives show various alterations of their carbon skeleton, some examples of which are given in Fig. 3. These so-called geohopanoids often contain an extended side chain or an additional methyl group on the nucleus. For this reason, they cannot have originated from plants.

After the detection of hopanoids in recent sediments such as coastal muds, it was consistent to find them also in bacteria. However, it was very surprising to find them in as many as 50% of the bacterial species studied. These bacterial hopanoids often contain an extended side chain [21,19] and resemble therefore, the geohopanoids. Thus it can be concluded that the geohopanoids are of bacterial origin.

The distribution of hopanoids in the bacterial kingdom does not follow a regular pattern [29]. In some groups every tested strain contains hopanoids, e.g. methylotrophic bacteria and Rhodospirillaceae (a group of photosynthetic bacteria). In other groups a significant part (Cyanobacteria) or only a few representatives (Streptomyces) or none (e.g. Archaeobacteria) possess hopanoids. Further examples of bacteria containing hopanoids are Bacillus acidocaldarius, a bacterial group isolated from acid volcanic springs and soil [30,31] and Zymomonas mobilis, an ethanol producing bacterium [32,33].

#### The Role of Hopanoids in Bacteria

We first ask the question can hopanoids substitute for the sterol function in the sterol dependent bacterium Mycoplasma? In experiments by other groups [3,34], it has been shown that the sterol requirement fulfilling the bulk membrane function is not very specific. Lanosterol, cycloartenol and different demethylated sterols can support growth. In our experiments diplopterol also supports growth [35]. The much bulkier hopane glycolipid (compound VI in Fig. 2) is not growth promoting. No attempts were made to demonstrate a synergism between diplopterol and cholesterol.

Bacillus acidocaldarius which lives in acid hot springs grows best at 60°C and pH 3. Similar species live in high titers in soil [31]. This bacterium has hopanoids and lipids containing  $\omega$ -cyclohexane fatty acids in its membrane [30,36]. The most prominent hopanoids in this organism are tetrahydroxy-bacteriohopane and a hopane glycolipid (compounds I and VI in Fig. 2). We were interested to know whether the hopanoid content of the cells changes with different environmental conditions. We have observed that the hopanoid content is dependent on growth temperature [37]; especially between 60 and 65°C, the hopanoid content increased dramatically, reaching 16% of the cellular lipids (Fig. 4). The lowest hopanoid content was 4%. The dependence of hopanoids on pH of the growth medium was small [37]. This result corresponds to



the finding with higher organisms where the sterol content in membranes of *Neurospora* [38], carp [39] or Chinese hamster ovary cells [40] is elevated after growth at higher temperature.

*Zymomonas mobilis* can produce up to 14% ethanol in its culture fluid. Normally these amounts kill bacteria due to a disintegration of the cellular membrane [41]. In continuous culture conditions by feeding different concentrations of glucose, thereby determining ethanol concentration in the cell, we demonstrated that the hopanoid content is increased with increasing ethanol concentration (Fig. 5). In this case hopanoids can be viewed as membrane strengtheners [33]. Due to their strong van der Waals interaction with phospholipids, the membrane dissolving property of ethanol is counteracted. This result is in good agreement with results in yeast where ergosterol has a growth promoting effect or shows an enhancement of survival in the presence of ethanol [42,43,44].

#### The Function of Hopanoids in Model Membrane Systems

From the experiments with cells as shown in the previous section we can surmise that hopanoids strengthen membranes as sterols do. Can this function be proven in model membrane systems? The best defined membrane in physical terms is a monolayer membrane. Such a membrane is artificial in a biological sense but very valuable in providing exact molecular data. Without these data the biological importance of a molecule cannot be understood.

Hopanoids (compounds I, II and VI in Fig. 2) form a monomolecular layer on a water surface. If one compresses this layer, one observes a low compressibility of the film (Fig. 6). This observation is in accordance with the rigid structure of the molecules. In contrast a phospholipid (e.g. dipalmitoylphosphatidylcholine) possesses a high compressibility correlated to a high molecular area at low surface pressure. A mixture of a hopanoid and a phospholipid shows at low surface pressure a lower area requirement as compared to the individual molecules [45,46]. Phospholipid molecules are packed more densely in combination with a hopanoid. This phenomenon is known as condensation [13].

With isobars of monolayers (Fig. 6) one can demonstrate the phase transition diminishing effect of hopanoids. The individual phospholipid shows a significant phase transition, a sudden increase in molecular area at a certain temperature. If one adds a hopanoid to the phospholipid, this increase is diminished.

Equally well, one can demonstrate this effect by differential scanning calorimetry (Fig. 7). The phospholipid shows an endothermic transition at a certain temperature. The enthalpy of transition is lowered by adding a hopanoid to the phospholipid. Furthermore, one can observe a broadening of the peak, meaning a lower cooperativity of the acyl chains at the phase transition [47].

In black lipid membranes containing phospholipid in combination with hopanoid, the mobility of a potassium ion complex can be measured [48]. The mobility of this complex is decreased by increasing the molecular fraction of the hopanoid. These experiments demonstrate viscosity enhancing property of hopanoids.

By a fourth physical method complementing the others one can show that hopanoids diminish the permeability of membranes. Small

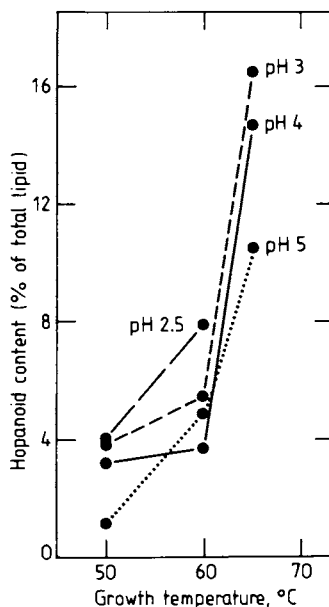


Fig. 4: Hopanoid content in *Bacillus acidocaldarius* measured as the acetate of 1-hydroxyethane-29-hopane as a function of growth temperature and pH of the medium.

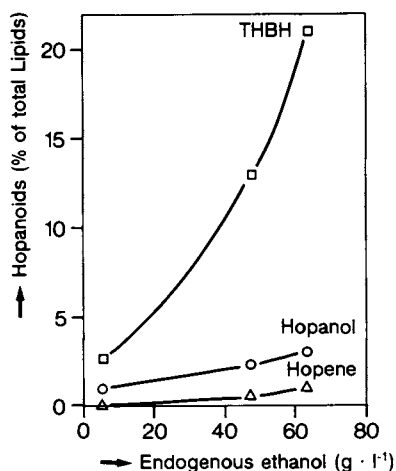


Fig. 5: Hopanoid content of *Zymomonas mobilis* in continuous cultures under different concentrations of ethanol produced. Hopanol = diplopterol, THBH = 32,33,34,35-tetrahydroxybacteriohopane.

American Chemical Society  
Library  
1155 16th St., N.W.

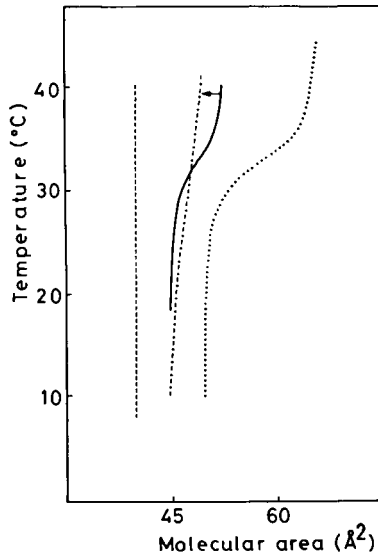


Fig. 6: Isobars from monolayer films at a surface pressure of 25 dyn/cm. ---, tetrahydroxybacteriohopane; ...., dipalmitoylphosphatidylcholine; - - -, 1 : 1 molecular mixture of these compounds; \_\_\_\_, calculated isobar of the 1 : 1 molecular mixture. The arrow indicates the condensation.

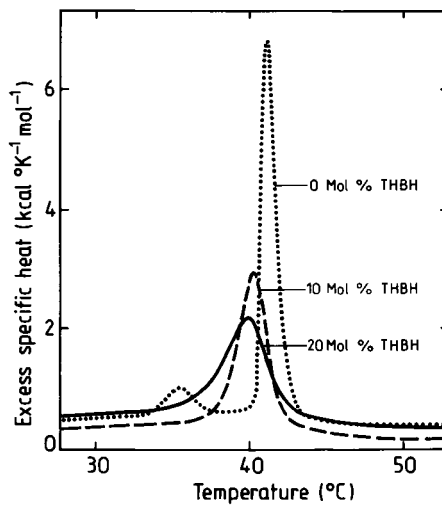


Fig. 7: Differential scanning calorimetry traces illustrating the thermotropic phase behaviour of multilamellar dispersions of dipalmitoyl-phosphatidylcholine containing 0 - 20 mol% tetrahydroxybacteriohopane (= THBH).

molecules such as glycerol permeate liposomal membranes containing hopanoids more slowly. Thus it can be concluded that lipids in liposomes are aligned more densely in the presence of hopanoids [49].

By following the swelling of phospholipid unilamellar vesicles by stopped-flow transmittance measurements, it was shown that hopanoids reinforce the mechanical properties of a membrane [50]. Tetrahydroxybacteriohopane has an effect similar to cholesterol, though not quite as large.

By these model experiments, it was shown that hopanoids act in a manner similar to sterols on lipid membranes. Often the magnitude of the influence is about the same on condensation, suppression of phase transition, enhancement of viscosity and reduction of permeability. Thus nature has invented at least one additional molecular type besides sterols exerting the above described properties. It should be mentioned that other molecules, e.g.,  $\omega$ -dihydroxycarotenoids have a similar effect on membranes [50].

### Conclusions

There exists ample evidence by physiological experiments and by different physico-chemical methods that hopanoids in bacteria possess membrane properties similar to sterols, especially cholesterol, in higher organisms. We are aware that one line of evidence in this context is still missing. Until now mutant analysis on hopanoids has not been done. Also the influence on enzymatic membrane processes has not been measured.

A more theoretical problem is of major importance. Why does one seldom find sterols in bacteria and why are hopanoids, as membrane constituents, hardly ever found in higher organisms? One can enumerate some disadvantages for hopanoids. There exists a solubility problem. Liposomal membranes can be loaded with cholesterol to a higher molecular fraction than hopanoids. Secondly, the value for the different physical effects on membranes are often slightly higher for cholesterol. From a purely structural viewpoint the sterol molecule is a combination between a hopanoid and a fatty acid. The apolar side chain imparts to the sterol molecule a partial flexibility. This flexibility can eventually be of value for specific requirements in a biomembrane of higher organisms. In this context it is valuable to consider the relationship of the protozoan Tetrahymena to sterols. This organism can utilize sterols from the medium. But if the medium is devoid of sterols, the biosynthesis of pentacyclic triterpenoid tetrahymanol is derepressed [51]. This organism has not yet acquired sterol-dependent functions in its membrane. Also a study of the above mentioned bacterium Methylococcus capsulatus would be rewarding. This organism synthesizes both sterols and hopanoids [24,52]. In this case, auxotrophic mutants for sterols and hopanoids should clarify for which special processes these polycyclic triterpenoids are necessary.



Literature Cited

1. Nes, W. R.; McKean, M. L. "Biochemistry of Steroids and other Isopentenoids"; University Park Press, Baltimore, 1977.
2. Lipke, H.; Fraenkel, G. Ann. Rev. Entomol. 1956, 1,17.
3. Smith, P. F. J. Lipid Res. 1964, 5, 121.
4. Razin, S., pp. 183 in Current Topics in Membranes and Transport, Vol. 17, Academic Press, New York, 1963.
5. Bird, C. W.; Lynch, J. M.; Pirt, F.J.; Reid, W.W.; Middleditch, C. J. W. Nature 1971, 230 473.
6. Bouvier, P.; Rohmer, M.; Benveniste, P.; Ourisson, G. Biochem. J. 1976, 159, 267.
7. Kohl, W.; Gloe, A.; Reichenbach, H. J. Gen. Microbiol. 1983, 129, 1629.
8. Hayami, M.; Okabe, A.; Sasai, K.; Hayashi, H.; Kanemasa, Y. J. Bacteriol. 1979, 140, 859.
9. Ono, T.; Bloch, K. J. Biol. Chem., 1975, 250, 1571.
10. Gibbons, G. F.; Goad, L. J.; Goodwin, T. W.; Nes, W. R. J. Biol. Chem. 1971, 246, 3967.
11. Benveniste, P.; Hirth, L.; Ourisson, G. Phytochemistry 1966, 5, 45.
12. Poralla, K. FEMS Microbiol. Lett. 1982, 13, 131.
13. Demel, R.A.; De Kruyff, B. Biochim. Biophys. Acta 1976, 457, 109.
14. Bloch, K. E. CRC Crit. Rev. Biochem. 1983, 14, 47.
15. De Gier, J.; Mandersloot, J.G.; Van Deenen, L. L. M. Biochim. Biophys. Acta 1968, 150, 666.
16. Dahl, C.E.; Dahl, J.S.; Bloch, K. Biochemistry 1980, 19, 1467.
17. Dahl, C. E.; Dahl, J. S.; Bloch, K. J. Biol. Chem. 1983, 258, 11814.
18. Pinto, W. J.; Lozano, R.; Sekula, B.C.; Nes, W. R. Biochem. Biophys. Res. Commun. 1983, 12-47.
19. Ramgopal, M.; Bloch, K. Proc. Nat. Acad. Sci. U.S. 1983, 80, 712.
20. Nes, W. R. Lipids 1974, 596.
21. Rohmer, M.; Bouvier, P.; Ourisson, G. Proc. Nat. Acad. Sci. U. S. 1979, 76, 847.
22. Ourisson, G.; Albrecht, P.; Rohmer, M.; Pure Appl. Chem. 1979, 51, 709.
23. Neunlist, S.; Holst, O.; Rohmer, M. Eur. J. Biochem. 1985, 147, 561.
24. Rohmer, M.; Bouvier, P.; Ourisson, G. Eur. J. Biochem. 112, 557.
25. Seckler, B. Doctoral Thesis, University of Tuebingen, 1980.
26. Zander, J. M.; Greig, J. B.; Caspi, E. J. Biol. Chem. 1970, 245, 1247.
27. Devon, T. K.; Scott, A. I. "Handbook of Naturally Occurring Compounds", Academic Press, New York, 1972; Vol. 2.
28. Van Dorsselaer, A.; Ensminger, A.; Spyckerelle, C.; Dastillung, M.; Sieskind, O.; Arpino, P.; Albrecht, P.; Ourisson, G.; Brooks, P. W.; Gaskell, S. J.; Kimble, B. J.; Philip, R. P.; Maxwell, J. R.; Eglinton, G. Tetrahedron Lett. 1974, 1349.

29. Rohmer, M.; Bouvier-Nave, P.; Ourisson, G. J. Gen. Microbiol. 130, 1137.
30. Langworthy, T. A.; Mayberry, W. R. Biochim. Biophys. Acta 1976, 431, 570.
31. Hippchen, B.; Roell, A.; Poralla, K. Arch. Microbiol. 1981, 129, 53.
32. Barrow, K. D.; Collins, J. G.; Rogers, P. L.; Smith, G. M. Biochim. Biophys. Acta 1983, 753, 324.
33. Bringer, S.; Haertner, T.; Poralla, K.; Sahm, H. Arch. Microbiol. 140-312.
34. Dahl, C. E.; Dahl, J. S.; Bloch, K. Biochemistry 1980, 19, 1462.
35. Kannenberg, E.; Poralla, K. Arch. Microbiol. 1982, 133, 100.
36. De Rosa, M.; Gambactorta, A.; Minale, L.; BuLock, J. D. Chem. Commun. 1971, 1334.
37. Poralla, K.; Haertner, T.; Kannenberg, E. FEMS Microbiol. Lett. 1984, 23, 253.
38. Aaronson, L. A.; Johnson, A. M.; Martin, C. E. Biochim. Biophys. Acta 1982, 713, 456.
39. Wodtke, E. Biochim. Biophys. Acta 1978, 529, 280.
40. Anderson, R. L.; Minton, K. W.; Li, G. C.; Hahn, G. M. Biochim. Biophys. Acta 1981, 641, 334.
41. Ingram, L. O.; Buttke, T. M. Advanc. Microbial. Physiol. 25, 253.
42. Thomas, J. D. A.; Hossack, J. A.; Rose, A. H. Arch. Microbiol. 117, 239.
43. Ohta, K.; Hayashida, S. Appl. Environ. Microbiol. 1983, 46, 821.
44. Janssens, J. H.; Burrell, N.; Woodward, A.; Bailey, R. B. Appl. Environ. Microbiol. 1983, 45, 598.
45. Poralla, K.; Kannenberg, E.; Blume, A. FEBS Lett. 1980, 113, 107.
46. Kannenberg, E.; Poralla, K.; Blume, A. Naturwissenschaften 1980, 57, 458.
47. Kannenberg, E.; Blume, A.; McElhaney, R. N.; Poralla, K. Biochim. Biophys. Acta 1983, 733, 111.
48. Benz, R.; Hallmann, D.; Poralla, K.; Eibl, H. Chem. Phys. Lipids 1983, 34, 7.
49. Kannenberg, E.; Blume, A.; Geckeler, K.; Poralla, K. Biochim. Biophys. Acta 1985, 814, 719.
50. Bissere, P.; Wolff, G.; Albrecht, A. M.; Tanaka, T.; Nakatami, Y.; Ourisson, G. Biochem. Biophys. Res. Commun. 1983, 110, 210.
51. Conner, R. L.; Landrey, J. R.; Burns, C. H.; Mallory, F. E. Protozool.
52. Bird, C. W.; Lynch, J. M.; Pirt, F. J.; Reid, W. W. Tetrahedron Lett. 1971, 3189.

RECEIVED June 9, 1986

## Chapter 16

# Structure-Function Relationships for Sterols in *Saccharomyces cerevisiae*

William R. Nes

Department of Bioscience and Biotechnology, Drexel University, Philadelphia, PA 19104

Ergosterol has two kinds of function which can be distinguished from each other by differing sensitivities to the  $24\beta$ -methyl moiety of the sterol's structure. The "bulk membrane" function which requires most of the sterol, is only slightly influenced either by the presence, absence, or size of the substituent at C-24 or by the chirality of C-24, while the "regulatory" function has an absolute requirement for the  $24\beta$ -methyl group. In agreement with the assignment of most of the ergosterol to an architectural role in membranes, the sterol's overall length is close to the distance from the polar to the nonpolar side of the lipid monolayer when the sterol's side chain is in the staggered conformation with C-22 positioned to the right. However, when the side chain is altered so that it has significantly different spatial characteristics, *S. cerevisiae* is unable to utilize the resulting sterol. Deleterious changes are brought about for instance by removal of the carbon atoms on the right by direct chain shortening or by shifting them to the left rigidly with a  $17(20)$ -double bond or conformationally by inversion of C-20.

### Variability of Sterol Structure

Cholesterol, sitosterol, and ergosterol are among the names of structurally different sterols which are not only widely recognized but are also commonly associated with animals, plants, and fungi, respectively. This illustrates an important point: The structure of sterols is variable, and there is some sort of association between structure and biology. Actually, the structural variability turns out to be quite large when one examines the problem in detail (1-3). More than a hundred sterols have been found in living systems. They fall into two major categories in terms of amount. There are those which accumulate and presumably play a functional role (1) and secondly those which do not accumulate and remain in trace amounts because they are only transient precursors to the functional

0097-6156/87/0325-0252\$06.00/0  
© 1987 American Chemical Society

compounds. On occasion there is some overlap between the two categories, especially with respect to saturated-unsaturated pairs when one of them is a precursor to the other. This latter situation is probably represented by accumulation of the pairs isofucoesterol/sitosterol and sitosterol/stigmasterol, but for the most part accumulated or dominant sterols tend to be end-products rather than intermediates.

Good examples of dominant sterols are cholesterol and its homologs, 24 $\alpha$ -methylcholesterol (campesterol) and 24 $\alpha$ -ethylcholesterol (sitosterol). These along with 24 $\beta$ -methylcholesterol and the 22E-dehydro derivative of sitosterol (stigmasterol) comprise the main sterol component of most tracheophytes. However, there are many more sterols to consider than just these three even among higher plants. If we concentrate only on the sterols which accumulate, there are as many as three dozen of them which are known to be present in terrestrial (*i.e.*, non-marine) living systems (Table I). Most of them vary

Table I. Variable Features of the Principal Sterols of Terrestrial Living Systems<sup>a</sup>

---

Nuclear double bond:	$\Delta^5$ or $\Delta^7$
Carbon addition to side chain:	24-CH <sub>3</sub> or 24-C <sub>2</sub> H <sub>5</sub>
Configuration of substituent at C-24:	$\alpha$ or $\beta$
E-Double bond at C-22,C-23	
Double bond at C-24,C-28 <sup>b</sup>	
Double bond at C-25,C-27 (only 24 $\beta$ -Series)	

---

a) The features listed are added cumulatively (except where indicated by "or" or "only") to 5 $\alpha$ -cholestan-3 $\beta$ -ol. Thus, addition of  $\Delta^5$  gives cholesterol, or cumulative addition of  $\Delta^7$ , 24 $\alpha$ -ethyl, and  $\Delta^{22E}$  gives spinasterol. Thirty-two sterols (including cholesterol and lathosterol) can be constructed from this list. Stanols, especially 5 $\alpha$ -cholestan-3 $\beta$ -ol and 24 $\alpha$ -ethyl-5 $\alpha$ -cholestan-3 $\beta$ -ol (sitostanol), also occur. In addition,  $\Delta^{5,7}$ -sterols accumulate especially in many fungi and in some algae. The most common one is in the 24 $\beta$ -methyl-22E-dehydro series (ergosterol). Others include ergosterol's 24 $\beta$ -ethyl analog, 7-dehydroporiferasterol.

b) In the 24-ethylidene series both the E- and the Z-configurations are known.

by changes in unsaturation in ring B and the side chain and by substitution at C-24. For instance, we have isolated fifteen of the possible  $\Delta^5$ - and  $\Delta^7$ -sterols from the seeds of a single plant (the squash, *Cucurbita maxima*) (Table II) (4,5). Although in most plants a smaller number of sterols accumulate, it is interesting that as many as five sterols of the squash comprising 81% of the mixture are each present at levels > 10.0% of the total. Six more sterols are present at levels from 1.0 to 6.4%. This suggests a function for each of the sterols. The list of functional sterols is made still longer by the addition of sterols from the marine environment where variation can also be based on lengthening or shortening of the side chain as well as on additions of methyl and cyclopropyl groups to several positions in the side chain (6-8). Changes also occur in the skeleton of ring A.

Table II. The 4-Desmethylsterols of *Cucurbita maxima* (squash) Seeds  
(Garg and Nes, refs. 4 and 5)

Sterol	Amount (mg/100 g)	% of Sterol
24 $\alpha$ -Ethyl-5 $\alpha$ -cholesta-7,22E-dien-3 $\beta$ -ol	10.55	26.2
24 $\beta$ -Ethyl-5 $\alpha$ -cholesta-7,25(27)-dien-3 $\beta$ -ol	6.96	17.3
24 $\beta$ -Ethyl-5 $\alpha$ -cholesta-7,22E,25(27)-trien-3 $\beta$ -ol	6.59	16.4
24 $\alpha$ -Ethyl-5 $\alpha$ -cholest-7-en-3 $\beta$ -ol	4.29	10.7
24Z-Ethylidene-5 $\alpha$ -cholest-7-en-3 $\beta$ -ol	4.19	10.4
24 $\beta$ -Methylcholesta-5,25(27)-dien-3 $\beta$ -ol	2.56	6.4
24 $\beta$ -Ethylcholesta-5,25(27)-dien-3 $\beta$ -ol	1.43	3.6
24 $\alpha$ -Ethylcholesta-5-en-3 $\beta$ -ol	1.20	3.0
24 $\alpha$ -Ethylcholesta-5,22E-dien-3 $\beta$ -ol	0.79	2.0
24Z-Ethylidenecholest-5-en-3 $\beta$ -ol	0.54	1.3
24 $\alpha$ -Methylcholest-5-en-3 $\beta$ -ol	0.41	1.0
24 $\xi$ -Methyl-5 $\alpha$ -cholest-7-en-3 $\beta$ -ol	0.33	0.8
24 $\beta$ -Ethylcholesta-5,22E,25(27)-trien-3 $\beta$ -ol	0.31	0.7
24 $\beta$ -Methyl-5 $\alpha$ -cholesta-7,25(27)-dien-3 $\beta$ -ol	0.07	0.1
24 $\beta$ -Methylcholest-5-en-3 $\beta$ -ol	trace	-

### Implications of Sterol Variability

Now, the question which has motivated much of the recent work in my laboratory, and which I should like to discuss here, is this: What really is the reason why certain sterols accumulate? Is one sterol as good as another? Are we just observing a more or less random distribution based on chance mutations? Or is a selection operative, a mating of structure to function, suggesting, for instance, in squash the existence of multiple functions for sterol?

One way to get at this problem would be to delineate what sterols actually do, to find a way to quantitate their value, and then to determine the extent to which deviations in structure affect function. It was this course that was settled on, but the question which arose immediately was: What living system should be used experimentally?

### Early Work With Yeast

It so happens that at the time I was first thinking seriously about this problem (the early to mid 1970s) the literature already revealed an apparent solution. The primary sterol (ergosterol) of the fungus, *Saccharomyces cerevisiae* (the common yeast of bakers and brewers), has a 24 $\beta$ -methyl group, as we have confirmed using <sup>1</sup>H-NMR (9), and the way this group is introduced biosynthetically is fairly complicated (1,2,10). It requires preservation (rather than reduction) of the terminal double bond of squalene ( $\Delta^{24}$  of sterols) so that transfer of a C<sub>1</sub>-group can occur to it from S-adenosylmethionine with elimination of a proton from the incoming C<sub>1</sub>-group (C-28) and 1,2-hydride migration (C-24 to C-25). This gives a 24-methylene group which then undergoes a stereospecific reduction to yield the 24 $\beta$ -methyl group. Other alternatives could have occurred, e.g., elimination from C-27 instead of C-28, reduction to the  $\alpha$ - rather than  $\beta$ -methyl, or a second C<sub>1</sub>-transfer to the 24-methylene group might have occurred.

All of the biosynthetic steps which actually produce the 24 $\beta$ -methyl group are well documented (1,2,10), and much more than a single point mutation in fungal evolution would be necessary to account for this involved sequence of reactions. Despite this, wild type *S. cerevisiae* seemed, according to the literature (11-13), to have little use for the 24 $\beta$ -methyl group. Ergosterol could be replaced, for instance, by cholesterol which has no substituent at C-24 or by sitosterol which has an ethyl group at C-24 and no serious loss of function was observable. In particular, growth still occurred with cholesterol or sitosterol and almost at the same rate as with ergosterol. This strongly suggested either that the manner in which the experiments were carried out left the functional question more or less moot or that biosynthesis and function (and by implication evolutionarily surviving mutants and function) are not closely correlated. Yeast thus presented an intriguing challenge. It was chosen for study both for this reason and because it has some characteristics which make it very useful as an experimental tool.

Useful characteristics of yeast. Yeast, *i.e.*, *S. cerevisiae*, is, first of all, eukaryotic and at least to some degree should be representative of higher forms of life in general. Secondly, it is naturally single celled and can be grown in a liquid culture. If needed, large quantities (100 g) can be obtained. A great deal is known about this microorganism, which is not pathogenic, and it can be easily obtained in an axenic condition, for instance from the American Type Culture Collection.

Perhaps of most importance is yeast's unusual ability among eukaryotes to be able to live anaerobically and to derive its ATP by nonrespiratory glycolysis. Under these conditions, as seen under the electron microscope, the mitochondria change morphologically to what are called promitochondria (14), and the cells become auxotrophic for sterol (11) and unsaturated fatty acid such as oleate (15). Aerobically yeast derives its sterol and unsaturates by biosynthesis, and for both types of compound there are biosynthetic steps involving mixed function oxidases, *e.g.*, for the formation of squalene oxide (1,2). This means that molecular oxygen is required, and, when availability of oxygen is denied, it is obvious that neither sterol nor unsaturates can be formed. By operating anaerobically we can therefore dictate what sterol is in the cells simply by adding the sterol of our choice to the medium. Andreassen and Stier (11) were the first to demonstrate that this could be done and they were the first also to show that without sterol (11) and unsaturated fatty acid (15) yeast will not grow. This was an elegant demonstration that these lipids play a vital role.

#### The Function of Sterols

There is extensive evidence, which I have reviewed earlier (1,16), to indicate that the principal role of sterols throughout nature is to act as architectural components of some though not necessarily all membranes. The plasma membrane is frequently the principal residence of the sterol (13,17,18).

Eukaryotic as well as most prokaryotic membranes are bilayers of phospholipid and protein. Each monolayer, which is about 2.1 nm thick (19,20), is believed to contain the sterol in a nonhomogeneous distribution, and at least in some cases sterol can move between the monolayers. This process is called "flip-flop". Sterol has been found both in the mitochondria (21) and the plasma membrane of *S. cerevisiae* (13,18), and the ability to support the growth of anaerobic yeast presumably is associated with its membranous function.

It is not fully clear how sterol is important architecturally, but much evidence points to a modulation of the physical properties of the membrane, especially of the transition from the gel to the liquid crystal state of the phospholipid thereby controlling the rigidity, usually expressed in the reverse as fluidity, of the membrane. This has been called the "bulk membrane function" (22) of sterol to distinguish it from another function or functions which require(s) less sterol. The nature of the latter function(s) is more elusive, although we (23,24) and others (7,18,25) have presented good evidence for its existence in yeast, and a similar phenomenon exists also in prokaryotic mycoplasmas (22,26). We have called the function

requiring less sterol "regulatory" (23), while Parks and his group use the word "sparking" (25), and Bloch and his associates apply the term "synergistic" (18).

### The Experimental System

We discovered very early that the growing of yeast anaerobically, i.e., really anaerobically, is quite difficult. Or, to put it another way, it is far from easy to remove enough oxygen to prevent at least some growth from occurring on a synthetic medium containing no sterol. We achieved success by carefully sealing inlets into our growth chamber with paraffin wax and prior to inoculation by using a prolonged flush of electronic grade nitrogen (< 0.2 ppm of oxygen) which had been passed through a solution of chromous chloride to reduce residual oxygen to the water stage. Under these conditions it became possible to take a log phase inoculum of yeast from a continuous culture and find it would not grow (<  $10^6$  from  $10^5$  cells) in the presence of Tween-80 (as a source of unsaturated fatty acid). At the same time, growth ensued well ( $10^8$  cells/ml from  $10^5$  cells/ml in 3 days) when ergosterol (5 mg/liter of medium) had been emulsified in the Tween-80 and then dispersed in the medium.

Our initial plan was to make a systematic investigation of the structural features of ergosterol by replacing the ergosterol with other sterols which either lacked a particular feature, e.g., the  $\Delta^7$ -unsaturation, or had some other interesting aspect to them, e.g., altered stereochemistry. The contribution of the feature was then to be assessed by evaluation of the number of cells after three days. The cell population could be measured both visually and by means of a 16-channel Coulter counter which also permits determination of the distribution of cell sizes and volumes. In addition, extraction of the cells, or subcellular fractions, would permit us to examine the amount, distribution, and metabolism of the added sterol.

This experimental design (27,28) proved adequate to show a great deal about what is and what is not important about the sterol molecule. However, some growth and it should be added variable growth in the presence of cholesterol occurred. By examining the sterol distribution in the neutral lipid fraction of the yeast grown in the manner described we found that there were still sufficient traces of oxygen left in the medium to allow very small but significant amounts of endogenous sterol synthesis to occur. We could identify (28) not only ergosterol but also lanosterol, zymosterol, 24-methylene-7-dehydrocholesterol, and 22-dihydroergosterol all of which are intermediates in the ergosterol pathway (1). Such biosynthesis presumably accounts for growth on cholesterol in the experiments of Hossack and Rose (13) who reported that ergosterol was also present. Ergosterol produced biosynthetically of course complicates the interpretation of the results obtained with other sterols. In order to reduce biosynthesis to an insignificant level in the oxygen-deprived yeast we added 2,3-iminosqualene in our more recent experiments (29). This compound is a competitive inhibitor of 2,3-oxidosqualene cyclase (30), and when it was incorporated into the medium along with cholesterol no growth then occurred although on replacement of cholesterol with ergosterol growth proceeded well.

Thus, stepwise we were able to achieve a no-growth condition



without sterol by using prolonged flushing with highly purified nitrogen and then by adding 2,3-imosqualene we also achieved a no-growth condition in the presence of cholesterol. One final experimental thing must be mentioned. If we want to have both ergosterol and some other sterol present, it is obvious we could add both, and we have done just that. Such a method has the advantage of allowing various relative amounts to be added, but we also found for some purposes generation of ergosterol in situ by the addition of air was a simple alternative. With 10 ml of air added to the headspace (ca. 100 ml) of our growth chamber in the presence of the iminosqualene but with no sterol we (23) obtained  $24 \times 10^6$  cells/ml which is a quarter or a fifth of what we obtained with added ergosterol. The effect of added sterol could then be determined by measuring the increase in cell count.

#### The Positioning of C-21 and C-22 in Space

It has been possible to correlate several physical properties with the configuration at C-20 of sterols. The configuration at C-20 of cholesterol (Figure 1) is known to be R from degradation of the  $\Delta^{14}$ -analog and correlation of the product containing C-20 with D-(+)-glyceraldehyde (30,31). Cholesterol is therefore a primary standard. We prepared 20-epicholesterol and both for it and for cholesterol we determined  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR spectra, the melting points, the optical rotations, and the chromatographic rates of movement (32-34). In some cases this was also done for a series of other sterols (33-35) such as 20R- and 20S-halosterol (35) which is the  $\text{C}_{26}$ -analog of cholesterol which has one less  $\text{CH}_2$ -group in the side chain and occurs in the marine environment (1). There were several consistent shifts. The 20R-isomers moved slower in both adsorption and gas-liquid chromatography, showed a downfield shift in the C-21 proton NMR-signal, a greater separation of the terminal gem-dimethyl proton signals, and a more positive (actually less negative), optical rotation at the D-line of sodium. There was also a tendency for the S-isomer to have a lower melting point, although this was not so for the epimeric cholesterol pair. This information along with x-ray diffraction permitted a survey of the C-20 configuration of natural sterols, e.g., ref. 32. With one well documented but curious exception (36) along with one dubious exception lacking strong physical evidence (37), all sterols in both the marine and terrestrial environments have the 20R-configuration. Why should this be so? It is not necessary from a biosynthetic point of view, since reasonable mechanisms are possible to give both the R- and the S-epimers (32).

I believe the answer to why the R-configuration at C-20 is chosen naturally lies in the mating of the shape and size of the sterol to the dimensions of the monolayer in the lipid leaflet of membranes. In order to keep the smallest group on C-20 adjacent to C-18 which lies on the front or  $\beta$ -face of the nucleus, rotation of C-20 about the 17(20)-bond should occur preferably so that the 20-H-atom is in front. This means that in the R-series C-22 is on the right ("right-handed") and C-21 on the left ("left-handed") when the sterol is viewed in the usual way, i.e., with C-3 to the left and C-18 and C-19 toward the observer. X-ray data for many natural sterols corroborates not only the R-configuration but the existence

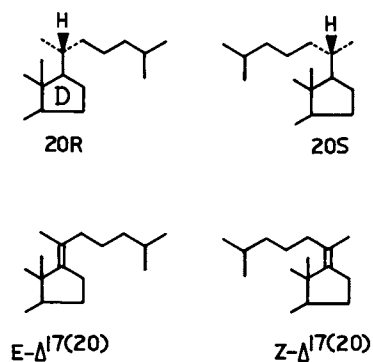


Figure 1. Stereochemistry of sterols at C-20. Structures illustrate ring-D and the cholesterol side chain. The carbon bearing the H-atom shown is C-20. In halosterol the isoheptyl moiety  $[(\text{CH}_2)_3\text{-CH-(CH}_3)_2]$  on C-20 is replaced by an isopentyl group  $[(\text{CH}_2)_2\text{-CH-(CH}_3)_2]$ . Other replacements were made with n-alkyl groups.

of the side chain in the right-handed conformation. We have also been able to show from x-ray diffraction with the tetracyclic triterpenoids euphol and tirucallol that the orientation of C-22 really is dependent on the configuration of C-20 and the presence of C-18 (38). The right-handed conformation of sterols is probably chosen in an evolutionary sense because with C-22 on the right and the side chain in the staggered conformation the long dimension of cholesterol (and other sterols with the same chain length) coincides almost exactly with that of the thickness of the monolayer. However, the sterol length is much shorter than the latter when C-22 is on the left as we think it would be in the 20S-epimer.

Direct evidence for the above interpretation was obtained as follows. When incubated with our oxygen-deprived yeast in the absence of 2,3-iminosqualene, cholesterol, it will be recalled, permitted some growth. However, when cholesterol was replaced by 20-epicholesterol no growth at all occurred (28). Similarly, while cholesterol or halosterol is metabolized by the protozoan *Tetrahymena pyriformis*, the 20-epi-analogs are not (39,40) and cholesterol but not its epimer at C-20 induced oospores in the fungus *Phytophthora cactorum* (41). In order to show that this negative effect of inversion at C-20 correlates with the positioning of C-22 on the left, we synthesized E- and Z-17(20)-dehydrocholesterol (42). In the E-isomer C-22 is rigidly oriented to the right, and to the left in the Z-isomer. The E-isomer permitted some growth of yeast, but as expected the Z-isomer did not (28). Again in agreement with results with the yeast system, only the E-isomer was metabolized by *T. pyriformis* (39), the Z-isomer being recovered unchanged from the cells (39). Similarly, only the E-isomer induced formation of oospores in *P. cactorum* (43).

#### The Length of the Side Chain

A direct test of the importance of the long dimension of the sterol molecule was obtained by our synthesizing androst-5-en-3 $\beta$ -ol, which is a  $\Delta^5$ -C<sub>19</sub>-sterol with no side chain at all, and by also making a variety of  $\Delta^5$ -sterols in which the isoheptyl group (C-22 to C-27) on C-20 of cholesterol was replaced either with hydrogen (pregn-5-en-3 $\beta$ -ol) or with longer or shorter arrays of carbon atoms than occur in cholesterol, i.e., a series of 20R-n-alkylpregn-5-en-3 $\beta$ -ols (34). The epimeric 20S-sterols were also prepared and as already mentioned 20R- and 20S-halosterol were made (35). We also prepared 21-isopentylcholesterol (44) which we called "wingsterol", since it has an isoheptyl group on both sides of C-20. Neither the C<sub>19</sub>-sterol nor wingsterol permitted growth of yeast (44). The C<sub>19</sub>-sterol also prevented growth of *T. pyriformis* (44) as well as of the larvae of the insect *Heliothis zea* (44), although *H. zea* larvae grow well on cholesterol. In work which has only been partly published (45) we also found that as the length of the sterol side chain is lengthened growth of yeast rises to a maximum at five C-atoms on C-20, i.e., with 20R-n-pentylpregn-5-en-3 $\beta$ -ol and fell virtually to zero with more than seven C-atoms on C-20. The 20S-analogs were all inactive. The influence of length of the side chain was also assessed in oospore induction with *P. cactorum* (46), metabolism in *T. pyriformis* (47), esterification in *T. pyriformis* (44), inhibition of tetra-

hymanol production by *T. pyriformis* (44), and esterification with mammalian ACAT (44). In all cases maximal activity occurred either with the 20R-n-pentyl or 20R-n-hexyl derivative of pregn-5-en-3 $\beta$ -ol agreeing well with the yeast results. The observed length for maximal activity (5 or 6 C-atoms on C-20) correlates well with the length of natural sterols (8).

#### Importance of the 24 $\beta$ -Methyl Group in Yeast

While the stereochemistry at C-20 and the length of the side chain should and actually do appear to have general significance in terms of the size of the lipid leaflet in membranes, it is not as easy a priori to assign a role to the 24 $\beta$ -methyl group. This is especially difficult, since from data on occurrence (1) the methyl group certainly does not seem to have general importance. Geometrically it should thicken the side chain and probably make it more rigid. This sort of phenomenon might be interpreted to imply that it might protect a membrane against too high a fluidity at elevated temperatures. However, yeast, with an optimum temperature of 28-36° and a maximum temperature of 40-42°, does not grow very well at temperatures much above those which are normal for mammals which have cholesterol, and there is no correlation between the optimum temperature for a fungus and the sterol it makes. Nevertheless, the early work of ourselves (27,28) and of Andreasen and Stier (11) showed that ergosterol is better for *S. cerevisiae* than is cholesterol. More recently Ramgopal and Bloch (18) concurred in this using the GL7 mutant which is auxotrophic for sterol aerobically having lost the oxidosqualene cyclase. We went on to show that this difference in effectiveness is not due to the  $\Delta^7$ -bond, since neither lathosterol nor 7-dehydrocholesterol were any better than cholesterol in supporting the growth of our semi-anaerobic wild type yeast (28). On the other hand, 24 $\beta$ -methyl-22E-dehydrocholesterol (brassicasterol) was virtually as effective as was ergosterol, and when the double bond was removed to give 24 $\beta$ -methylcholesterol the compound was still 75% as active as ergosterol (28), although under the same conditions cholesterol was only 23% as active.

The mystery deepened when we showed recently (48) that growth on cholesterol or on other sterols lacking a 24 $\beta$ -methyl group (Table III) can be brought to zero by using 2,3-iminosqualene even though the yeast retains its ability to grow well on ergosterol, brassicasterol, 24 $\beta$ -methylcholesterol, and desmosterol which we were able to show was converted in high yield in the yeast to 24 $\beta$ -methylcholesterol (48). Interestingly, neither 24 $\alpha$ -methylcholesterol nor 24 $\beta$ -ethylcholesterol supported growth indicating that both the exact size and configuration of the substituent at C-24 are important. Thus, the 24 $\beta$ -methyl group itself is essential and the idea that ergosterol is just better than cholesterol is not quite right. In some vital way cholesterol can not replace ergosterol at all. Yet, it appeared that in some way it could, because we (27,28) and others (11,13) had gotten yeast to grow in the absence of 2,3-iminosqualene to variable degrees on cholesterol. Since in our system without the inhibitor there was some ergosterol present, though not enough by itself to let growth occur (28), it appeared that it must be this small amount of endogenous sterol which was responsible for the ability of chole-

Table III. Growth Response of Oxygen-Deprived *S. cerevisiae* to Sterols with 2,3-Iminosqualene (50  $\mu$ M) Present (From refs. 23, 24, and 48)

Sterol (5 mg/liter)	Cell Count (Millions of Cells/ml)	
	No air <sup>a</sup>	Added air <sup>b</sup>
A. Sterols lacking an HO-group		
Cholest-5-ene	0.1	21
5 $\alpha$ -cholestan-3-one	0.2	61
B. 24-Desalkylsterols		
Cholesterol	0.1	112
Lathosterol	0.2	97
7-Dehydrocholesterol	0.5	100
24-Dehydrocholesterol (desmosterol)	99	-
5 $\alpha$ -Cholestanol	0.1	85
E-17(20)-Dehydrocholesterol	0.2	80
Z-17(20)-Dehydrocholesterol	0.1	16
C. 24 $\alpha$ -Alkylsterols		
24 $\alpha$ -Methylcholesterol	0.2	107
24 $\alpha$ -Ethylcholesterol	0.2	89
D. 24 $\beta$ -Alkylsterols		
24 $\beta$ -Methylcholesterol	95	-
24 $\beta$ -Methyl-22E-dehydrocholesterol	108	-
24 $\beta$ -Methyl-7,22E-bisdehydrocholesterol (ergosterol)	114	-
24 $\beta$ -Ethylcholesterol	0.2	103

a. After 3 days, average of 3 experiments. Cells adapted to oxygen-deprivation were used.

b. 10 ml of air added to cultures which had not grown after initial three days and cell count obtained after another 3 days. Without sterol, the cell count was 24 million. Data are averages for 3 experiments.

terol to be active. This in turn suggested the existence of a dual (or perhaps multiple) role for sterol in yeast. In one role cholesterol could replace ergosterol, but in the other, requiring less sterol, cholesterol must be ineffectual.

#### Evidence for a Dual Role of Sterol

The supposition discussed above that 24 $\beta$ -methylsterol in an amount too small to support growth by itself would permit sterols lacking the methyl group to induce growth was demonstrated directly in two ways. In the first place, in the presence of 2,3-iminosqualene after three days following inoculation, cultures containing the sterols listed in Table III which had not grown were oxygenated with 10 ml of air to generate ergosterol *in situ* (23,24). After an additional three days the only cultures which had not now grown more than the control (lacking sterol) were the ones which contained cholest-5-ene and Z-17(20)-dehydrocholesterol (23,24). Except for 5 $\alpha$ -cholestan-3-one which was reduced to 5 $\alpha$ -cholestan-3 $\beta$ -ol (49), none of the sterols was metabolized, or more precisely all except the ketone were recovered unchanged in substantial quantity, and no metabolites were observed (23). The growth data after addition of air (Table III) show that quite a variety of sterols will support growth if a small amount of 24 $\beta$ -methylsterol is present. This kind of effect was first found in insects by Clark and Bloch (50) for cholesterol and cholestanol, and cholestanol was said to "spare" (partly replace) the cholesterol. We (23) have adopted this term, *i.e.*, cholesterol, for instance, will spare ergosterol. Table III reveals that sparing activity occurs with sterols not only lacking a substituent at C-24 but also with those having 24 $\alpha$ -methyl, 24 $\alpha$ -ethyl, or 24 $\beta$ -ethyl groups. Unsaturation in ring B is unnecessary, as is also the case in insects (50), either  $\Delta^5$ -,  $\Delta^7$ -, or  $\Delta^5,7$ -unsaturation can be present and C-22 must be oriented to the right in the usual view of the molecule.

We have also constructed growth curves using mixtures of cholesterol and ergosterol in various ratios from 0-100% of each (23) instead of generating ergosterol with air. The results are shown in Table IV in an abbreviated form. It will be seen that neither a lot (5 mg/l) of cholesterol alone nor a little (< 0.5 mg/l) ergosterol alone was supportive of growth, yet maximal growth could be obtained when the sterols were combined at these levels, *e.g.*, 4.5 mg/l of cholesterol and 0.50 mg/l of ergosterol. Since it takes 1.50 mg/l of ergosterol alone for the yeast to grow maximally, cholesterol is clearly sparing most (about two-thirds) but not all of the ergosterol. Under these circumstances, the cholesterol is presumably fulfilling the bulk membrane function.

Since the ergosterol (or other 24 $\beta$ -methylsterol) is required in only a small amount, so long as another sterol is present in large amount, we think 24 $\beta$ -methylsterols play some regulatory function. This may have something to do with controlling ratios of saturated and unsaturated fatty acid (50,51), although the exact nature of the function needs more exploration.

Table IV. Sparing Activity of Cholesterol for Ergosterol in *S. cerevisiae* with 50  $\mu\text{M}$  2,3-Iminosqualene Present (From ref. 23)

Sterol Conc. (mg/l)		Cell Count (Millions of Cells/ml)
Ergosterol	Cholesterol	
0.00 <sup>a</sup>	5.00	0.2
0.05 <sup>a</sup>	4.95	0.2
0.10 <sup>a</sup>	4.90	12
0.20 <sup>a</sup>	4.80	48
0.30 <sup>b</sup>	4.70	71
0.50 <sup>c</sup>	4.50	107
1.00 <sup>d</sup>	4.00	108

- a) This conc. without cholesterol gave  $0.2 \times 10^6$  cells/ml.  
 b) This conc. without cholesterol gave  $0.3 \times 10^6$  cells/ml.  
 c) This conc. without cholesterol gave  $0.8 \times 10^6$  cells/ml.  
 d) This conc. without cholesterol gave  $54.0 \times 10^6$  cells/ml.

Use of mutants. Ramgopal and Bloch (18) have come to conclusions similar to ours using the yeast mutant GL7, but for reasons not really clear neither they nor others (25,53) have been able to obtain a no-growth-condition with GL7 on cholesterol. However, Ramgopal and Bloch (18) find that the mutant grows better on ergosterol than on cholesterol, and a small amount of ergosterol substantially enhances growth on cholesterol which these authors refer to as a synergistic effect. Bloch and his associates have also found a related synergism for cholesterol and lanosterol in mycoplasmas (26). Similarly, with GL7 and a mutant (FY3) with much the same defects Parks and his associates (25) find ergosterol is necessary in small amount in order for  $5\alpha$ -cholestan- $3\beta$ -ol to support growth. However, Parks and his group (25,54) interpret the results only to mean a  $\Delta^5$ -grouping is being supplied by ergosterol for what they call the sparking function, and they feel a  $24\beta$ -methyl group is not necessary (54) in view of the ability of cholesterol alone to permit growth of GL7 and FY3. The reason for this growth remains unclear. Since the mutants are grown aerobically and require much less sterol than our wild type, e.g., 0.3 vs. 1.5 mg/l of ergosterol to give maximum growth for GL7 (18) and wild type (23), respectively, slight leakiness through the block of sterol biosynthesis in the mutants might account for the apparent lack of an absolute requirement for a  $24\beta$ -methylsterol. That is, a minute amount of regulatory sterol may actually be biosynthesized. It of course is also possible that the mutants actually do have a less stringent requirement for  $24\beta$ -methylsterol.

#### Structural Effects on Feedback Inhibition of Sterol Biosynthesis

Another way to gain evidence on whether the structure of ergosterol is especially important to yeast, would be to measure the effect of ergosterol and other sterols on the ability of the organism to

biosynthesize sterols. Under oxygen-deprived conditions squalene accumulates in yeast, because it can not proceed on to squalene oxide. The squalene level, therefore, should be a measure of the flow of carbon into the sterol pathway. Ergosterol is already known by other parameters, to depress sterol biosynthesis in yeast, and in agreement we found squalene accumulation was increasingly depressed as we raised the concentration of ergosterol in the medium (55). However, cholesterol was much less effective (55). Since cholesterol might have been less effectively absorbed, we also measured uptake (and esterification). Based on the amount of free sterol in the cells, ergosterol was about four times as effective as cholesterol in depressing squalene accumulation (55). This agrees with the supposition that the choices made in the biosynthetic process are determined by a mating of the 24 $\beta$ -methyl structure with function (23,48).

### Sterol Uptake

Since the concentration and distribution of sterol within the cell is important to an understanding of function, we have been measuring among other things the relationship of structure to uptake from the medium. Many different steroids were absorbed (56). They included not only the 3 $\beta$ -hydroxysterols in Table III which support growth with added air but also steroids such as 5 $\alpha$ -cholestan-3-one which do not have an HO-group at C-3 (49). Especially interesting was the observation that when a small amount of growth was induced by addition of air (10 ml), the sterols with the wrong, left-handed orientation of C-22 (20-epicholesterol and Z-17(20)-dehydrocholesterol) were not absorbed at all (56) even though sterols with the natural right-handed orientation (cholesterol and E-17(20)-dehydrocholesterol) were absorbed well under the same conditions (23,56)). This means that the reason why the left-handed sterols failed to support growth (23, 28) was that they actually never even entered the cells. This in turn implies the existence of a structurally specific gate for absorption, and this gate seems to be stereochemically related to function, since we can relate right- and left-handedness to membrane geometry (see earlier). The existence of a gate is also manifested in the fact that yeast cells can turn absorption on and off. For instance, while ergosterol is readily absorbed anaerobically, it is not absorbed aerobically (56,57). This means that absorption can simply not be a passive entry of sterol into the plasma membrane (56). A carrier protein may conceivably be involved (56). Such a protein is well known to exist in mammals (58) and evidence also exists in yeast (58). The triggering of the entry mechanism by oxygen-deprivation is believed to occur via elimination of an oxygen-requiring step in heme biosynthesis (59). The loss of heme then sets in motion events leading to sterol auxotrophy (59).

### Acknowledgments

I should like to thank the National Institutes of Health for support of this work through Research Grant AM-12172.



Literature Cited

1. Nes, W. R., McKean, M. L. "The Biochemistry of Steroids and Other Isopentenoids"; University Park Press: Baltimore, 1977.
2. Nes, W. R. Adv. Lipid Res. 1977, 15, 233.
3. Goad, L. J.; Goodwin, T. W. In "Progress in Phytochemistry"; Reinhold, L.; Liwschitz, Y., Eds.; Interscience: New York, 1972; Vol. III, p. 113.
4. Garg, V. K.; Nes, W. R. Phytochemistry 1984, 23, 2919.
5. Garg, V. K.; Nes, W. R. Phytochemistry 1984, 23, 2925.
6. Djerassi, C. Pure and Appl. Chem. 1981, 53, 873.
7. Barbier, M. In "Marine Natural Products"; Scheuer, P. J., Ed.; Academic Press: New York, 1981; Vol. IV, p. 147.
8. Nes, W. R. In "Isopentenoids in Plants: Biochemistry and Funktion"; Nes, W. D.; Fuller, G.; Tsai, L-S., Eds.; Marcel Dekker, Inc.: New York, 1984; p. 325.
9. Adler, J. H.; Young, M.; Nes, W. R. Lipids 1977, 12, 364.
10. Lederer, E. Quart Rev. 1969, 23, 453.
11. Andreasen, A. A.; Stier, T. J. B. J. Cell Comp. Physiol. 1953, 41, 23.
12. Proudlock, J. W.; Wheeldon, L. W.; Jollow, D. J.; Linnane, A. W. Biochim. Biophys. Acta 1968, 152, 434.
13. Hossack, J. A.; Rose, A. J. J. Bacteriol. 1976, 127, 67.
14. Morpurgo, G.; Serlupi-Crescenzi, G.; Tecce, G.; Valente, F.; Venettacci, D. Nature (London) 1964, 201, 897.
15. Andreasen, A. A.; Stier, T. J. B. J. Cell. Comp. Physiol. 1954, 43, 271.
16. Nes, W. R. Lipids 1974, 9, 596.
17. Lange, Y.; Ramos, B. V. J. Biol. Chem. 1983, 258, 15130.
18. Ramgopal, M.; Bloch, K. Proc. Nat'l Acad. Sci., U.S.A. 1983, 80, 712.
19. Huang, C.; Mason, J. T. Proc. Nat'l Acad. Sci., U.S.A. 1978, 75, 308.
20. Dalton, A. J.; Haguenu, F. "The Membranes"; Academic Press: New York, 1968.
21. Bottema, C. K.; Parks, L. W. Lipids 1980, 15, 987.
22. Dahl, J. S.; Dahl, C. E.; Bloch, K. J. Biol. Chem. 1981, 256, 87.
23. Pinto, W. J.; Lozano, R.; Sekula, B. C.; Nes, W. R. Biochem. Biophys. Res. Commun. 1983, 112, 47.
24. Pinto, W. J. Ph.D. Dissertation, Drexel University, Philadelphia, 1982.
25. Rodriguez, R. J.; Taylor, F. R.; Parks, L. W. Biochem. Biophys. Res. Commun. 1982, 106, 435.
26. Dahl, J. S.; Dahl, C. E.; Bloch, K. Biochemistry 1980, 19, 1467.
27. Nes, W. R.; Adler, J. H.; Sekula, B. C.; Krevitz, K. Biochem. Biophys. Res. Commun. 1976, 71, 1296.
28. Nes, W. R.; Sekula, B. C.; Nes, W. D.; Adler, J. H. J. Biol. Chem. 1978, 253, 6218.
29. Corey, E. J.; de Montellano, P. R. O.; Lin, K.; Dean, P. D. G. J. Am. Chem. Soc. 1967, 89, 2797.
30. Riniker, B.; Arigoni, D.; Jeger, O. Helv. Chim. Acta 1954, 37, 546.

31. Conforth, J. W.; Youhotsky, I.; Popjak, G. Nature (London) 1954, 173, 536.
32. Nes, W. R.; Varkey, T. E.; Krevitz, K. J. Am. Chem. Soc. 1977, 99, 260.
33. Zarembo, J. E. Ph.D. Dissertation, Drexel University, Philadelphia, 1980.
34. Joseph, J. M. Ph.D. Dissertation, Drexel University, Philadelphia, 1980.
35. Joseph, J. M.; Nes, W. R. J. Chem. Soc. Chem. Commun. 1981, 367.
36. Vanderah, D. J.; Djerassi, C. Tetrahedron Letters 1977, 693.
37. Ikekawa, N.; Tsuda, K.; Morisaki, N. Chemistry and Industry 1966, 1179.
38. Nes, W. D.; Wong, R. Y.; Benson, M.; Landrey, J. R.; Nes, W. R. Proc. Nat'l Acad. Sci., U.S.A. 1984, 81, 5896.
39. Nes, W. R.; Joseph, J. M.; Landrey, J. R.; Conner, R. L. J. Biol. Chem. 1978, 253, 2361.
40. Nes, W. R.; Joseph, J. M.; Behzadan, S.; Conner, R. L. J. Lipid Res. 1981, 22, 770.
41. Nes, W. D.; Patterson, G. W.; Bean, G. A. Plant Physiol. 1980, 66, 1008.
42. Nes, W. R.; Varkey, T. E.; Grump, D. R.; Gut, M. J. Org. Chem. 1975, 41, 3429.
43. Nes, W. D.; Stafford, A. E. Lipids 1984, 19, 544.
44. Nes, W. R.; Adler, J. H.; Billheimer, J. T.; Erickson, K. A.; Joseph, J. M.; Landrey, J. R.; Marcaccio-Joseph, R.; Ritter, K. S.; Conner, R. L. Lipids 1982, 17, 257.
45. Nes, W. R.; Joseph, J. M. Fed. Proc. 1981, 40, 1561.
46. Nes, W. D.; Nes, W. R. Experientia 1983, 39, 276.
47. Nes, W. R.; Joseph, J. M.; Landrey, J. R.; Conner, R. L. J. Biol. Chem. 1980, 255, 11815.
48. Pinto, W. J.; Nes, W. R. J. Biol. Chem. 1983, 258, 4472.
49. Pinto, W. J.; Nes, W. R. Abstracts of the Ann. Meet. of the Am. Soc. Microbiol., Las Vegas 1985, p. 247.
50. Clark, A. J.; Bloch, K. J. Biol. Chem. 1959, 234, 2583.
51. Buttke, T. M.; Jones, S. D.; Bloch, K. J. Bacteriol. 1980, 144, 124.
52. Buttke, T. M.; Reynolds, R.; Pyle, A. L. Lipids 1982, 17, 361.
53. Kumari, S. N.; Ranadive, G. N.; Lala, A. L. Biochim. Biophys. Acta 1982, 692, 441.
54. Rodriguez, R. J.; Parks, L. W. Arch. Biochem. Biophys. 1983, 225, 861.
55. Pinto, W. J.; Lozano, R.; Nes, W. R. Biochim. Biophys. Acta 1985, 836, 89.
56. Nes, W. R.; Dhanuka, I. C.; Pinto, W. J. Lipids 1985, in press.
57. Trocha, P. J.; Sprinson, D. B. Arch. Biochem. Biophys. 1976, 174, 45.
58. Dempsey, M. E. Current Topics in Cellular Regulation 1984, 24, 63.
59. Lewis, T. A.; Taylor, F. R.; Parks, L. W. J. Bacteriol. 1985, 163, 199.

RECEIVED September 10, 1986

## Chapter 17

# Mechanism of Fungal Growth Suppression by Inhibitors of Ergosterol Biosynthesis

John D. Weete

Department of Botany, Plant Pathology, and Microbiology, Alabama Agricultural  
Experiment Station, Auburn University, AL 36849

There are a large number of chemically diverse nitrogenous substances that block the C-14 demethylation of lanosterol (or 24-methylene dihydrolanosterol), and have proven to be effective antifungal compounds. Although the biochemical site of action is well-defined, precisely how blocking the formation of ergosterol or functionally equivalent sterol results in growth inhibition is not known. It appears from the available evidence that growth inhibition may be the result of a deterioration in membrane function, mainly the plasma membrane, due to an alteration in physical properties caused by changes in sterol content directly, or other modifications in membrane lipid composition brought about by changes in sterol content.

Inhibitors of various reactions in the pathway of sterol biosynthesis have been known for many years, but more recently there has been considerable interest in a large and widely diverse group of such chemicals synthesized during the past 15 years that block several reactions in the later stages of this pathway. Although not technically correct in all cases, the term "sterol inhibitor" (SI) is often used to refer collectively to these substances. The reason for the high level of interest in these substances is that most of them are potent growth inhibitors of a broad spectrum of agriculturally and medically important fungi, most of which have ergosterol as the principal sterol; thus, they are also called "ergosterol biosynthesis inhibitors" (EBI). Most of these inhibitors have a common biochemical site of action, which seems remarkable in view of the wide diversity of chemical structures. At their lowest biologically active concentrations, they inhibit the demethylation of lanosterol (or 24-methylene dihydrolanosterol) at C-14, which is the first step in the metabolism of this sterol to ergosterol or other functionally equivalent sterols in fungi. Inhibitors with this mode of action include certain azole, pyridine, pyrimidine, and piperazine derivatives. Azoles are 5-membered cyclic molecules with one or more heteroatoms in the ring, at least one of which must be nitrogen, and the maximum number of noncumulated double bonds (1). They are the largest

0097-6156/87/0325-0268\$06.00/0  
© 1987 American Chemical Society

group of such inhibitors, and include compounds with imidazole and triazole rings.

In addition to the inhibitors that block C-14 demethylation, certain morpholine derivatives inhibit the reduction at C-14(15) in an intermediate formed by demethylation at C-14 of lanosterol (2,3), or  $\Delta^8 \rightarrow \Delta^7$  isomerization (4). Azasterols are another group of antifungal substances that interfere with sterol metabolism. The site of action of these substances depends on the location of the nitrogen atom in the steroid molecule; for example, 15-azasterol blocks C-14(15) reduction. Unlike the above substances which are all xenobiotics, the azasterols are products of the fungus *Geotrichum flavo-brunneum* (5). A group of xenobiotics that truly inhibit sterol biosynthesis are the allylamines which block squalene epoxidation and hence the conversion of squalene to lanosterol (6). The names and structures of some of the most common sterol inhibitors of current interest are given in Table 1 and Figure 1, respectively.

It is well known that ergosterol, or a functionally equivalent sterol, is required for the optimum growth of most fungi. This is based on the following evidence: 1) a sterol is required for the anaerobic growth of *Saccharomyces cerevisiae* (7), 2) a variety of chemicals that block various steps in the pathway of sterol biosynthesis also inhibit fungal growth (see below), and 3) the growth rates of mutants defective in various aspects of ergosterol biosynthesis are lower than those of corresponding wild-type strains (see below). This requirement accounts for the antifungal properties of the sterol inhibitors.

Since research on the growth and biochemical responses, as well as resistance, to sterol inhibitors has been recently reviewed (8-14) this information will be covered here only insofar as necessary to establish the background for a discussion on the molecular basis for fungal growth inhibition by these substances. Since most of the work has been conducted with C-14 demethylation inhibitors, emphasis will be given to them, particularly the azoles.

#### Inhibition of Demethylation of Lanosterol at C-14 and Other Effects on Lipid Metabolism

The cyclization of 2,3-epoxidosqualene, which is formed from mevalonic acid via the isoprenoid pathway, to lanosterol is the process that constitutes sterol synthesis. The lanosterol molecule then undergoes considerable modification to form a functionally competent sterol, such as cholesterol in animals and ergosterol in most fungi, as reviewed by Mercer (15). The first of these modifications in certain yeasts (*S. cerevisiae* and *Torulopsis glabrata*) involves the oxidative removal of methyl groups at the C-4 and C-14 positions, whereas in most fungi alkylation at C-24 occurs prior to demethylation. Regardless of when alkylation occurs, the C-14 methyl group is removed prior to those at C-4. Although some of the details of C-14 demethylation are not well established, the overall reaction involves a P-450 cytochrome-catalyzed oxidation of the methyl group to the corresponding hydroxymethyl which is subsequently oxidized to a formyl group and removed from the sterol molecule as formic acid. The product of the demethylation would be

TABLE I. Representative antifungal azoles and other sterol inhibitors

TRIVIAL NAME	SYSTEMATIC NAME	TRADE NAME
<b>A. IMIDAZOLE</b>		
1. Bifonazole	1-[(4-biphenyl)-phenylmethyl]-1H-imidazole	Mycospor
2. Enilconazole (Imazalil)	1-[2-(2,4-dichlorophenyl)-2-(2-propenyloxy)]-1H-imidazole	Fungaflo/ Fecunda
3. Miconazole	1-[2-(2,4-dichlorophenyl)-2-[(2,4-dichlorophenyl)methoxy]ethyl]-1H-imidazole mononitrate	
4. Clotrimazole	bis-phenyl[1,2-chlorophenyl]-1-imidazolyl methane	Canesten
<b>B. TRIAZOLE</b>		
1. Dicloubutrazol	[(2RS,3RS)-1-(2,4-dichlorophenyl)-4,4-dimethyl-2-(1H-1,2,4-triazol-1-yl)pentan-3-ol]	Vigil
2. Propiconazole <sup>a</sup>	1-[2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2-ylmethyl]-1H-1,2,4-triazole	Tilt
3. Triadimefon <sup>b</sup>	1-(4-chlorophenoxy)-3,3-dimethyl-1-(1,2,4-triazol-1-yl)butanone	Rayleton
<b>C. PYRIDINE</b>		
1. Buthiolate	S-n-butyl-S'-p-tert-butylbenzyl-N-3-pyridyl dithiocarbonyl imidate	Denmert

D. PYRIMIDINE			
1. Fenarimol	$\alpha$ -(2-chlorophenyl)- $\alpha$ -(4-chlorophenyl)-5-pyrimidine-methanol	Rubigan/Rimidin	
E. MORPHOLINE			
1. Tridemorph	N-tridecyl-2,6-dimethylmorpholine	Calixin	
2. Fenpropimorph	{ $\pm$ -cis-4-[3-(4-tert-butylphenyl)-2-methylpropyl]-2,6-dimethylmorpholine}	Corbel	
F. PIPERAZINE			
1. Triforine	1,4-di-(2,2,2-trichloro-1-formamidoethyl) piperazine	Funginex/Saprol	
G. AZASTEROL	15-aza-24-methylene-cholesta-8,14-dien-3-ol	--	
H. ALLYLAMINE			
1. Naftifine	(E)-N-methyl-N-(1-naphthylmethyl)-3-phenyl-2-propen-1-amine hydrochloride	--	

betaconazole = the ethyl homologue of propiconazole, Vangard.

btriadimenol = the reduced form of triadimefon, Baytan.

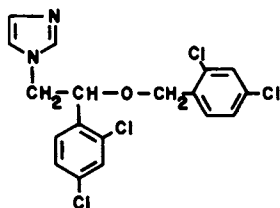
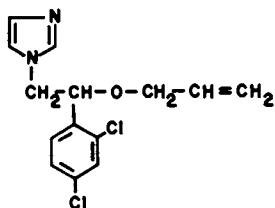
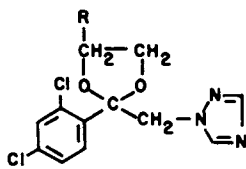
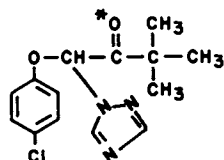
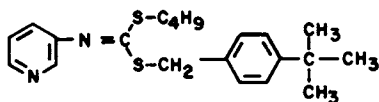
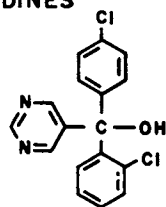
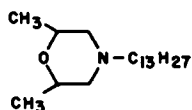
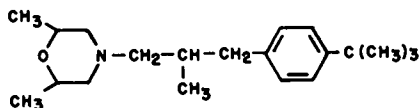
**A. IMIDAZOLES****MICONAZOLE****IMAZALIL****B. TRIAZOLES**R = C<sub>3</sub>H<sub>7</sub> = **PROPICONAZOLE**R = C<sub>2</sub>H<sub>5</sub> = **ETACONAZOLE****TRIADIMEFON**(\*OH = **TRIADIMENOL**)**C. PYRIDINES****BUTHIOBATE****D. PYRIMIDINES****FENARIMOL**

Figure 1. Structures of representative sterol inhibitors.

## E. MORPHOLINES

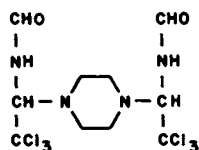


TRIDEMORPH



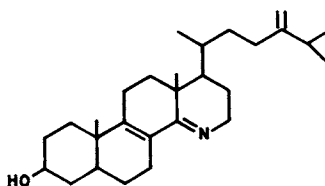
FENPROPIMORPH

## F. PIPERAZINE



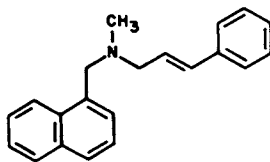
TRIFORINE

## G. AZASTEROL

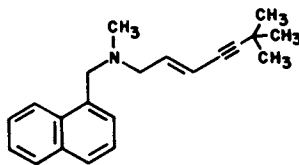


15 - AZASTEROL

## H. ALLYLAMINES



NAFTIFINE



Sf-86-327

Figure 1. Continued. Structures of representative sterol inhibitors.



either 4,4-dimethyl-ergosta-8,14,24(28)-trienol or 4,4-dimethyl-cholesta-8,14,24-trienol depending on whether alkylation at C-24 occurs before or after demethylation at C-14. The double bond at C-14(15) in these intermediates is subsequently reduced in a reaction requiring NADPH.

The C-14 demethylase is a monooxygenase consisting of cytochrome P-450 and a cytochrome P-450 reductase that requires molecular oxygen and NADPH for activity (16). The sterol inhibitors block the first reaction catalyzed by this enzyme by binding the heme iron of cytochrome P-450 (17-22). No oxygenated intermediates in the demethylation process are known to accumulate in inhibitor-treated tissues. An unhindered nitrogen atom at position 3 to the main backbone of the inhibitor molecule is favorable for binding. Miconazole and ketoconazole are believed to show selectivity for the pathogen in clinical situations because they have a higher affinity for the cytochrome P-450 of the yeast enzyme than for that of the host (21).

Fungi treated with substances that bind cytochrome P-450 of the C-14 demethylase accumulate C-14 methyl sterols, which are usually only faintly detectable in most non-treated cells. With C-14 demethylation blocked, the methyl groups at C-4 are sequentially removed as would normally occur in the absence of the inhibitor. Thus, 24-methylene-dihydrolanosterol [24-methyl-lanosta-8,24(28)-dienol], obtusifoliol [ $4\alpha,14\alpha$ -dimethyl-ergosta-8,24(28)-dienol], and  $14\alpha$ -methyl-fecosterol [ $14\alpha$ -methyl-ergosta-8,24(28)-dienol] accumulate in most fungi treated with sterol inhibitors (Figure 2). It appears that the double bond transformations of the sterol molecule that typically occur in the later stages of ergosterol biosynthesis cannot proceed in the presence of the C-14 methyl group. Similar results have been obtained for the various sterol inhibitors and sensitive fungi tested (23-43). Sterols that accumulate in *S. cerevisiae* and *T. glabrata* treated with the C-14 demethylase inhibitors are the corresponding C-24 demethyl,  $\Delta 24(25)$  derivatives (Figure 2).

Some fungi treated with sterol inhibitors produce approximately twice the amount of sterol of non-treated cells (38,42). For example, the total sterol content of mycelium of the peanut leafspot pathogen *Cercospora arachidicola* treated with 0.1 ppm of propiconazole is 1.5 ug/mg dry weight, 80% of which is C-14 methyl sterols, compared to 0.7 ug/mg in non-treated mycelium, (42). The ergosterol content is reduced 50% on a dry weight basis by treatment with the inhibitor. This increase in total sterol may be due to a reduction in feedback inhibition of sterol biosynthesis by ergosterol or one of its metabolites. However, the increase in total sterol does not occur in some fungi (e.g. *S. cerevisiae*). This may be accounted for by the fact that the activity of the key regulatory enzyme of sterol biosynthesis, 3-hydroxy-3-methyl-glutaryl-CoA reductase, is reduced in this yeast by C-14 methyl sterols such as lanosterol that accumulate in response to treatment with these inhibitors (44,45).

Since other vital activities tested (protein synthesis, nucleic acid synthesis, and respiration) are not affected to the same extent, or as soon after treatment, by sub-MIC (minimum inhibitor concentration) levels of the antifungal azoles and related compounds (25,26,28,32,35,39), it is generally agreed that the

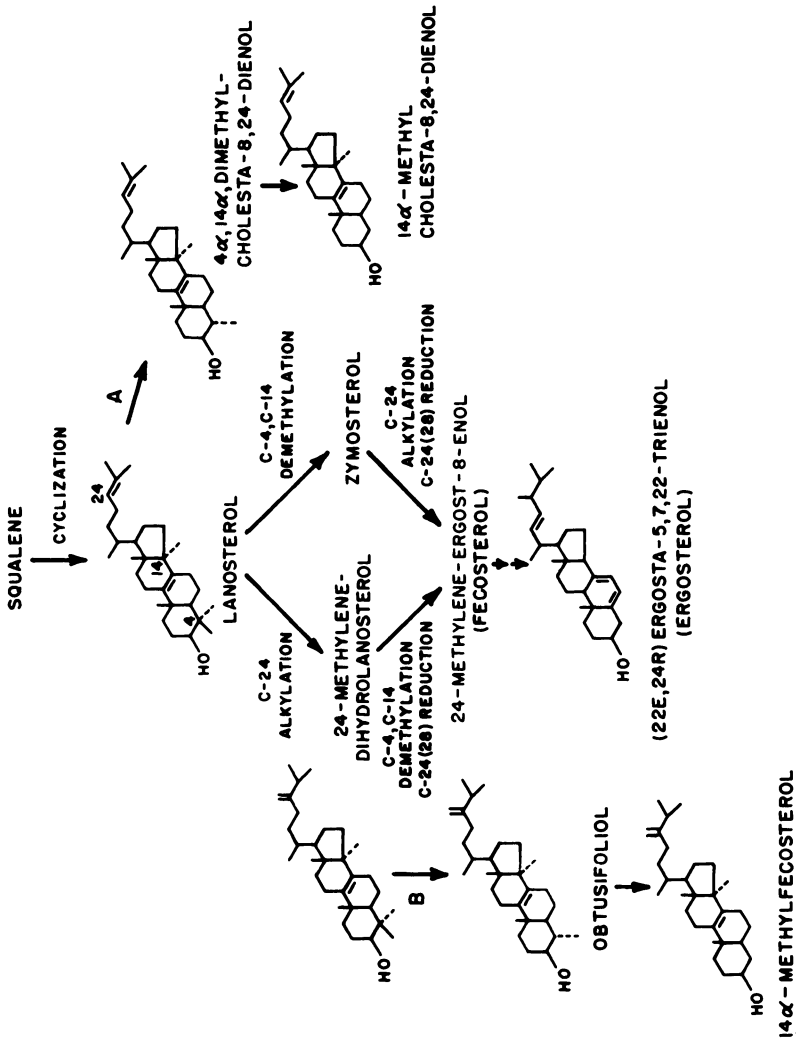


Figure 2. Later stages of the pathway of ergosterol biosynthesis (center) and locations of C-14 demethylase inhibitor action in most fungi (B) and certain yeasts (A).

primary site of their action is the C-14 demethylase. This is further supported by results obtained by Walsh and Sisler (46) who showed that a mutant of Ustilago maydis deficient in C-14 demethylation is not sensitive to representatives of several classes of sterol inhibitors (miconazole, etaconazole, and fenarimol). This mutant accumulates the same sterols as fungi treated with sterol inhibitors, and the doubling time is one-third that of the wild-type. However, a sterol auxotroph of S. cerevisiae supplied with exogenous ergosterol is reported to be as sensitive to miconazole and clotrimazole as the wild-type (47).

In addition to blocking the demethylation of 24-methylene dihydrolanosterol at C-14, there are several other alterations of lipid metabolism in fungi treated with C-14 demethylase inhibitors. These include: 1) an increase in free fatty acids, linoleic acid, and in some cases major saturated fatty acids, and 2) a decrease in oleic acid (8,22,38,39,42,47,48). The increase in free fatty acids has been attributed to an imbalance between the synthesis and degradation of acyl lipids (36), and appears to be associated with changes in sterol content rather than an alternate action of the inhibitors since it also occurs in a mutant of U. maydis deficient in C-14 demethylase (46). This increase seems to occur subsequent to detectable changes in sterol metabolism and is not considered to be a major initial factor in the growth inhibitory activity of the inhibitors. The increase in free fatty acids does not occur in all cases (42,50). The decrease in oleic acid, which is accompanied by an increase in linoleic and in some cases linolenic acid, is particularly pronounced in, but not restricted to, the polar lipid (21,38,42,48,49). These alterations in the relative proportions of fatty acids have been attributed to adaptive-type responses to changes in the fluid properties of membranes brought about by sterol inhibitor-induced modification of the sterol content (21,38,42,48,49). In some but apparently not all fungi, palmitic and in some cases stearic acid accumulate with treatment by anti-fungal azoles (see references cited above). van den Bossche et al. (21) have attributed this to a decrease in the activity of a membrane-bound desaturase.

#### Fungal Growth Inhibition and Tolerance

A large number of fungi have been screened in vitro for their sensitivity to the C-14 demethylation inhibiting substances. Although there are exceptions, fungi show growth reduction (about 50% inhibition) within one to several hours of treatment with 0.1 to 2.0 ppm of a sterol inhibitor (3,26,31,33,39,41,42,51-53). The human pathogen Candida albicans and the plant pathogenic Ustilago maydis, U. avenae, and Taphrina deformans, all of which grow as yeast-like or sporidial forms in vitro, and the filamentous Monilinia fructigena, Penicillium italicum and Aspergillus nidulans are some of the species used for more in depth mode of action studies on the sterol inhibitors. A few of the most active azoles, with their ED<sub>50</sub> concentrations, and fungi most sensitive to them are given in Table II.

TABLE II. Azole sterol inhibitors and their ED<sub>50</sub> concentrations for selected sensitive fungi

Fungal SPECIES	INHIBITOR	APPROXIMATE ED <sub>50</sub> <sup>a</sup> CONCENTRATION	REFERENCE
<i>Candida albicans</i>	miconazole	10 <sup>-8</sup> to 10 <sup>-7</sup> M	(37)
<i>Penicillium italicum</i>	imazalil	0.01 ppm	(35)
<i>Taphrina deformans</i>	propiconazole	0.073 ppm	(51)
<i>Torulopsis glabrata</i>	bifonazole	0.125 ppm	(52)

<sup>a</sup>ED<sub>50</sub> = concentration giving 50% growth inhibition.

Cell division in yeast is more sensitive to sterol inhibitors than dry matter accumulation (32,51). Spore germination in most species is not inhibited at concentrations of the inhibitors that prevent hyphal elongation (26,34). Also, growth inhibition by these substances is either not or only partially reversed by ergosterol supplied in the growth medium (34,51,54); however, a variety of unsaturated lipophilic substances, such as Tween 20 and 40, oleic acid, tocopherol,  $\beta$ -carotene, farnesol, trilinolenin and several others can alleviate the growth inhibitory effects of azoles (34,54-56) as well as morpholines (58). The sterol inhibitors are generally considered to be fungistatic rather than fungicidal (51).

The C-14 demethylation inhibitors are generally considered to be single-site inhibitors, and there has been concern about the development of field resistance or tolerance to them. (The terms resistance and tolerance are used interchangeably here to conform to the terminology used by authors of the work being cited. To my knowledge, true resistance to a sterol inhibitor has not been reported.) Indeed, fungal isolates with a high degree of tolerance to these substances can be readily selected for in laboratory culture (33,34,59), or induced with mutagenic agents (60,61). There had been no reports of field resistance to these inhibitors up to 1982 (62), but more recently there have been reports in Europe (63). The absence of confirmed resistance in the field has been attributed to reduced fitness or pathogenicity of resistant strains relative to the wild-type strains (11). Generally, mutants to one C-14 demethylase inhibitor show cross-resistance to others (62).

Studies on the mechanism of resistance have been conducted with a strain of *Aspergillus nidulans* that carries genes for resistance to the pyrimidine inhibitor fenarimol (60) and the imidazole imazalil (64). Resistance to fenarimol has been attributed to reduced uptake of the inhibitor (65). Both the wild-type and resistant strains possess energy-dependent efflux mechanisms with different efficiencies to excrete fenarimol, the rate of excretion being higher in the resistant strain (66). The mechanism for resistance to imazalil is, at least in part, similar to that for fenarimol (59).

Certain Mucorales examined have shown a relatively high degree of tolerance to the sterol inhibitors. For example, 80 ppm of propiconazole is required for 50% growth inhibition of *Mucor rouxii* in liquid shake culture (57). Growth inhibition by this relatively high concentration of the inhibitor is probably due to direct interaction of the triazole with the plasma membrane, and not to

inhibition of the C-14 demethylase, as has been shown for miconazole in *C. albicans* (21). In fact, C-14 demethylation in *Mucor* is inhibited by 2 ug/ml propiconazole. The basis for the observed tolerance to propiconazole is not known, but preliminary results suggest that it is not due to metabolism or reduced uptake of the inhibitor (57). Apparently not all of the Mucorales possess the same degree of tolerance to these inhibitors since the growth of *Rhizopus stolonifer* is inhibited 50% by 5 to 8 ug/ml propiconazole (51). Also, other phycomycetes (Oomycetes) are sensitive to fenarimol (4 and cited in 65).

Likewise, species of the pythiaceous genera *Pythium* and *Phytophthora* show a high degree of tolerance to the sterol inhibitors. For example, 50 ppm of propiconazole is required for 50% growth inhibition of *Phytophthora cinnamomi* (57). The relatively high tolerance to sterol inhibitors by species of these genera can be accounted for by the fact that they neither produce sterols nor require them for vegetative growth. Growth inhibition at relatively high concentrations is again probably due to direct interaction with membranes.

#### Morphology, Ultrastructure, and Permeability

There are several common morphological responses by various fungi and yeasts to treatment with sterol inhibitors (3 and references cited therein). While spore germination is generally not inhibited, germ tube and hyphal extension is curtailed, frequently with swelling and bursting of the hyphal tips. Yeast cells may swell, become vacuolated, and form chains or aggregates of 2 to 6 cells resulting from failure of buds to separate from mother cells. Yeast-hypha conversion is preferentially inhibited in *C. albicans* by bifonazole (67). These results are suggestive of abnormal cell wall formation, but there is little difference in the chitin content of the cell walls of *Cercospora* and *Cercosporidium* (42), or several other species (57) from propiconazole-treated and non-treated cultures; in fact, cell walls from treated cultures usually contain slightly more chitin/chitosan than those from non-treated cultures. Furthermore, buthiolate (S-1358) has little effect on the incorporation of [<sup>14</sup>C] labeled glucose and glucosamine into the cell wall of *Monilinia fructigena* (26). Yet, alteration of cell wall structure has been detected with scanning and transmission electron microscopy (68,69). Using a specific fluorescent marker, it has been shown that bifonazole- and imazalil-treated cells of *C. albicans* and *Torulopsis glabrata* lack septa and have irregular depositions of chitin in their walls (69,70).

The most obvious ultrastructural effects of the sterol inhibitors have been found in cell walls and membrane systems. Except for mitochondrial swelling, fungistatic levels of clotrimazole have little effect on the cytology of *C. albicans* cells, whereas fungicidal levels ( $1.5 \times 10^{-4}$  M) result in a proliferation of membranes by invagination of the plasma membrane and the development of membrane particles between the retracted cell membrane and cell wall (71). Deterioration of the internal structure of mitochondria and nuclei also has been observed. In addition, cell wall and septum thickening, accumulation of lipid bodies, and various changes in membrane organization within the cells have been observed in

sporidia of *U. avenae* (72) and other fungi (71a,71b) treated with several C-14 demethylase inhibitors.

As might be expected for cells in which the plasma membrane has been altered, leakage of cellular constituents (5,73,74) and altered substrate uptake (35,51, and cited in 37,75) including an increased permeability to protons (75), have been observed in sterol inhibitor-treated cells.

#### Mechanism of Growth Inhibition

The mechanism of growth inhibition by sterol inhibitors must be explained in the context of specific sterol functions. Unfortunately, our knowledge of this area is somewhat limited. In fungi, sterols such as ergosterol exercise their principal biological activity in membranes (76) where they are believed to participate in the modulation of fluid properties (77). In addition to satisfying this "bulk" function, there is recent evidence for additional, perhaps metabolic, roles for sterols in fungal membranes. These roles have been described independently for ergosterol in yeast sterol auxotrophs and mutants and referred to as "sparking" (78,79) and "sterol synergism" (80), both of which probably reflect the same functions. The different roles for sterols have certain structural requirements (79) and distinct stereospecificities (81). The synergistic role of sterols can be satisfied by considerably lower amounts of ergosterol than are required to affect the bulk fluid properties of membranes (80). It is well-documented that C-14 methyl sterols (i.e. lanosterol) will not fully substitute for ergosterol in supporting the growth of *S. cerevisiae* (82). Moreover, endogenous lanosterol in a heme deficient mutant of *S. cerevisiae* is detrimental to cell growth, and adaptation for growth on media supplemented with cholesterol is correlated with the absence of lanosterol (83). Bloch (84) postulated that the reason lanosterol cannot fully satisfy the sterol requirement for growth is that the axial C-14 methyl group interferes with van der Waals interactions of the sterol with fatty acyl chains of membrane lipid. Furthermore, the  $\Delta^8(9)$  double bond present in C-14 methyl sterols is responsible for a bend in the molecule that is apparently unfavorable for packing in the lipid bilayer of the membrane. Nevertheless, C-14 methyl  $\Delta^8(9)$  sterols are generally not considered to be lethal because several C-14 demethylase mutants are able to grow without exogenous sterol (46,85,86). C-14 methyl sterols are believed to satisfy only the bulk requirement and not the more specific role for sterol (78,78a). With this limited background, some of the biological responses of fungi to treatment with sterol inhibitors, particularly the C-14 demethylase inhibitors, might be explained, and a hypothesis for the molecular basis for growth inhibition by these compounds proposed.

The separation in time of up to several hours between the inhibition of C-14 demethylation, or other reactions related to sterol biosynthesis, and the onset of growth reduction (i.e. 10 hours for *P. italicum* treated with fenpropimorph, (3)) suggests that either blocking ergosterol biosynthesis is not the growth inhibiting mode of action of these substances or, perhaps more likely in this case, the actual growth limiting activity(ies) is several steps removed from the primary biochemical site of action.

This delay might be explained in part by the time required for ergosterol-deficient membranes to become sufficiently integrated into the plasma membrane to have an impact on growth essential activities associated with this membrane. It is difficult to determine the nature, if any, or extent of the possible effects of C-14 methyl sterols on the apparent growth limiting effects of having little or no ergosterol in the membranes. They may tend to compound the problems associated with ergosterol-deficient membranes, a contention supported by several authors (13,21).

The perplexing inability to be able to reverse growth inhibition with ergosterol might be explained by the fact that exogenous sterol is not taken up by the cell and incorporated into the membrane. This is consistent with the fact that *S. cerevisiae* cells grown anaerobically readily take up sterols from the medium, and indeed require an exogenous source under this condition, but cells do not take up sterols from the medium when grown under aerobic conditions which are favorable for sterol formation. Growth inhibition by sterol inhibitors other than those that block C-14 demethylation also cannot be reversed by sterols; for example, growth inhibition of *Sordaria fimicola* by AY 9944 and SKF 3301-A cannot be reversed by sterols (87), and neither can the inhibition of *U. maydis* by 15-azasterol be reversed by ergosterol (88).

Although it has not been shown unequivocally, the specific changes in fatty acid composition (i.e. reduction in C<sub>18:1</sub> and increase in C<sub>18:2</sub>) are probably not caused directly by the C-14 demethylation inhibitors, but instead they may be adaptive responses to perturbation of the membrane by changes in sterol composition resulting from treatment with the inhibitors. In *Phytophthora cinnamomi*, which does not produce sterols, the shift to higher unsaturation was not observed when the fungus was treated with the ED<sub>50</sub> concentration of propiconazole (57). It is well-known that such changes occur in poikilothermic organisms undergoing adaptation to a temperature below the optimum for growth (89). It is not certain if the C<sub>18:1</sub> → C<sub>18:2</sub> shift in sterol inhibitor-treated fungi is due to the increase in C-14 methyl sterols, decrease in ergosterol, or a combination of both. This shift has been observed in *T. deformans* grown in a medium containing very high levels (250 µg/ml) of lanosterol (38); however, similar changes in fatty acid composition accompany reductions in brassicasterol caused by naftifine which does not cause C-14 methyl sterols to accumulate (90).

Contributions of the observed changes in fatty acids to the growth inhibitory process brought about by C-14 demethylase inhibitors are difficult to assess. The ability of a wide variety of unsaturated, lipophilic substances at relatively high concentrations to reverse growth inhibition by sterol inhibitors suggests that these lipids may substitute for the bulk role of sterols in membranes deficient in ergosterol. van den Bossche et al. (21) suggest that higher levels of saturated fatty acids, along with C-14 methyl sterols, in azole treated fungi disturb membrane permeability and the activity of membrane-bound enzymes. On the other hand, it appears that the sterol composition of the membranes may, at least in part, determine the optimum fatty acid composition favorable for growth. For example, cholesterol allows the sterol-requiring cells of *M. capricolum* to grow with a wide range of fatty

acid supplements to the medium, but lanosterol supports growth only when supplemented with certain fatty acid combinations (91). It is also clear that the nature of the sterol supplement for the yeast mutant GL 7 (deficient in squalene epoxide cyclase and heme synthesis) determines the fatty acid that best satisfies the growth requirement (80).

It is tempting to speculate that the decrease in oleic acid in the lipid, particularly in phospholipid, that occurs universally in fungi treated with C-14 demethylase inhibitors is relevant to the growth inhibitory process. There is experimental evidence that sterols are specifically involved in oleic acid metabolism (80,92). For example, the GL 7 mutant (see above) grown in a medium containing a synergistic mixture of ergosterol and cholesterol (1:3) incorporates more [<sup>14</sup>C] oleic acid into phospholipids than when grown on cholesterol alone (80). Based on oleic acid deprivation studies using the GL 7 mutant, it appears that this acid is required for normal lipid metabolism (93). A fivefold decrease in oleic acid has been found in the polar lipid of a plasma membrane-enriched fraction from *T. deformans* treated with 0.073 ug/ml propiconazole (49,94). It is not known whether the decrease in membrane oleic acid is sufficient to have harmful consequences to the growth of fungal cells, or even if the alteration in fatty acid composition in general resulting from treatment with a C-14 demethylase-inhibitor contributes to the growth inhibitory mechanism of these substances.

There are no plasma membrane-bound enzymes whose activities are known to be specifically and sufficiently altered to account for growth inhibition by the sterol inhibitors at sub-MIC doses. A likely candidate is chitin synthetase, but the activity of this enzyme is not reduced; in fact, sterol inhibitor-treated fungi contain more glucosamine polymers than controls. However, the altered deposition of chitin (see above references) may reflect a discontinuity between cytoskeletal elements which are believed to be involved in cell wall formation and the plasma membrane, but it is unlikely that this is at the root of growth inhibition since a wall-less slime mutant of *Neurospora crassa* is as sensitive as the wild-type strain to propiconazole (57). Cytochrome oxidase and microsomal ATPase are inhibited by high concentrations (10<sup>-5</sup>, 10<sup>-4</sup> M) of miconazole and ketoconazole, but this has been attributed to their direct destructive action on the membrane systems required for the activity of these enzymes (56). The weight of evidence leans toward altered permeability of the plasma membrane as the growth-limiting factor in fungi treated with sterol inhibitors. Perhaps the most important element in this regard is the increased permeability to protons. Baldwin (13) has shown that the half-time for the entry of protons into *S. cerevisiae* cells treated for 18 hours with diclobutrazol is over three times higher than corresponding controls. Thus, he attributes the inability of inhibitor-treated cells to take up nutrients (e.g. amino acids) against the concentration gradient to the absence of sufficient electrochemical gradient across the plasma membrane, and therefore the continued expulsion of protons from the cell by the ATPase is a wasteful use of energy leading to cell death.



### Summary

The sterol inhibitors of current interest are a large and diverse group of substances that block various reactions in the later stages of sterol biosynthesis, and have potent antifungal properties against a wide variety of agriculturally and medically important species. Within the limits of our knowledge, the major biochemical responses by fungi to sublethal doses of these inhibitors, particularly the C-14 demethylase inhibitors, appear to be restricted to lipid metabolism. The molecular basis for the antifungal properties appears to relate to changes in the physical properties of membranes, particularly the plasma membrane, brought about initially by a decrease in ergosterol or functionally equivalent sterol and perhaps an increase in C-14  $\Delta^8$  sterols which in turn lead to subsequent other changes in lipid content. This results in impaired membrane functions, the most important of which may be permeability. The growth-limiting factors in fungi treated with sterol inhibitors are complex, probably interrelated, may vary with the individual fungus, and most likely collectively contribute to a reduction in growth potential of the treated organism (49).

### Acknowledgments

The work presented from this laboratory was supported by funds from the Alabama Agricultural Experiment Station (Project ALA-5-877), the Herman Frasch Foundation and the Ciba-Geigy Corporation. Contributors to this work are Dr. Michel Sancholle, H. Gary Hancock, Dory Kirby, Mitch Wise, Vivi Karathanasis, Sabrina Barber-Azzouz, Mieke Van Den Reek, and Scott McCraney.

### Literature Cited

1. Jager, G. In "Pesticide Chemistry"; Miyamoto, J.; Kearney, P.C., Eds.; Pergamon Press, New York, 1983; Vol. 3; pp. 55-65.
2. Kerkenaar, A.; Uchiyama, M.; Verslius, G. G. Pestic. Biochem. Physiol. 1981, 16, 97-104.
3. Kerkenaar, A.; van Roseum, M.; Versluis, G. G.; Marsman, J. W. Pestic. Sci. 1984, 15, 177-187.
4. Berg, L. R.; Patterson, G. W.; Lusby, W. R. Lipids 1983, 18, 448-452.
5. Boeck, L. D.; Hoehn, M. M.; Westhead, J. E.; Wolter, R. K.; Thomas, D. N. J. Antibiol. 1975, 29, 95-101.
6. Petrányi, G.; Ryder, N.S.; Stutz, A. Science 1984, 224, 1239-1241.
7. Andreasen, A. A.; Stier, J. B. J. Cell. Comp. Physiol. 1953, 41, 23-35.
8. Ragsdale, N. N. In "Antifungal Compounds"; Siegel, M. R.; Sisler, H. D., Eds.; Marcel Dekker, Inc., New York, 1977; Vol. 2; pp. 333-363.
9. Siegel, M. R. Plant Disease 1981, 65, 986-989.
10. Waard, M. A. de; Dekker, J. In "Pesticide Chemistry"; Matsunaka, S.; Hutson, D. H.; Murphy, S.D., Eds.; Pergamon Press, New York, 1982; pp. 43-49.

11. Fuchs, A.; Waard, M. A. de. In "Fungicide Resistance in Crop Protection"; Dekker, J.; Georgopoulos, S. G., Eds.; Tudoc, Wageningen, The Netherlands, 1982; pp. 71-86.
12. Schwinn, F. J. Pestic. Sci. 1983, 15, 40-47.
13. Baldwin, B. C. Brit. Biochem. Soc. Trans. 1983, 11, 659-663.
14. Sisler, H. D.; Ragsdale, N. N. In "Mode of Action of Antifungal Agents"; Trinci, A. P. J.; Ryley, J. F. Eds.; Brit. Mycol. Soc., Great Britain, 1984; pp. 258-282.
15. Mercer, E. I. Pestic. Sci. 1984, 15, 133-155.
16. Aoyama, Y.; Yoshida, Y.; Hata, S.; Nishino, T.; Katsuki, H.; Maitra, U.; Mohan, V.; Sprinson, D. B. J. Biol. Chem. 1983, 258, 9040-9042.
17. Gadher, P., Mercer, E.; Baldwin, B. C.; Wiggins, T. E. Pestic. Biochem. Physiol. 1983, 19, 1-10.
18. Wiggins, T. E.; Baldwin, B. C. Pestic. Sci. 1984, 15, 208-209.
19. Aoyama, Y.; Yoshida, Y.; Hata, S., Nishino, T., Katsuki, H. Biochem. Biophys. Res. Commun. 1983, 115, 642-647.
20. van den Bossche, H.; Willemsens, G. Arch. Int. Physiol. Biochem. 1982, 90, B218-219.
21. Van den Bossche, H.; Lauwers, W.; Willemsens, G.; Marichal, P.; Cornelissen, F.; Cools, W. Pestic. Sci. 1984, 15, 188-198.
22. Henry, M. J.; Sisler, H. D. Pestic. Biochem. Physiol. 1984, 22, 262-275.
23. Kato, T. In "Pesticide Chemistry"; Matsunaka, S.; Hutson, H.; Murphy, S. D., Eds.; Pergamon Press, New York, 1983; Vol. 3; pp. 33-44.
24. Kato, T. Neth. J. Plant Pathol. 1977, 83, 113-120.
25. Kato, T.; Shoani, M.; Kawase, Y. J. Pestic. Sci. 1980, 5, 69-79.
26. Kato, T.; Tanaka, S.; Ueda, M.; Kawase, Y. Agr. Biol. Chem. 1974, 38, 2377-2384.
27. Kato, T., Tanaka, S.; Ueda, M.; Kawase, Y. Agr. Biol. Chem. 1975, 39, 169-174.
28. Kato, T.; Kawase, Y. Agr. Biol. Chem. 1976, 40, 2379-2388.
29. Leroux, P.; Gredt, M. Ann. Phytopathol. 1978, 10, 45-60.
30. Ragsdale, N. N. Biochim. Biophys. Acta 1975, 380, 81-96.
31. Ragsdale, N. N.; Sisler, H. D. Biochem. Biophys. Res. Commun. 1972, 46, 2048-2053.
32. Ragsdale, N. N.; Sisler, H. D. Pestic. Biochem. Physiol. 1973, 3, 20-29.
33. Sherald, J. L.; Sisler, H. D. Pestic. Biochem. Physiol. 1975, 5, 477-488.
34. Sherald, J. L.; Ragsdale, N. N.; Sisler, H. D. Pestic. Sci. 1973, 4, 719-727.
35. Siegel, M.; Ragsdale, N. N. Pestic. Biochem. Physiol. 1978, 9, 48-56.
36. Sisler, H. D.; Ragsdale, N. N. Neth. J. Plant Physiol. 1977, 83, 81-91.
37. van den Bossche, H.; Willemsens, G.; Cools, W.; Lauwers, W. F. J.; LeJeune, L. Chem.-Biol. Interactions 1978, 21, 59-78.
38. Weete, J. D.; Sancholle, M. S.; Montant, C. Biochim. Biophys. Acta 1983, 752, 19-29.
39. Buchenauer, H. Pflanzenschutz-Nachrichten 1976, 29, 281-302.

40. Buchenauer, H. J. Pestic. Sci. 1978, 9, 507-512.
41. Ebert, E.; Gaudin, J.; Muecke, W.; Ramstriner, K.; Vogel, C.; Fuhrer, H. Z. Naturforsch. 1983, 38C, 28-34.
42. Hancock, H. G.; Weete, J. D. Pestic. Biochem. Physiol. 1985 (in press).
43. Henry, M. J.; Sisler, H. D. Pestic. Sci. 1981, 12, 98-102.
44. Berg, D.; Draber, W.; von Hugo, H.; Hummel, W.; Mayer, D. Z. Naturforsch. 1981, 36C, 798-803.
45. Berg, D.; Regel, E.; Harenberg, H. E.; Plempel, M. Arzneim.-Forsch. 1984, 34, 139-146.
46. Walsh, R. C.; Sisler, H. D. Pestic. Biochem. Physiol. 1982, 18, 122-131.
47. Taylor, F. R.; Rodriguez, R. J.; Parks, L. W. Antimicrob. Agents Chemother. 1983, 23, 515-521.
48. van den Bossche, H.; Willemsens, G.; Cools, W.; Lauwers, W. F. Arch. Int. Physiol. Biochim. 1981, 89, B134.
49. Weete, J. D.; Sancholle, M.; Touze-Soulet, J. M.; Bradley, J.; Dargent, R. Biochim. Biophys. Acta 1985, 812, 633-642.
50. Baldwin, B. C.; Wiggins, T. E. Pestic. Sci. 1984, 15, 156-166.
51. Sancholle, M.; Weete, J. D.; Montant, C. Pestic. Biochem. Physiol. 1984, 21, 31-44.
52. Barug, D.; de Groot, C. Arzneim.-Forsch. 1983, 33, 538-545.
53. Takano, H.; Oguri, Y.; Kato, T. J. Pestic. Sci. 1983, 8, 575-582.
54. Leroux, P.; Gredt, M.; Fritz, F. Phytiatrie-Phytopharmacie 1976, 25, 317-334.
55. Siegel, M. R.; Kerkenaar, A.; Sijpesteijn, A. K. Neth. J. Plant Pathol. 1977, 83, 121-133.
56. van den Bossche, H.; Ruyschaert, J. M.; Defrise-Quertain, F.; Willemsens, G.; Cornelissen, F.; Marichal, P.; Cools, W.; van Cutsen, J. Biochem. Pharmacol. 1982, 31, 2609-2617.
57. Weete, J. D., unpublished data.
58. Kerkenaar, A.; Barug, D.; Sijpesteijn, A. K. Pestic. Biochem. Physiol. 1979, 12, 195-204.
59. Siegel, M. A.; Solel, Z. Pestic. Biochem. Physiol. 1981, 15, 222-233.
60. Waard, M. A. de; Gieshes, S. A. Neth. J. Plant Pathol. 1977, 83, 177.
61. Barug, D.; Kerkenaar, A. Meded. Fac. Landbouw. Rijksuniv. Gent. 1979, 44, 121.
62. Waard, M. A. de; Fuchs, A. In "Fungicide Resistance in Crop Plants"; Dekker, J.; Geogopoulos, S. G., Eds.; Tudoc, Wageningen, The Netherlands, 1982; pp. 87-100.
63. Staub, T.; Sozzi, D. Plant Dis. 1984, 68, 1026-1031.
64. Van Tuyl, J. M. Neth. J. Plant Pathol. 1977, 83, 169.
65. Waard, M. A. de; van Nistelrooy, J. G. M. Pestic. Biochem. Physiol. 1979, 10, 219-229.
66. Waard, M. A. de; van Nistelrooy, J. G. M. Pestic. Biochem. Physiol. 1980, 13, 255-266.
67. Barug, D.; Samson, R. A.; Kerkenaar, A. Arzneim.-Forsch. 1983, 33, 528-537.
68. Nollin, S. de; Borgers, M. Sabouraudia 1974, 12, 341-351.
69. Nollin, S. de; Borgers, M. Antimicrob. Agents Chemother. 1975, 7, 704-711.

70. Kerkenaar, A.; Barug, D. Pestic. Sci. 1984, 15, 199-205.
71. Iwata, K.; Yamaguchi, H.; Hiratani, T. Sabouraudia 1973, 11, 158-166.
- 71a. Stiers, D. L.; Fellman, J. K.; LeTourneau, D. Environ. Exper. Botany 1980, 20, 181-189.
- 71b. Sancholle, M. Docteur D'Etat. Thesis, Universite Paul Sabatier, Toulouse, 1984.
72. Hippe, S. Pestic. Biochem. Physiol. 1984, 21, 170-183.
73. Iwata, K.; Kanda, Y.; Yamaguchi, H.; Osumi, M. Sabouraudia 1973, 11, 205-209.
74. Swamy, K. H. S.; Sirsi, M.; Rao, G. R. Antimicrob. Agents Chemother. 1974, 5, 420-425.
75. Thomas, P. G.; Haslam, J. M.; Baldwin, B. C. Brit. Biochem. Soc. Trans. 1983, 11, 713.
76. Nes, W. R. Lipids 1974, 9, 596-612.
77. Demel, R. A.; de Kruiff, B. Biochim. Biophys. Acta 1976, 457, 109-132.
78. Rodriguez, R. J.; Taylor, F. R.; Parks, L. W. Biochem. Biophys. Res. Commun. 1982, 106, 435-441.
- 78a. Taylor, F. R.; Rodriguez, R. J.; Parks, L. W. J. Bacteriol. 1983, 155, 64-68.
79. Rodriguez, R. J.; Parks, L. W. Arch. Biochem. Biophys. 1983, 225, 861-871.
80. Ramgopal, M.; Bloch, K. Proc. Natl. Acad. Sci. USA 1983, 80, 712-715.
81. Pinto, W. J.; Lozano, R.; Sekula, B. C.; Nes, W. R. Biochem. Biophys. Res. Commun. 1983, 112, 47-54.
82. Nes, W. R.; Sekula, B. C.; Nes, W. D.; Adler, J. H. J. Biol. Chem. 1978, 253, 6218-6225.
83. Taylor, F. R.; Parks, L. W. Biochem. Biophys. Res. Commun. 1980, 95, 1437-1445.
84. Bloch, K. E. CRC Crit. Rev. Biochem. 1979, 7, 1-5.
85. Trocha, P. J.; Jasne, S. J.; Sprinson, D. B. Biochemistry 1977, 16, 4721-4726.
86. Pierce, A. M.; Mueller, R. B.; Unrau, A. M.; Oehleslager, A. C. Can. J. Biochem. 1978, 56, 794-800.
87. Elliott, C. G. J. Gen. Microbiol. 1969, 56, 331-343.
88. Woloshuk, C. P.; Sisler, H. D.; Dutky, S. R. Antimicrob. Agents Chemother. 1979, 16, 98-103.
89. Martin, C. E.; Hiramitsu, K.; Kitajima, Y.; Nozawa, Y.; Skriver, L.; Thompson, G. A. Biochemistry 1976, 15, 5218-5227.
90. Weete, J. D.; van den Reek, M., unpublished data.
91. Dahl, J. S.; Dahl, C. E.; Bloch, K. Biochemistry 1980, 19, 1467-1472.
92. Dahl, J. S.; Dahl, C. E.; Bloch, K. J. Biol. Chem. 1981, 256, 87-91.
93. Buttke, T. M.; Pyle, A. Z. J. Bacteriol. 1982, 152, 747-756.
94. Sancholle, M.; Weete, J. D.; Touze-Soulet, J. M.; Dargent, R. In "Structure, Function, and Metabolism of Plant Lipids"; Siegenthaler, P. A.; Eichenberger, W., Eds.; Elsevier Science Publ., B.V., 1984; pp. 347-352.

RECEIVED January 10, 1986

## Chapter 18

# Antifungal Activity of Plant Steroids

James G. Roddick

Department of Biological Sciences, University of Exeter, Exeter, United Kingdom

Antifungal steroids in plants are represented mainly by the glycoalkaloids (especially the Solanum type) and the saponins. Both types of compound are strongly fungistatic/fungicidal in vitro apparently due to their ability to complex with membrane sterols and disrupt membrane integrity. The suggestion that the aglycone (rather than the glycoside) is the active moiety is questioned partly on the basis of data from studies on synthetic lipid membranes. Available evidence suggests that glycoalkaloids and saponins are not key factors in the resistance of vegetative organs to fungal infections although they may be of greater significance in reproductive structures and also contribute to the general defences of the plant.

Plants produce a wide range of steroids (Table I) and accumulate some in considerable quantities but our knowledge of the functions of most of these compounds is meagre (1). Probably the major exception is the sterols which are known to be important membrane components (2,3) as well as precursors of other steroids (4). Estrogens, androgens, corticosteroids (5,6) and brassinosteroids (7) may have growth regulatory activity although this is still not certain. For the remaining groups, no definite role has been established within the plant but the toxic nature of many of these steroids suggests they could have an ecological rather than metabolic function contributing to plant resistance to pathogens (especially fungi) and predators (especially insects) (8).

This article will review the antifungal activity of plant steroids but it should be noted that not all steroids act in an inhibitory capacity towards fungi. Certain sterols have a stimulatory effect on fungal development promoting growth, the differentiation of reproductive structures, and fertilisation in species of Pythium and Phytophthora which lack the ability to synthesize sterols (9). This subject is considered elsewhere in this volume.

Antifungal activity has been demonstrated mainly in two groups of plant steroids, glycoalkaloids of the Solanum type and saponins. Members of both groups are comprised of a steroidal aglycone

0097-6156/87/0325-0286\$06.00/0  
© 1987 American Chemical Society

attached to one or more carbohydrate moieties. In addition, alkaloids contain a basic nitrogen group which renders them much more toxic, especially to homiothermic organisms (10). Steroidal saponins are usually based on a spirostane skeleton (Figure 1). Various types of Solanum glycoalkaloids exist but those so far shown to be fungitoxic invariably possess a spirosolane- or solanidane-type aglycone (Figure 1). These glycoalkaloids and the monodesmosidic saponins are biologically-active amphipathic molecules with an oligosaccharide comprising up to five monosaccharides attached at C-3; bi-desmosidic saponins have an additional sugar moiety (usually one glucose) at C-26 and are biologically inactive. The chemistry of the Solanum alkaloids and saponins has been reviewed by various workers (11-14). Of the two groups, the glycoalkaloids have received more research attention undoubtedly because they are present in edible parts of the important food plants, potato and tomato, and have caused illness and death of humans and livestock on a number of occasions (15). Steroidal saponins are present in the non-edible parts of some less prominent food plants (e.g. oat) and have not caused serious incidences of poisoning. The biological properties of these compounds are detailed in a number of reviews (13,14,16-20).

Table I. Major Groups of Steroids found in Plants

Group	Example	Reference
Sterol	Sitosterol, stigmasterol	3, 96
Estrogen	Estradiol, estrone	5, 6
Androgen	Testosterone, androstenedione	5, 98
Corticosteroid	11-deoxycorticosterone	5, 102
Brassinosteroid	Brassinolide	7
Progesterone	Progesterone, pregnenolone	97, 98
Withanolide	Withaferin, nicandrenone	99
Ecdysteroid	Ecdysone, ecdysterone	100
Cardiac glycoside	Calotropin	101
Saponin	Digitonin, avenacosides A and B	13, 14
Glycoalkaloid	Solanine, jervine	11, 12

In the following section some of the more important *in vitro* studies which established the fungitoxicity of glycoalkaloids and saponins are described leading, in the two subsequent sections, to a critical assessment of how these compounds affect fungi at the biochemical level and what contributions they make to the resistance of plants to fungal pathogens.

#### Fungitoxicity of Glycoalkaloids and Saponins *in vitro*

A large number of studies have been made in this field with reports dating from at least 1933 when the potato glycoalkaloid 'solanine' (Figure 2) was reported to inhibit growth of Cladosporium fulvum (21,22). (At this time 'solanine' preparations probably contained both potato alkaloids,  $\alpha$ -solanine and  $\alpha$ -chaconine, the latter not being discovered until 1954). The finding around the same time that expressed juice from tomato plants inhibited Fusarium oxysporum f.sp. lycopersici (23) subsequently led to the discovery of the tomato glycoalkaloid,  $\alpha$ -tomatine (Figure 2), the active ingredient of the extracts. Further work (24,25) demonstrated the general toxicity of

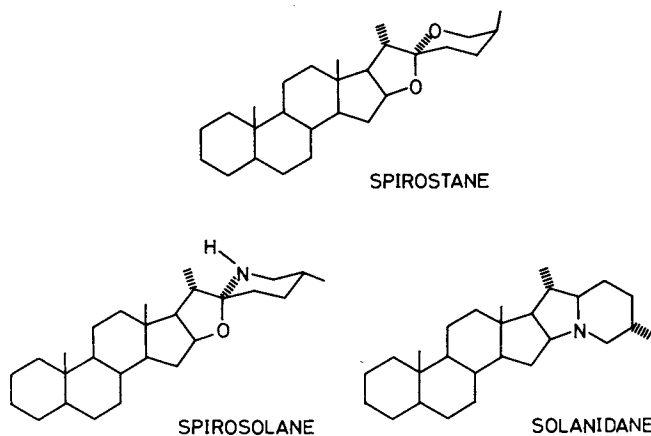
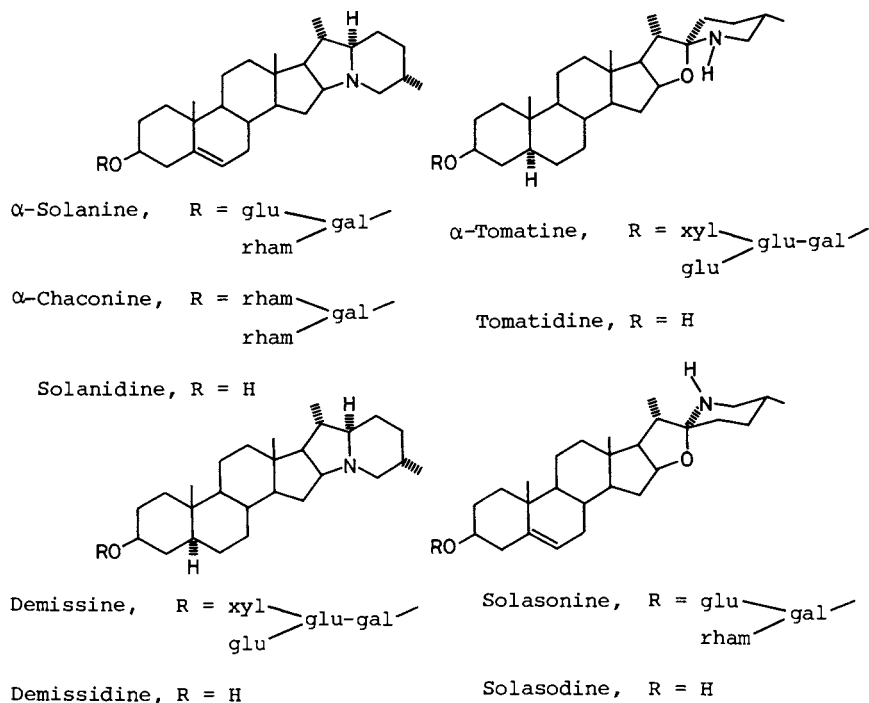


Figure 1. Aglycone skeletons of fungitoxic steroidal saponins and Solanum glycoalkaloids.

Figure 2. Solanum steroidal glycoalkaloids.



tomatine towards fungi, with *F. oxysporum* and various human dermatophytic fungi proving particularly sensitive. The differential susceptibility of fungi to tomatine was later confirmed in a more comprehensive investigation involving 30 species from 19 genera (26). The maximum and minimum concentrations of tomatine to completely inhibit mycelial growth ranged from 850 mM to 130  $\mu$ M, a factor of 6500.

An important feature of glycoalkaloid toxicity was highlighted by McKee (27) when he demonstrated the pH dependence of solanine-, tomatine- and demissine- (Figure 2) induced disruption of spores of a number of fungi, including *Fusarium caeruleum*. Disruption was greatest in alkaline conditions, the LD<sub>50</sub> of solanine at pH 5.6 being 100x greater than at pH 7.6. The saponin digitonin (Figure 3), although more toxic than these alkaloids, was relatively unaffected by pH (Table II). The pH dependence of glycoalkaloid action has since been shown in other fungi (e.g. 28-30) and to be a general effect. Although details remain uncertain, protonation of the alkaloid produces a form with less biological activity whereas dissociation in alkaline conditions yields the unprotonated, highly active form (28).

Table II. LD<sub>50</sub> (mg/l) of Glycoalkaloids and Saponins against Spores of *Fusarium caeruleum* in relation to pH. After McKee (27).

Steroid	pH							
	5.0	5.6	5.9	6.5	7.0	7.6	8.0	8.3
Tomatine	-	460	220	64	32	13	-	7
Solanine	-	2000	1100	260	85	20	11	8
Demissine	-	-	260	72	22	11	-	8
Digitonin	27	-	-	18	-	-	14	-

In the study by McKee (27), chaconine (Figure 2) proved more toxic than solanine with their common aglycone, solanidine (Figure 2), much less so. Similarly, tomatine (a tetraoside) was more effective than its trisaccharide hydrolysis products against *Helminthosporium turcicum*, *Septoria linicola* and *Colletotrichum orbiculare* with the aglycone, tomatidine (Figure 2), being least effective (28). The effect of tomatidine varied considerably with the test organism. The greater toxicity of glycosides compared with aglycones has also been shown with *Botrytis cinerea* (31) and *Phytophthora cactorum* (32), the latter authors also demonstrating this feature for solasonine/solasodine (Figure 2). Nevertheless, a few reports also exist of tomatidine and solanidine being more fungitoxic than their respective glycosides (33,34).

Virtually all the reports on fungal development in the presence of glycoalkaloids or saponins describe inhibitory effects, but recently tomatine was found to stimulate sporulation in *F. oxysporum* f.sp. *lycopersici* even though it depressed colony growth, spore germination and germ tube growth (35). Promotion of reproductive development, however, was not observed in *P. cactorum*; on the contrary, various steroidal alkaloids of both the *Solanum* (solanine, tomatine) and *Veratrum* (jervine, muldamine, Figure 4) types, as well as tomatidine, solanidine and solasodine, all inhibited sitosterol-induced spore production (32). However, inhibition of vegetative hyphae by these compounds was not so marked or consistent, and varied with the sitosterol content of the medium. McKee (27) also found

solanine less damaging towards hyphae than towards spores but attributed this to the ability of hyphae to degrade the alkaloid. The different response of vegetative hyphae and reproductive structures towards such compounds obviously has important implications for the experimental assessment of fungitoxicity. Probably the most widely-used method has been measurement of colony diameter but the validity of this parameter has recently been questioned (36).

Few *in vitro* studies with fungi make reference to the cellular nature of growth impairment. However, the disintegration of *Phytophthora infestans* zoospores (which lack a cell wall) by solanine (27) and the release of amino acids from digitonin-treated *Pythium ultimum* hyphae (37), point to damage to limiting (and possibly other) membranes.

#### Mode of Fungitoxic Action

Almost 60 years ago Fischer (38) and Boas (39) proposed that 'solanine' and digitonin caused haemolysis by interacting with steroids in the erythrocyte membrane, but this work appears to have been forgotten for the next 30 years. In that time, the surfactant properties of glycoalkaloids and saponins were elucidated and figured prominently in explanations of membrane lysis by these compounds. Consistent with this thinking was the observation that aglycones had lower surface activity and were less disruptive than glycosides. Resurrection of the steroid-binding hypothesis probably dates from 1957 when Schulz and Sander (40) demonstrated the formation of 1:1 molecular complex *in vitro* between tomatine and 3 $\beta$ -hydroxy steroids such as cholesterol. Since then, a large number of glycoalkaloids and saponins have been shown capable of complexing with various sterols (including the fungal sterol ergosterol) *in vitro* (41,42). Evidence was presented (28) that the reduced fungitoxicity of hydrolysis products of tomatine (including tomatidine) could not be explained solely on the basis of surfactant properties but was more likely related to their inability to complex with sterols. It was further reported (28,43) that binding to sterols *in vitro* only occurs to a significant degree with the unprotonated alkaloid in alkaline conditions (Figure 5). Such a mode of action is reminiscent of the sterol-binding polyene antibiotics (44), and species of *Pythium* and *Phytophthora* which are unaffected by polyenes because their membranes lack sterols are also relatively insensitive to tomatine (26) and digitonin (37,45). However, when grown in the presence of sterols, these fungi incorporate them into their membranes, a process which sensitizes them to polyenes, saponins, etc. (37,45). Work by this author (46) indicated that tomatine disrupts isolated organelles in a similar manner to polyenes such as filipin and nystatin, causing loss of lysosomal contents and inhibition of chloroplast PS II activity, but having no effect on the respiratory activity of mitochondria. Nystatin-resistant mutants of *Fusarium solani* with lower sterol levels were also less sensitive to tomatine (47,48). These mutants were unaffected by 800 ppm tomatine whereas wild-type strains succumbed to 100 ppm tomatine. Crossing experiments between mutants and wild-types showed that low sterol content and insensitivity to tomatine or digitonin were always inherited together (49). As with polyenes, the complexing of glycoalkaloids and saponins to membrane sterols is thought to lead to the formation of pores in membranes (50,51).

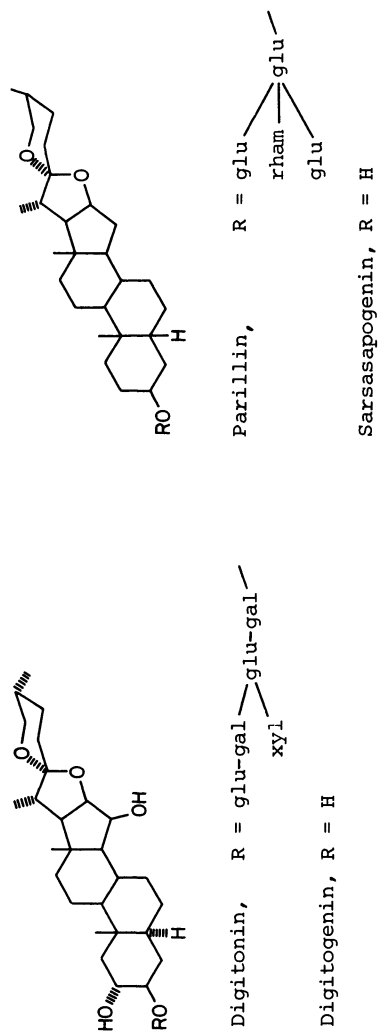
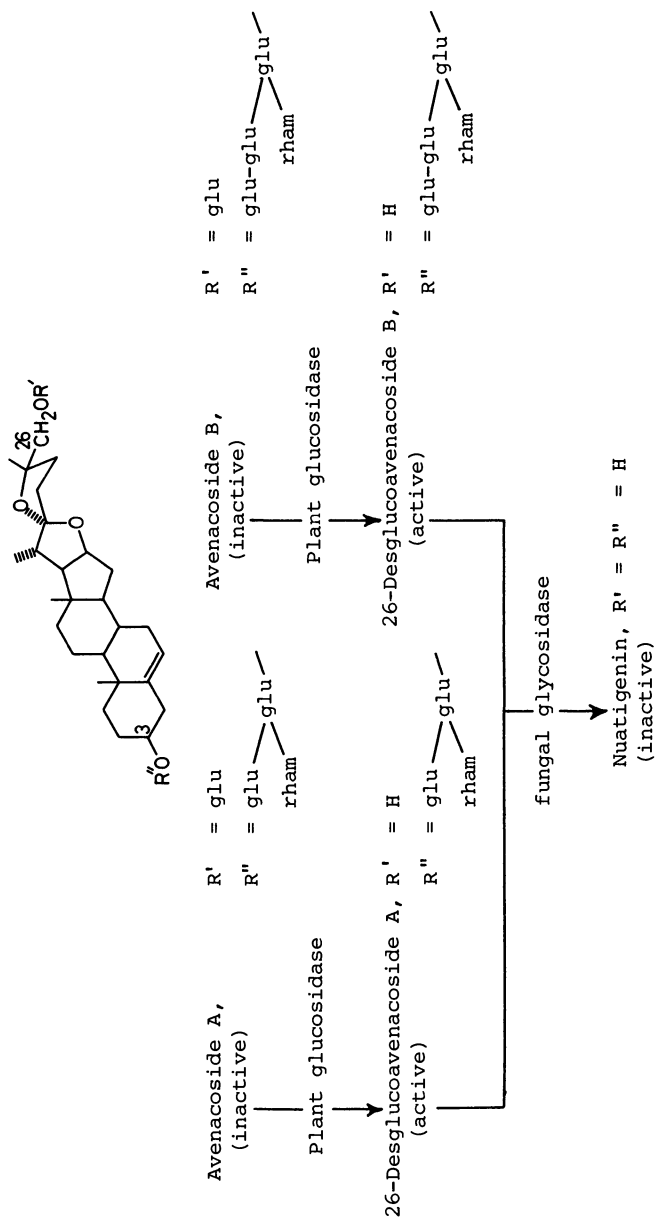


Figure 3. Steroidal saponins.

Figure 3. Continued. Steroidal saponins.

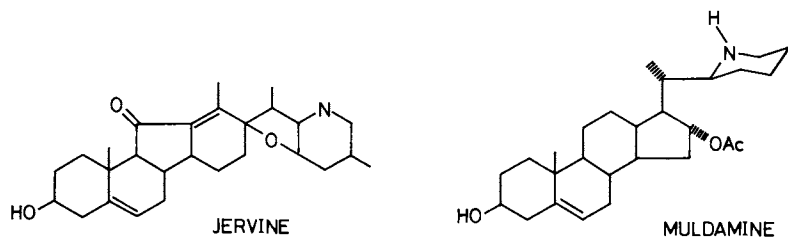


Figure 4. Veratrum steroidal alkaloids.

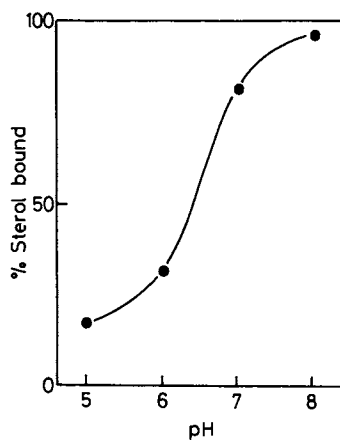


Figure 5. Effect of pH on binding of tomatine to cholesterol in vitro.

Freeze-fracture E.M. studies of natural and synthetic membranes treated with filipin, digitonin and tomatine revealed characteristic protuberances on the surfaces of membranes (52,53) but how these structures relate to the chemical complexes or to the formation of pores is not understood.

Despite the evidence supporting a sterol-binding mechanism, a number of doubts still remain. For example, no quantitative relationship could be established between the *in vitro* sterol-binding capacity of glycoalkaloids and saponins and their haemolytic action (41). Nor has the greater antifungal activity of aglycones than glycosides observed by Wolters (33) and Sinden *et al.* (34) yet been satisfactorily explained. Also, although less potent than tomatine, tomatidine was still capable of interfering with mycelial growth of *H. turcicum*, *S. linicola* and (particularly) *C. orbiculare* (28). The minimum concentrations of the aglycone required to cause complete growth inhibition were respectively 400x, 2000x and only 3x the minimum concentration of tomatine. Tomatidine and solanidine have also been shown to reduce sporulation in *P. cactorum* (32). Another proposed weakness of the sterol-binding hypothesis is the lack of direct evidence of incorporation of glycoalkaloids or saponins into the fungal mycelium or membrane (36). In evidence, solanine did not influence sporulation in *P. cactorum* and, in tritiated form, was not incorporated into mycelium whereas [<sup>3</sup>H]-solanidine was both deleterious to sporulation and incorporated into mycelium. The ability of this fungus to hydrolyse solanine to solanidine suggested to the authors that their findings might be explicable on the basis of a quite different hypothesis for mechanism of action which laid emphasis on the aglycone rather than the glycoside.

This hypothesis has its origins in haemolysis experiments carried out by Segal *et al.* (54). Following treatment of erythrocytes with digitonin, solanine or tomatine, aglycones but not glycosides could be detected in haemolysed ghosts whereas both aglycones and glycosides were associated with non-haemolysed cells. Aglycones alone were also haemolytic. It was proposed that the aglycone was the active moiety being released from the glycoside by a membrane glycosidase. Consistent with this claim was the subsequent finding (55) that the addition of gluconolactone or galactonolactone (reputedly specific inhibitors of glycosidases) inhibited tomatine- and (to a lesser extent) digitonin-induced haemolysis. When these experiments were repeated on *B. cinerea* and *Rhizoctonia solani*, essentially similar results were obtained (56). Thus, in addition to the requirement for membrane sterol to bind the glycoside, this hypothesis necessitates a second prerequisite in the form of a membrane glycosidase. How the aglycone brings about lysis was not made clear. The glycoside was thus considered to be simply the "water-soluble transport form" (56), but such a role has recently been disputed (57,58).

Although there are good grounds for accepting that inactive, non-sterol-binding bidesmosidic saponins (e.g. avenacosides A and B, Figure 3) are enzymically hydrolysed in damaged cells to active, monodesmosidic saponins (59), a number of problems arise when attempting to explain the toxicity of monodesmosidic saponins and glycoalkaloids on the basis of a similar hydrolytic activation process. The principal objections come from work on synthetic lipid membranes which lack glycosidases. Elferink (60), for

example, found that digitonin caused significant leakage of  $K^+$  from phosphatidylcholine liposomes only when cholesterol was also present. A similar observation was reported recently by this author (43) using tomatine (Table III). The extent of liposome disruption was directly related to the concentration of both the sterol and the alkaloid (with a strong interaction between the two) as well as to pH. The aglycone was not active in this system. In experiments on planar lipid bilayers and monolayers (61), digitonin was particularly effective in causing channel-like conductance changes in membranes containing sterols; in sterol-free membranes, effects could only be achieved with significantly higher concentrations. Obviously, caution is essential when attempting to extrapolate from artificial membranes to cell membranes but the similarities in responses/susceptibility of the two systems to glycoalkaloids and saponins in relation to pH, sterol content, etc. suggest that such comparisons have some validity.

Table III. Effect of Tomatine on Release of Peroxidase from Liposomes containing different Phospholipids and Sterols

Phospholipid	Treatment	Sterol			
		Chole-sterol	Stigma-sterol	Ergo-sterol	No Sterol
Phosphatidylcholine	Control	19.5	26.0	19.2	19.6
	Tomatine	52.0	51.4	69.2	15.4
Sphingomyelin	Control	6.3	10.6	7.4	48.4
	Tomatine	29.8	31.8	24.9	50.0

Values are % of liposome peroxidase activity released into supernatant. Liposomes were treated with 150  $\mu$ M alkaloid at pH 7.2 for 1 hour. Adapted from Roddick and Drysdale (43).

Some aspects of the experimentation which support the aglycone hypothesis are also open to doubt. For instance, aglycones were apparently dissolved in 20-25% DMSO which is itself strongly haemolytic, and no control data were presented. With tomatidine in 1% (or less) DMSO and proper controls, no haemolysis could be attributed to either of these components (Roddick, unpublished). High concentrations of sugar lactones can depress pH in weakly-buffered solutions and thus possibly also the action of pH-dependent glycoalkaloids. The inability of sugar lactones to inhibit aglycone-induced haemolysis could be explained by lysis being caused by DMSO, as indicated above. The fact that haemolysed erythrocyte ghosts were washed whereas non-haemolysed cells were not could account for the failure to detect glycosides in the former but their presence in the latter. The situation is further confused by claims that a fungal membrane glycosidase activates tomatine (56) whereas a fungal wall glycosidase, effecting an identical hydrolysis, inhibits tomatine action (62). In neither case was the proposed location of these enzymes established using unequivocal (e.g. fractionation) techniques.

However, this is not to refute the existence or activity of such glycosidases. Numerous reports exist of the hydrolysis of glycoalkaloids by fungal glycosidases (e.g. 27, 31, 63-65) but in virtually every case hydrolysis was viewed as an inactivation of a toxic glycoside. Nor do the doubts attaching the aglycone hypothesis necessarily

mean that membrane destabilization by glycoalkaloids and saponins can be explained in all cases by sterol-binding. Many anomalies still exist in this area e.g. the lack of a quantitative relationship between sterol binding in vitro and haemolysis (41); the greater disruption of sterol-free than sterol-containing liposomes by the saponin parillin (Figure 3) (60). It may be that different membrane-active steroidal glycosides operate in slightly, or even markedly, different ways. Even so, the weight of evidence points to sterol binding as being an important factor, perhaps qualitatively rather than quantitatively, in the destabilization of membranes by the compounds in question. Interactions, of either a direct or indirect nature, between glycoalkaloids/saponins and membrane phospholipids and/or proteins may also be involved. Opinions have been voiced both in favour (36,52) and against (37) such possibilities. Obviously more work is required in this field.

#### Glycoalkaloids and Saponins as Resistance Factors

The toxicity of steroidal glycoalkaloids and saponins to various parasitic fungi in vitro has naturally led to suggestions that these compounds might also operate in a similar capacity in the intact, infected plant. Early studies (24,33,66) gave some support to this idea with tomato pathogenic fungi apparently less susceptible to tomatine than non-pathogens. From a more critical investigation (26) under standard conditions with 30 species of fungi, including tomato pathogens, non-pathogens and general saprophytes, a ranking order of increasing susceptibility to tomatine was produced in which tomato pathogens occupied 14 of the first 16 places. The probability of such a ranking occurring by chance was calculated as 1 in  $10^9$ . However, demonstration of a correlation between susceptibility in vitro and pathogenicity is not in itself sufficient evidence that compounds like tomatine play a major role in resistance to fungal pathogens.

Although only one of many factors, differential susceptibility of fungi to toxic plant metabolites is undoubtedly not without significance. A probable explanation for this in Pythium and Phytophthora spp. is the lack of membrane sterols, a supposition which finds support in the fact that growth in a sterol-containing medium sensitizes these fungi to glycoalkaloids and saponins (37,45). An alternative explanation is that these fungi may not assimilate the glycosides (36) although the significance of assimilation has yet to be ascertained. Detoxification of glycoalkaloids or saponins by extracellular glycosidases which hydrolyse glycosides to aglycones may also contribute to the relative insensitivity of these species (32,67) although this mechanism may be more important in other species of fungi. The leaf-spot fungus Septoria lycopersici, for example, inactivates tomatine by removing one glucose to produce  $\beta_2$ -tomatine (63,64) whereas F. oxysporum f.sp. lycopersici (65) and B. cinerea (31) both remove the whole tetrasaccharide moiety. In Alternaria solani (62) and F. caeruleum (27) there is evidence of a stepwise removal of all the monosaccharide units of glycoalkaloids. In damaged oat leaves, the inactive, bidesmosidic saponins avenacosides A and B are hydrolysed at C-26 by leaf enzymes to yield the active, monodesmosidic derivatives. However on infection by Helminthosporium avenae, the monodesmosidic saponins are further degraded to the inactive aglycone, nuatigenin, by a fungal glycosidase which removes the carbo-



hydrate moiety at C-3 (Figure 3) (59). In potato tubers, the conversion of solanine to solanidine observed in tissue damaged by P. infestans or the bacterium Erwinia atroseptica was reported to be due to the release of host-cell hydrolases (68). In infected, but non-damaged, resistant varieties the aglycone was not detectable.

The proposal that tomatine might be a factor in the resistance of tomato varieties to F. oxysporum f.sp. lycopersici (23,69) was not confirmed by Kern (66) who considered the alkaloid neither sufficiently inhibitory to the fungus nor sufficiently abundant in lower stems and roots. Drysdale and coworkers have argued that the tomatine concentration in these organs is adequate to inhibit growth and spore germination of F. oxysporum (70) but rule out a primary resistance role for this alkaloid because it was not detectable in fluid from xylem (where this wilt fungus is located) and because its levels increased to the same extent in both resistant and susceptible cultivars following infection (71). Others (35) have detected tomatine in tracheal fluid but again did not observe differences in alkaloid concentration in resistant and susceptible cultivars before or after infection. Further evidence against a primary role for tomatine was that the alkaloid stimulated sporulation of F. oxysporum f.sp. lycopersici (35), a process which the authors point out is more important in invasion by this fungus than mycelial growth. On the other hand, some workers have arrived at the opposite conclusion regarding the importance of tomatine in fusarium wilt of tomato. Sarhan and Király (72) concluded that infection was related to soil nutrients and fungicide treatments and that resistance was mediated via elevated tomatine levels. A similar claim was made from a study of the fungitoxicity of extracts from infected and non-infected tomato plants of resistant and susceptible cultivars (73); differences from the conclusions of other workers (e.g. 70) were explained by varietal differences. The above information together with the fact that F. oxysporum f.sp. lycopersici is a fungus of low (or zero) tomatine regions capable of detoxifying this alkaloid (65) suggest that tomatine is probably not a major factor in resistance to fusarium wilt. Claims that tomatine may contribute to resistance to bacterial wilt (Pseudomonas solanacearum) in roots of Lycopersicon pimpinellifolium (74) require substantiation.

Glycoalkaloid levels in whole potato tubers tend to be similar to the low levels found in roots and stems although the skin and peel of tubers have much higher concentrations (75). Nevertheless, glycoalkaloids are not thought to be important in combatting tuber infections by F. caeruleum (76) or R. solani (77). It may be significant that F. caeruleum is able to degrade potato glycoalkaloids (27). Similarly, no correlations were observed between alkaloid levels in potato roots/stems and Verticillium wilt (V. albo-atro), or between tuber alkaloids and the bacterial disease, common scab (Streptomyces scabies) (78). Nor are glycoalkaloids apparently involved in resistance of potatoes to bacterial ring rot caused by Corynebacterium sepedonicum (79). Much work into the role of glycoalkaloids in potato tuber resistance to the late-blight fungus (P. infestans) has been done by Kuć and coworkers and has recently been reviewed by Kuć (80). Early investigations (81,82) pointed to glycoalkaloids as being a possible factor in tuber resistance but the subsequent demonstration that alkaloid accumulation (which increases in damaged

tubers) is suppressed by compatible and incompatible races of P. infestans (83) questioned the involvement of these compounds in R gene and hypersensitive resistance. A study of this disease in 15 potato clones (84) also produced no evidence of a glycoalkaloid contribution to multigenic (field) resistance. Despite these conclusions, Kuć (80) is of the opinion that tuber glycoalkaloids may still play a role in general resistance to disease.

In view of their higher concentrations in leaves and reproductive structures, it has been proposed (63) that glycoalkaloids might be more important against leaf- or fruit-infecting fungi. The leaf pathogen C. fulvum was adversely affected by tomatine but it was not clear whether this fungus, which grows in intercellular spaces, could cause tomatine leakage from cells (30). In potato leaves, elevated resistance to the early blight fungus A. solani observed in continuous light was not attributable to glycoalkaloids (85). Sinden et al. (34) concluded likewise for older (120 day) leaves which had a lower alkaloid content (260 ppm) but suggested that in younger (30 day) leaves, higher alkaloid levels (1570 ppm) might restrict development of A. solani. In a study of leaf infection by A. solani and P. infestans in 10 potato cultivars, no correlations were apparent between glycoalkaloids and resistance to disease (78) although this might be explained by the age of the plants (approx. 8 weeks) in the case of A. solani.

Tomato fruits are interesting organs for phytopathological studies being sites of tomatine synthesis and high tomatine accumulations (in small, green fruits) as well as tomatine degradation and low to zero alkaloid levels (in large green to ripe fruits) (58,86). Of interest in this respect is the finding that B. cinerea germ tubes penetrated epidermal cells of green fruits but no further development of the fungus occurred (31). However, the authors expressed doubt that this was due to tomatine as the fungus is able to degrade this alkaloid and also did not resume growth in ripe fruits, though still alive. More clear-cut results have been reported for F. solani, low sterol mutants of which caused severe rot of green tomato fruits whereas wild type strains did not (48). Both types were equally aggressive on ripe tomatoes. The authors suggested that tomatine could be a major resistance factor in fruits (at least to this fungus). Further evidence in support of this claim was that in crossing experiments the ability to rot green fruits was inherited along with insensitivity to tomatine (49). Ripening tomato fruits show a decline in their resistance to Colletotrichum phomoides (87) but it is not known if this is related to the concomitant decrease in fruit tomatine. A study of colonisation of tomato fruits (of different developmental stages) by the fruit rot pathogens Corticium rolfsii, B. cinerea, Monilia fructigena and Gloeosporium fructigenum revealed an order of pathogenicity (as shown) which corresponded to that of insensitivity to tomatine *in vitro* (29). Colonization was explained, not by secretion of hydrolytic enzymes, but by alteration to the pH at the inoculation sites. The most successful pathogen (C. rolfsii) lowered the pH from 5.7 to 3.6, the least successful (G. fructigenum) increased the pH to 6.4, and the two intermediate pathogens gave intermediate pH changes (to 4.4). The relationship between pH and resistance was explained on the basis of the pH dependence of tomatine toxicity. In another report, the same author (62) showed that invasion of tomato fruits by various species and races of Alternaria, pathogenic and non-

pathogenic to tomato, was related, not to pH changes (all test fungi increased this equally), but to their ability to secrete tomatine-hydrolysing enzymes. The species/strains which did not produce such enzymes were incapable of successful colonization.

The major factors which influence the toxicity of glycoalkaloids and saponins to fungi *in vivo* can thus be identified as the level of glycoside, fungal sterol content, secretion of fungal hydrolases, production of suppressors of glycoside synthesis and alteration of pH. To this list could be added a number of other factors which may be important in moderating the above or having a bearing in their own right, but which have been little researched. For example, glycoalkaloid level can be influenced by age (34) and/or developmental stage (86) as well as by variety, environmental factors and cultural practices (88,89). Consideration should also be given to the identity (and ratios) of compounds present as some are more toxic than others (27,34). In view of the differential sensitivity of vegetative hyphae and reproductive structures to alkaloids (27,32), developmental aspects of the fungal pathogen could be important. A number of compounds of host origin liberated along with glycoalkaloids or saponins following damage to their main storage site, the vacuole (90,91), or to other cell compartments are known to enhance, ameliorate or even nullify toxicity of these compounds. These include sugars (27), ions such as  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Ca}^{2+}$  (27), sterols (32) and glycosidases (92-94).

### Conclusions

Despite their potent antifungal action *in vitro*, steroidal glycoalkaloids and saponins do not appear to be key factors in the resistance of roots, stems and leaves to fungal disease, although they may play a greater role in resistance to fruit infections. Nevertheless, many authors still believe these compounds play some role in the general defences of the plant against fungi. The fact that many successful fungal parasites have means of degrading or inactivating glycoalkaloids or saponins or of suppressing their synthesis, or do not synthesize their target molecules, etc. suggests that in evolutionary, if not ecological, terms these compounds do have a potentially protective function. Many parasitic fungi are not pathogenic to plants which elaborate glycoalkaloids or saponins (26), a situation which could be due to the presence of these compounds. In the co-evolution of plants and fungal parasites, any equilibrium is usually a dynamic one and the relative importance of a particular protective device in a plant undoubtedly changes with the evolution of its parasites. Such flexibility necessitates the maintenance of a variety of defence mechanisms only one, or a few, of which may operate as the 'front-line' system at any one time and/or against any one pathogen, with the others acting in an auxiliary or 'back-up' capacity. Thus, although it may not be possible to demonstrate a primary role for a particular compound, it is not unreasonable to assume that its synthesis and accumulation still represent an important, and possibly vital, investment in evolutionary terms.

Finally, it must be borne in mind that chemical defences in plants have evolved in response to a number of biotic pressures, not least those from herbivores, and particularly insects. The rapidity with which damage is caused by feeding is such that, to be effective,

counter measures must usually be equally rapid and the release of preformed inhibitors, toxins or repellants is one way of achieving this. The possibility therefore that steroidal glycoalkaloids and saponins evolved primarily in response to predation pressures mainly from insects is a very real one (95). Even so, the general toxicity of glycoalkaloids and saponins renders it unlikely that any deterrent role played by these compounds would be a highly specific one.

#### Literature Cited

1. Heftmann, E. Phytochemistry 1975, 14, 891.
2. Demel, R.A.; de Kruyff, B. Biochim. Biophys. Acta 1976, 457, 109.
3. Nes, W.D.; Heftmann, E. J. Nat. Prod. 1981, 44, 377.
4. Heftmann, E. Lipids 1971, 6, 128.
5. Geuns, J.M.C. Phytochemistry 1978, 17, 1.
6. Hewitt, S.; Hillman, J.R.; Knights, B.A. New Phytol. 1980, 85, 329.
7. Mandava, N.G.; Thompson, M.J. In "Isopentenoids in Plants"; Nes, W.D.; Fuller, G.; Tsai, L.-S., Eds.; Marcel Dekker: New York, 1984; p. 401.
8. Swain, T. Ann. Rev. Plant Physiol. 1977, 28, 479.
9. Elliott, C.G. Adv. Microbiol. Physiol. 1977, 15, 121.
10. Roddick, J.G. In "Secondary Plant Products"; Bell, E.A.; Charlwood, B.V., Eds.; ENCYCLOPAEDIA OF PLANT PHYSIOLOGY, NEW SERIES, Springer-Verlag: Berlin, Heidelberg, 1980; Vol. 8, p. 167.
11. Schreiber, K. In "The Alkaloids. Chemistry and Physiology"; Manske, R.H.F., Ed.; Academic: New York, 1978; Vol. X, p. 1.
12. Schreiber, K.; Ripperger, H. In "The Alkaloids. Chemistry and Physiology"; Manske, R.H.F.; Rodrigo, R.G.A., Eds.; Academic: New York, 1981; Vol. XIX, p. 81.
13. Tschesche, R.; Wulff, G. Fortschr. Chem. Org. Naturstoffe 1973, 30, 462.
14. Mahato, S.B.; Ganguly, A.N.; Sahu, N.P. Phytochemistry 1982, 21, 959.
15. Morris, S.C.; Lee, T.H. Food Technol. (Australia) 1984, 36, 118.
16. Roddick, J.G. Phytochemistry 1974, 13, 9.
17. Défago, G. Ber. Schweiz. Bot. Ges. 1977, 87, 79.
18. Schönbeck, F.; Schösser, E. In "Physiological Plant Pathology"; Heitefuss, R.; Williams, P.H., Eds.; ENCYCLOPAEDIA OF PLANT PHYSIOLOGY, NEW SERIES, Springer-Verlag: Berlin, Heidelberg, 1976; Vol. 4, p. 653.
19. Jadhav, S.J.; Sharma, R.P.; Salunkhe, D.K. Crit. Rev. Toxicol. 1981, 9, 21.
20. Roddick, J.G. In "The Biology and Systematics of the Solanaceae"; D'Arcy, W.G.; Hawkes, J.G., Eds.; Columbia University: New York, In press.
21. Agerburg, L.S.; Schick, R.; Schmidt, M.; Sengbusch, R.V. Züchter 1933, 5, 172.
22. Schmidt, M. Planta 1933, 20, 407.
23. Fisher, P.L. Maryland Agr. Expt. Sta. Bull. 1935, 374.

24. Irving, G.W.; Fontaine, T.D.; Doolittle, S.P. J. Bacteriol. 1946, 52, 601.
25. Fontaine, T.D.; Irving, G.W.; Ma, R.M.; Poole, J.B.; Doolittle, S.P. Arch. Biochem. 1948, 18, 467.
26. Arneson, P.A.; Durbin, R.D. Phytopathology 1968, 58, 536.
27. McKee, R.M. J. Gen. Microbiol. 1959, 20, 686.
28. Arneson, P.A.; Durbin, R.D. Plant Physiol. 1968, 43, 683.
29. Schlösser, E. Z. Pflanzenkr. Pflanzensch. 1975, 82, 476.
30. Dow, J.M.; Callow, J.A. Phytopath. Z. 1978, 92, 211.
31. Verhoeff, K.; Liem, J.I. Phytopath. Z. 1975, 82, 333.
32. Nes, W.D.; Hanners, P.K.; Bean, G.A.; Patterson, G. Phytopathology 1982, 72, 447.
33. Wolters, B. Arch. Pharm. 1964, 297, 748.
34. Sinden, S.L.; Goth, R.W.; O'Brien, M.J. Phytopathology 1973, 63, 303.
35. Smith, C.A.; MacHardy, W.E. Phytopathology 1982, 72, 415.
36. Nes, W.D.; Saunders, G.A.; Heftmann, E. Phytochemistry 1983, 22, 75.
37. Schlösser, E. Phytopath. Z. 1972, 74, 91.
38. Fischer, R. Biochem. Z. 1927, 209, 319.
39. Boas, F. Naturwiss. u. Landwirtsch. 1928, 14, 82.
40. Schulz, G.; Sander, H. Hoppe-Seyler's Z. Physiol. Chem. 1957, 308, 122.
41. Tschesche, R.; Wulff, G. Planta Medica 1964, 12, 272.
42. Roddick, J.G. Phytochemistry 1979, 18, 1467.
43. Roddick, J.G.; Drysdale, R.B. Phytochemistry 1984, 23, 543.
44. Norman, A.W.; Spielvogel, A.M.; Wong, R.G. Adv. Lip. Res. 1976, 14, 127.
45. Schlösser, E.; Gottlieb, D. J. Bact. 1966, 91, 1080.
46. Roddick, J.G. J. Exp. Bot. 1978, 29, 1371.
47. Défago, G. Ann. Phytopathol. 1978, 10, 157.
48. Défago, G.; Kern, H. Physiol. Plant Path. 1983, 22, 29.
49. Défago, G.; Kern, H.; Seđlar, L. Physiol. Plant Path. 1983, 22, 43.
50. Dourmashkin, R.R.; Dougherty, R.M.; Harris, R.J.C. Nature 1962, 194, 1116.
51. Dennis, V.W.; Stead, N.W.; Andreolli, T.E. J. Gen. Physiol. 1970, 55, 375.
52. Elias, P.M.; Friend, D.S.; Goerke, J. J. Histochem. Cytochem. 1979, 27, 1247.
53. Severs, N.J.; Simons, H.L. Nature 1983, 303, 637.
54. Segal, R.; Shatkovsky, P.; Milo-Goldzweig, I. Biochem. Pharmacol. 1974, 23, 973.
55. Segal, R.; Milo-Goldzweig, I. Biochem. Pharmacol. 1975, 24, 77.
56. Segal, R.; Schlösser, E. Arch. Microbiol. 1975, 104, 147.
57. Roddick, J.G. Experientia 1982, 48, 460.
58. Eltayeb, E.A.; Roddick, J.G. Phytochemistry 1985, 24, 253.
59. Lüning, H.U.; Schlösser, E. Z. Pflanzenkr. Pflanzensch. 1975, 82, 699.
60. Elferink, J.G.R. Pharm. Weekbl. 1976, 112, 1.
61. Gögelein, H.; Huby, A. Biochim. Biophys. Acta 1984, 73, 32.
62. Schlösser, E. Acta Phytopath. Acad. Sci. Hung. 1975, 10, 77.
63. Arneson, P.A.; Durbin, R.D. Phytopathology 1967, 57, 1358.
64. Arneson, P.A.; Uchytıl, G.F. Biochim. Biophys. Acta 1969, 191, 176.

65. Ford, J.E.; McCance, D.J.; Drysdale, R.B. Phytochemistry 1977, 16, 545.
66. Kern, H. Phytopathol. Z. 1952, 19, 351.
67. Holland, H.L.; Taylor G.J. Phytochemistry 1979, 18, 437.
68. Zacharius, R.M.; Kalan, E.B.; Osman, S.F.; Herb, S.F. Physiol. Plant Path. 1975, 6, 301.
69. Gottlieb, D. Phytopathology 1943, 33, 1111.
70. Langcake, P.; Drysdale, R.B.; Smith, H. Physiol. Plant Path. 1972, 2, 17.
71. McCance, D.J.; Drysdale, R.B. Physiol. Plant Path. 1975, 7, 221.
72. Sarhan, A.R.T.; Király, Z. Acta Phytopath. Acad. Sci. Hung. 1981, 16, 133.
73. Hammerschlag, F.; Mace, M.E. Phytopathology 1975, 65, 93.
74. Mohanakumaran, N.; Gilbert, J.C.; Buddenhagen, I.W. Phytopathology 1969, 59, 14.
75. Lampitt, L.H.; Bushill, J.H.; Rooke, H.S.; Jackson, E.M. J. Soc. Chem. Ind. 1943, 62, 48.
76. McKee, R.K. Ph.D. Thesis, University of Nottingham, 1956.
77. Morrow, L.S.; Caruso, F.L. Am. Potato J. 1983, 60, 403.
78. Frank, J.A.; Wilson, J.M.; Webb, R.E. Phytopathology 1975, 65, 1045.
79. Paquin, R. Am. Potato J. 1966, 43, 349.
80. Kuć, J. Am. Potato J. 1984, 61, 123.
81. Locci, R.; Kuć, J. Phytopathology 1967, 57, 1272.
82. Allen, E.H.; Kuć, J. Phytopathology 1968, 58, 776.
83. Shih, M.; Kuć, J.; Williams, E.B. Phytopathology 1973, 63, 821.
84. Deahl, K.L.; Young, R.J.; Sinden, S.L. Am. Potato J. 1973, 50, 248.
85. Goth, R.W.; Sinden, S.L.; O'Brien, M.J. Phytopathology 1969, 59, 1556 (Abstr.).
86. Eltayeb, E.A.; Roddick, J.G. J. Exp. Bot. 1984, 35, 252.
87. Allison, P.B. Phytopathology 1952, 42, 1 (Abstr.).
88. Sinden, S.L.; Webb, R.E. Am. Potato J. 1972, 49, 334.
89. Sinden, S.L., Sanford, L.L.; Webb, R.E. Am. Potato J. 1984, 61, 141.
90. Roddick, J.G. Phytochemistry 1977, 16, 805.
91. Urban, B.; Laudenbach, U.; Kesselmeier, J. Protoplasma 1983, 118, 121.
92. Petrochenko, E.I. Dokl. Akad. Nauk. SSSR 1953, 90, 1091.
93. Prokoshev, S.M.; Petrochenko, E.I.; Paseshnikchenko, V.A. Dokl. Akad. Nauk SSSR 1956, 106, 313.
94. Swain, A.P.; Fitzpatrick, T.J.; Talley, E.A.; Herb, S.F.; Osman, S.F. Phytochemistry 1978, 17, 800.
95. Tingey, W.M. Am. Potato J. 1984, 61, 157.
96. Grunwald, C. In "Secondary Plant Products"; Bell, E.A.; Charlwood, B.V. Eds.; ENCYCLOPAEDIA OF PLANT PHYSIOLOGY, NEW SERIES, Springer-Verlag: Berlin, Heidelberg, 1980; Vol. 8, p. 221.
97. Šaden-Krehula, M.; Tajić, M.; Kolbah, D. Phytochemistry 1979, 18, 345.
98. Šaden-Krehula, M.; Tajić, M.; Kolbah, D. Biol. Zbl. 1976, 95, 223.
99. Kirson, I.; Glotter, E. J. Nat. Prod. 1981, 44, 633.
100. Jones, C.G.; Firn, R.D. J. Chem. Ecol. 1978, 4, 117.
101. Heftmann, E. "Steroid Biochemistry": Academic: New York, 1970.
102. Geuns, J.M.C. Z. Pflanzenphysiol. 1983, 111,141.

RECEIVED May 1, 1986

In Ecology and Metabolism of Plant Lipids; Fuller, G., et al.;  
ACS Symposium Series; American Chemical Society: Washington, DC, 1987.

## Chapter 19

# Biosynthesis and Requirement for Sterols in the Growth and Reproduction of Oomycetes

W. David Nes

Plant and Fungal Lipid Group, Plant Development and Productivity Research Unit,  
Western Regional Research Center, Agricultural Research Service, U.S. Department of  
Agriculture, Albany, CA 94710

All Oomycetes fungi possess an obligatory requirement for sterols to complete the sexual stage of their life cycle. Growth is mediated by sterol and in the non-sterol synthesizing Oomycetes additionally by "sterol-like" pentacyclic and tetracyclic triterpenoids. The structural features and levels which are determinant for bioregulator activity differ amongst the fungi. Herein the biosynthesis and sterol function(s) which operate to control growth are examined and compared with the involvement of sterol in reproduction.

Oomycetes are less-advanced fungi which are grouped apart from the lineage to the more-advanced fungi, *eg.* Basidiomycetes (Fig. 1), based on their having coenocytic mycelia and double-walled oospores. The Oomycetes may be divided into 2 groups - those which synthesize sterols and those which do not (1-10). While sterols are not obligatory for the growth of all the Oomycetes, both groups require sterols for sexual reproduction (oospore production). As discussed in what follows, the sterol utilized to mediate growth may be, at a later period in the vegetative cycle, responsible for regulating oosporogenesis without necessarily having undergone metabolism. With certain Oomycetes, "sterol-like" molecules, *eg.* tetracyclic and pentacyclic triterpenoids (Fig. 2), may produce the same physiological end response as sterols (2). The growth response to these polycyclic isopentenoids is intimately associated with their 3-dimensional geometry, molecular features and subcellular quantity. In order to discuss sterol function for what we assume is an evolutionary determinant of developmental change, a brief overview of sterol occurrence and biosynthesis is given first.

### Sterol occurrence.

McCorKindale, *et al.*, were the first to structurally and quantitatively examine the sterol composition of a variety of Oomycetes (8). From their study it was evident that: the Oomycetes produced a sterol profile dissimilar from the more-advanced fungi,

This chapter not subject to U.S. copyright.  
Published 1987, American Chemical Society

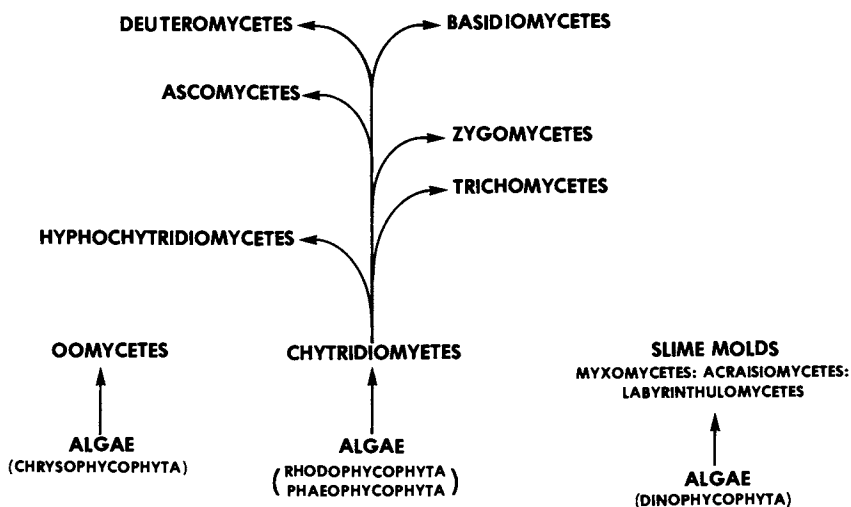


Figure 1. Phylogenetic relationships of fungi (based on classic considerations of Morphology, Life Cycle, Cell Wall Chemistry, and Amino Acid Pathways).



Figure 2. As shown.



eg. the ergosterol-producing Ascomycetes; some members failed to synthesize detectable levels of sterols; and the predominant end products characterized by GC-MS, included both 24-desalkyl and 24-alkylidene sterols. In the intervening 15 years additional Oomycetes have been examined for their sterol content (11-21, Table I), and in select fungi the sterol identification has been confirmed by proton magnetic resonance (PMR) spectroscopy (4,11). The sterol content ranged from .01 to .25% of the mycelial dry weight. The lower value may in some cases represent differences in the details of extraction and isolation. For instance, as we have found, mycelia extracted as fresh and freeze dried versus oven dried material generate about 5 to 10 times as much sterol unless water is added back in the extraction of the latter material. If digitonides are made then long chain fatty alcohols would not contaminate the TLC purified samples and hence would not interfere with GLC quantification. From these studies, cf. Table I, three findings are significant. Firstly, the absence of sterols is not confined to the Peronosporales. Secondly, lanosterol has been isolated without the accompaniment of cycloartenol (albeit, see Sterol Biosynthesis and Metabolism Section). Thirdly, some investigators have found ergosterol in numerous Oomycetes (12,13,21), although this observation has not been confirmed by others in which the same fungus, precursor material, eg. cycloartenol and lanosterol fed to Phytophthora and Lagenidium, and culture conditions were used (1,18,20). The occurrence of ergosterol, however, can be explained. It has been our experience that Phytophthora is readily contaminated by other fungi (spores) during poor culturing practices. Consequently, we routinely monitor the sterol content of our fungal collection by GLC and HPLC. When ergosterol is detected, especially in the Oomycetes sterol profile, the culture is destroyed and the parent strain regenerated, from material maintained under an envelope of liquid nitrogen. Another possibility is that ergosterol was derived from the media, eg. yeast extract. As shown in Table II, sterols and particularly - cholesterol, are widely distributed in commercial sources. Cholesterol, like ergosterol, has been isolated in various Oomycetes, although, like ergosterol, it may not be an endogenous component depending on the composition of the synthetically-compounded media.

When P. cactorum (a sterol-less fungus, cf. Table I) was cultured with [2-<sup>14</sup>C]mevalonic acid (MVA) on a synthetic media and inoculated with agar plugs, sufficient non-radioactive cholesterol was absorbed by the mycelia to be chemically identified, although cholesterol failed to possess <sup>14</sup>C-label (20). Fryberg et al., similarly found an unusual sterol in yeast ie., sitosterol (22), and concluded for biosynthetic reasons that the sterol must have been dietary (present in the oil supplement) rather than endogenous. We (1) and others (20) have attributed the trace levels of cholesterol present in P. cactorum cultured on agar-supplemented media to be derived from the agar plug - agar is an industrial by-product of red algae; the dominant sterol of red algae is cholesterol.

The solubility characteristics of sterols in the various dispersing agents also have an influence on the amount of dietary sterol made available to the fungus, thereby affecting quantitative

differences in content and development. For instance, a 1.0 ppm level of cholesterol dispersed in ether, ethanol (1,5) or no dispersing agent may not be as readily accumulated as sterol dispersed in the same concentration in lecithin. Thus, differences in growth-response and oospore numbers may vary although the amount of the sterol supplement is similar in the separate treatments. It follows that growth stimulation of pythiaceous fungi (24-28) and production of oospores (23,29,30) by Tweens, lecithins and vegetable oils, without (additional) sterol supplementation, can be rationalized not in terms of an obligatory requirement for a fatty acid but due to the contaminant sterol in the media preparation. This trace sterol may also permit other compounds, eg. cycloartenol (6,31), to act in a synergistic or sparking (32) capacity allowing for growth stimulation and oogonia induction (cf. Sterol Function Section). Concerning the cholesterol identified in other Oomycetes (Table 1), such as S. ferax (8), when [<sup>14</sup>C]acetate is incubated with S. ferax, [<sup>14</sup>C]cholesterol is isolated in radiochemically pure form, thus demonstrating its biosynthesis de novo (11). Apparently, those fungi which synthesize cholesterol fail to actively accumulate the dietary sterol precluding a significant contamination effect in situ, unlike P. cactorum, which readily absorbs exogenous cholesterol.

The steroid biosynthetic pathway of Oomycetes may be operationally divided into 4 stages: (1) acetate conversion to squalene-oxide; (2) polycyclization of squalene-oxide to a tetracyclic product; (3) metabolism of the tetracycle to a series of amphipathic neutral sterols having a free 3 $\beta$ -OH, planar nucleus and intact side chain of 8 to 10 C-atoms; and (4) side chain hydroxylation and reduction followed by additional nuclear metabolism of specifically the 24-alkylidenesterol-fucosterol to polar steroids (Fig.3).

Phytophthora and related "sterol-less" Oomycetes possess an enzymic defect in Stage I. They synthesize squalene (9,19) but fail to produce squalene-oxide (9,10,18). While some fungi cyclize squalene-oxide to pentacyclic triterpenoids it remains unknown whether the pythiaceous fungi similarly perform this kind of cyclization.

S. ferax and related sterol-producing Oomycetes synthesize lanosterol rather than cycloartenol (10) in Stage II. The mechanism of squalene-oxide cyclization to lanosterol involves a nonconcerted process in which a terminal intermediate, chair-boat-chair-boat-"left-handed" unfolded (33,34) side chain, proceeds through stereoelectronic ring and side chain inversion (Fig. 4) to yield a product with an all-chair nucleus and "right-handed" unfolded side chain (34, and ref. cited therein).

There are suggestions in the literature, however, that Oomycetes (13,36) may synthesize cycloartenol, and metabolize it to a 4-desmethylsterol, such as fucosterol; if the fungus cannot synthesize or metabolize the cyclopropyl sterol, then it may use the compound unaltered as a unique structural membrane insert (31) due to the butterfly or bent shape of the cyclopropyl sterol (32). Our original interest in cycloartenol utilization by Oomycetes was phylogenetic rather than functional. The utilization of a cycloartenol-versus a lanosterol-based pathway is known to

Table I. Sterol composition of Oomycetes\*

Group	Sterol Content*						
	1	2	3	4	5	6	7
<u>Saprolegniales</u>							
<i>Saprolegnia ferax</i>	4	13	68	15			
<i>S. Ferax</i>	1	37	43	12			
<i>S. Ferax</i>	-	-	-	-	18	55	26
<i>Achlya carolina</i>	72	27	1	-			
<i>A. hypogyna</i>	21	2	28	49			
<i>A. bisexualis</i>	3	-	17	70			
<i>A. americana</i>	4	2	22	67			
<i>Pythiopsis cymosa</i>	-	-	73	4			
<i>P. intermedia</i>							
<i>Dictyuchus monosporus</i>	4	-	14	39	-	-	-
<u>Leptomitales</u>							
<i>Apodachlya mimima</i>	85	1	6	1			
<i>A. brachynema</i>	68	2	8	22			
<i>Apodachylella completa</i>	3	-	41	56			
<i>A. completa</i>	tr	3	6	88			
<u>Peronosporales</u>							
<i>Phytophthora cactorum</i>							
<i>Phytophthora infestans</i>							
<i>P. cinnanomi</i>							
<i>Pythium ultimum</i>							
<i>P. graminicola</i>							
<i>P. debaryanum</i>							
<i>Zoophagus insidians</i>							
<u>Lagenidiales</u>							
<i>Atkinsiella dubia</i>	tr.	18	69	13			
<i>Lagenidium callinectes</i>	11	-	67	13			
<i>Lagenidium giganteum</i>							
<i>Haliphthoros milfordensis</i> strain, HAL - 223							

\* As per cent of total sterol: 1, cholesterol; 2, desmosterol; 3, 24-methylene cholesterol; 4, fucosterol; 5, zymosterol; 6, fecosterol; 7, stigmasta-8, E-24(28)-dienol; 8, lathosterol; 9, ergosta-7, 24(28)-dienol; 10, stigmasta-7, E-24(28)-dienol; 11, 7-dehydrofucosterol, 12, lanosterol.

\*\* These cultures may have been contaminated yielding ergosterol in otherwise a non-sterol synthesizing fungus. See text for further queries.

8	9	10	11	12	Sterol Absent	Ref.
						1
				7		15
				1		11,15
						8
						12
			10			17
				4		15
						8
					Yes	13
11	-	7	25	1		15
						8
						8
						8
				2		15
					Yes	1,20
					Yes	8
					Yes	9,18
					Yes	8,19
					Yes	19
					Yes	19
					? **	21
						16
						14,15
					Yes	10,14
					Yes	16

Table II. Sterols isolated from commercial sources

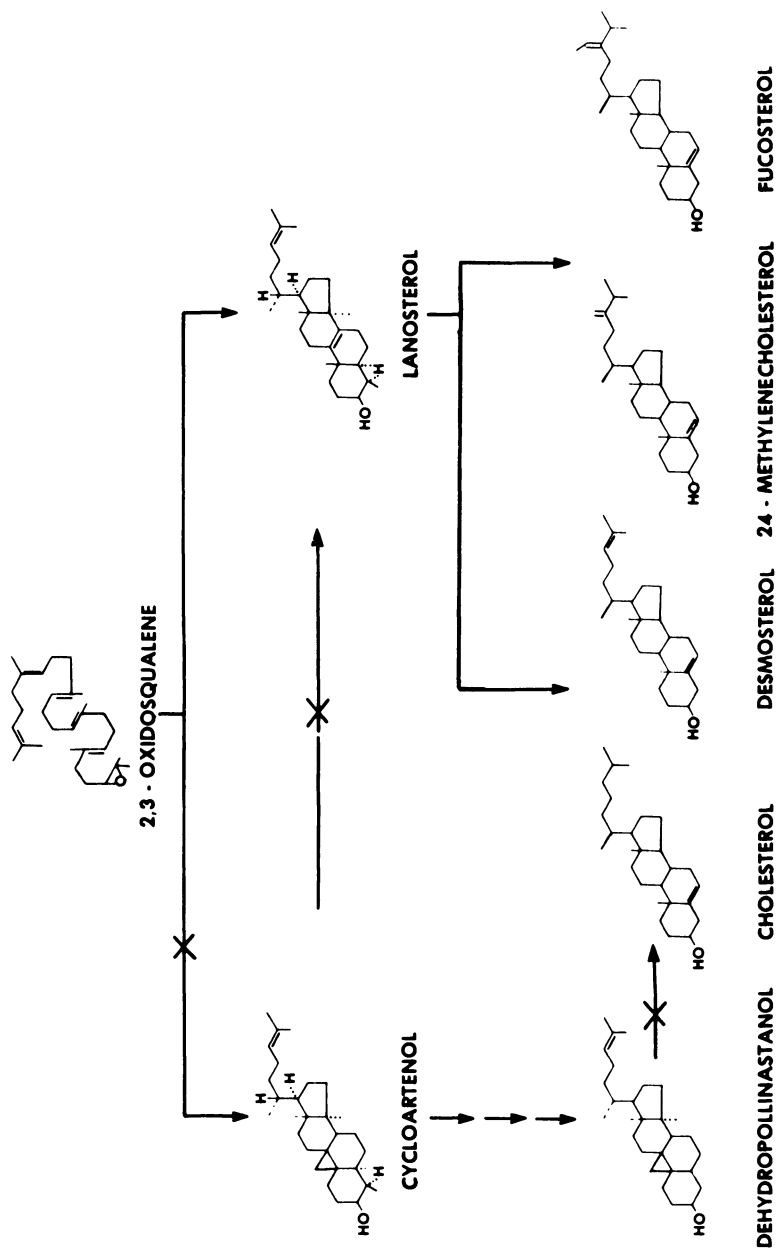
Sterol Content	Tween 80	Soybean Lecithin (ICN)	Soybean Lecithin (Sigma)	Noble Agar
Cholesterol	37 <sup>1</sup>	1	1	99
Campesterol	16	22	15	tr.
Sitosterol	31	52	68	tr.
Stigmasterol	16	25	16	tr.
Ergosterol	--	--	--	--
Unknown	--	--	--	--
Total <sup>3</sup>	13.4µg/100g	150µg/100g	212µg/100g	5µg/63g

<sup>1</sup> As per cent total sterol; tr - trace; dash indicates not detectable; unpublished observation from this laboratory.

<sup>2</sup> Total represents the amount of free sterol quantified per unit material extracted with 5% aq. acetone. Quantification was assessed using GLC (3% SE and 3% OV-17 packed columns) and RP-HPLC (detector set at 205 nm or 282 nm). Tween 80 and yeast extract were saponified with 10% methanolic KOH while V-8 juice was centrifuged and the clarified V-8 juice extracted with diethyl ether; V-8 juice and the other samples were chromatographed without prior saponification.

<sup>3</sup> Confirmation of sterol structure was by GC-MS.

Difco Agar	Yeast Extract (Difco)	Corn meal Agar (Difco)	Potato Dextrose Agar (Difco)	V-8 Juice (Campbell)
99	--	54	71	1
tr.	--	7	10	13
tr.	--	21	12	41
tr.	--	17	7	45
--	88	--	--	--
--	12	--	--	--
16 $\mu$ g/70g	100 $\mu$ g/100g	22 $\mu$ g/90g	14 $\mu$ g/108g	664 $\mu$ g/143mL (4.6 $\mu$ g/ml)



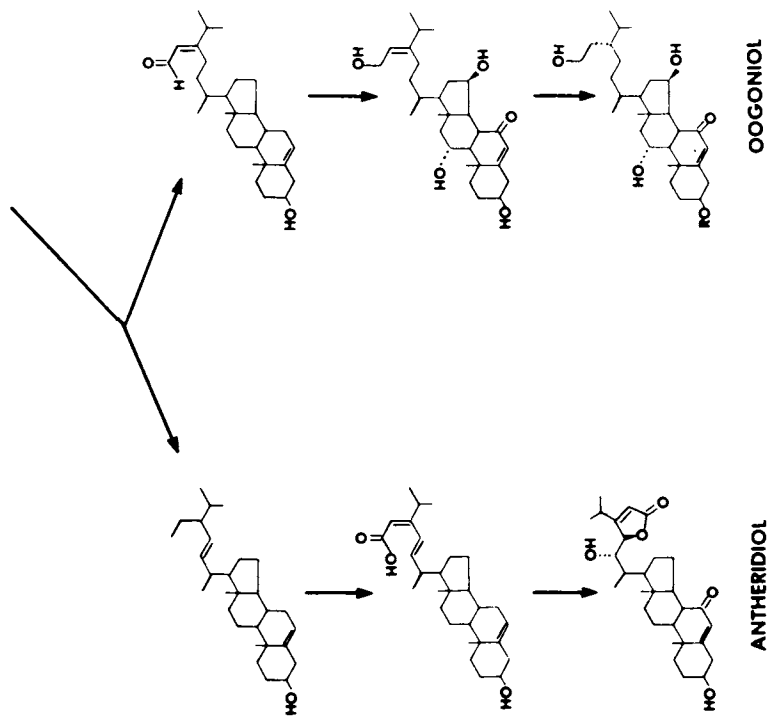


Figure 3. Biosynthesis and metabolism of sterols in Oomycetes.



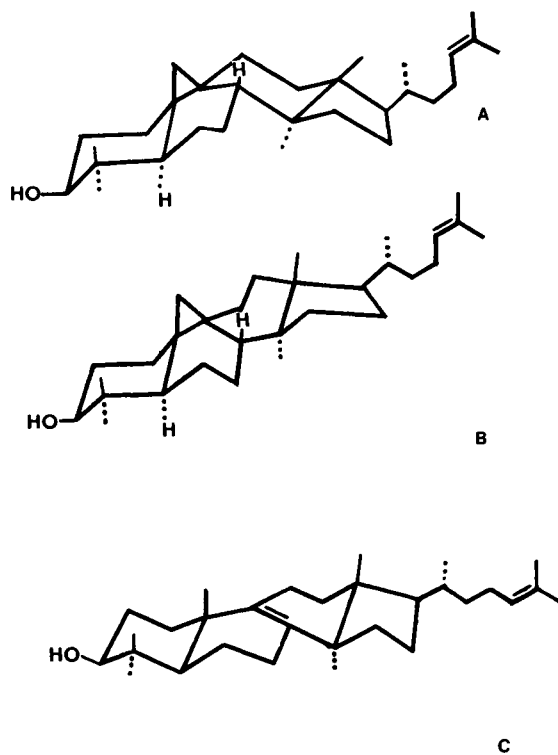


Figure 4. Two possible conformations of cycloartenol, (A) and (B); established conformation of lanosterol, (C).

disassociate organisms with a photosynthetic (cycloartenol) and a non-photosynthetic (lanosterol) ancestry. We have recently determined by feeding and trapping experiments (Table III) that cycloartenol is not formed in situ by the Oomycetes, although in S. ferax (and presumably related sterol synthesizers) it may be metabolized to the cyclopropyl 4-desmethyl sterol-dehydropollinastanol (8). We have interpreted the results to imply that Oomycetes are not of algal origin. In light of the functional implications, we continued our conformational analysis of cyclopropyl sterols. We have deduced by PMR (in particular with nuclear Overhauser effects) that the conformational preference of cycloartenol and other cyclopropyl sterols in solution (35) is chair-boat-boat-chair "right-handed" unfolded (conformer B in Fig. 4) which confirms the solid state X-ray data (37). This new information has permitted us to reassess the sequence of events in the terminal stages of squalene-oxide cyclization to cycloartenol (38-40) and the contribution of the cyclopropane ring to membrane function. With regards to the latter point, Bloch (32) has suggested that the bent conformer exists in membrane systems. However, it seems unlikely that a membrane which has physiochemical properties intermediate between pure solution and solid state would induce cyclopropyl sterols to adopt a different conformer than chair-boat-boat (A,B,C). Alternatively, it seems that the cyclopropyl group itself, without concurrently having the cyclosterols adopt an energetically unfavorable bent conformer, can perturb the membrane bilayer in a fashion similar to that shown for the cyclopropyl group introduced into fatty acids (41).

We (11) and Berg, et al. (42), have studied the sequencing in stage III. Additionally, we (unpublished observation) have determined the mechanism of C-24 transalkylation (Fig 5). The results are as follows: the substrate in situ for C-24 alkylation is zymosterol, rather than lanosterol, analogous to some higher-fungi, eg., yeast (43), but not others, eg., Gibberella fujikuroi (44,45); by incubation with inhibitors and labeled substrates we have shown that reduction of the (zymosterol) C-24 bond, leading to cholesterol formation, was found to precede the  $\Delta^8 \rightarrow \Delta^7$  isomerization step. Feeding and trapping experiments have confirmed the steps:  $\Delta^{8(9)} \rightarrow \Delta^7 \rightarrow \Delta^{5(7)} \rightarrow \Delta^5$ . Similar sterolic enzyme systems have been shown for P. cactorum (Nes, unpublished and (46)) indicating that P. cactorum presumably possessed a complete sterol pathway at one point in its evolutionary history, then lost it (2). As shown in Fig. 5, through incubations with 24-tritolanosterol, the mechanism by which S. ferax alkylates the  $\Delta^{24(25)}$ -bond is by a 1, 2-hydride shift analogous to yeast (47) and G. fujikuroi (Le and Nes unpublished observations). Since the second alkylation can be completely blocked by a carbonium ion high energy intermediate enzyme blocker (48), i.e., 25-azacholesterol, in addition to the loss of tritium at C-25 following iodine isomerization, it is assumed the first and second alkylations proceed through a similar mechanism. Presumably the other Oomycetes which synthesize these same sterols biosynthesize them in an analogous manner.

Stage IV has been studied primarily by McMorris. His group has over a period of 20 years identified the various male and female

Table III. Radiolabelling feeds to *Saprolegnia ferax*

Substrates	Starting specific activity (DPM/ $\mu$ G/50 mL Medium)	Incorporation $^{14}$ C or $^3$ H Into Sterol Mixture (DPM)
[ $^3$ H]Cholesterol		Not Examined
[ $^3$ H]Desmosterol	$2.1 \times 10^6/500\mu\text{G}$	$1.0 \times 10^6$
[ $^3$ H]24-Methylene cholesterol	$1.0 \times 10^6/400\mu\text{G}$	$4.0 \times 10^5$
[ $^{14}$ C]Zymosterol	$2.0 \times 10^5/250\mu\text{G}$	$1.0 \times 10^5$
[ $^{14}$ C]Fecosterol	$3.1 \times 10^5/205\mu\text{G}$	$1.3 \times 10^5$
[ $^3$ H]Lanosterol <sup>2</sup>	$8.0 \times 10^6/500\mu\text{G}$	$1.1 \times 10^3$
[ $^3$ H]Cycloartenol <sup>2</sup>	$2.5 \times 10^6/500\mu\text{G}$	$8.5 \times 10^3$
[ $^3$ H]Dihydro-lanosterol	$4.2 \times 10^6/600\mu\text{G}$	$3.9 \times 10^3$
[ $^3$ H]Dehydro-pollinastanol	$1.0 \times 10^7/500\mu\text{G}$	$3.5 \times 10^6$
[ $^{14}$ C]SO + Tridemorph <sup>3</sup>	$8.3 \times 10^5/450\mu\text{G}$	$7.1 \times 10^3$
[ $^{14}$ C]Acetate <sup>2</sup>	$1.4 \times 10^7$	$1.5 \times 10^5$

1. The label was introduced into the sterol by base-catalyzed exchange with  $^3\text{H}_2\text{O}$ , or by incubating the GL-7 yeast sterol mutant or *S. ferax* with [ $^{14}$ C]acetate in the presence and absence of tridemorph.
2. Recrystallized products to constant specific activity.
3. Accumulated  $\Delta^8$ -sterols; structures confirmed by MS and PMR SO = squalene oxide.

Ratio of activity in HPLC purified end products					
Cholesterol	Desmosterol	24-Methylene cholesterol	Fucosterol	Dehydropollin- astanol	Ref.
$1.0 \times 10^4$	$8.0 \times 10^5$	$2.5 \times 10^3$	$1.5 \times 10^2$	---	11
0	0	$2.6 \times 10^5$	$0.6 \times 10^3$	---	11
$0.1 \times 10^3$	$1.1 \times 10^3$	$0.8 \times 10^3$	$0.9 \times 10^3$	---	11
0	0	$1.8 \times 10^4$	$0.8 \times 10^3$	---	11
$2.0 \times 10^3$	$3.2 \times 10^3$	$4.1 \times 10^3$	$1.6 \times 10^4$	---	11
0	0	0	0	$05. \times 10^3$	10
$0.9 \times 10^3$	0	0	0	---	10
0	0	0	0	$2.8 \times 10^6$	11
0	0	0	0	---	10
$3.8 \times 10^3$	$1.0 \times 10^4$	$1.2 \times 10^4$	$3.4 \times 10^4$	---	10,11

gametangia inducing factors (GIF) from Achlya (4). GIF are steroid hormones biosynthetically derived from fucosterol (4). Our ability to block fucosterol metabolism in S. ferax with 25-azacholesterol, thereby inhibiting sporulation (unpublished data), leads us to conclude S. ferax synthesizes similar steroid (GIF) hormones.

#### Sterol Functions.

The classical view, reviewed in ref. 32,49, for how sterols regulate microbial growth envisages an asymmetric subcellular distribution of sterol aligned with fatty acyl chains in discrete domains along the plane of the monolayer such that changes in sterol levels within localized areas in each of the bilayer halves should sufficiently perturb the physicochemical properties (gel  $\rightleftharpoons$  liquid phase transitions) of the membrane lipid core to alter its permeability, thereby stimulating, as a cascading response to the influx of nutrients, the growth dynamics. Bloch (32) has referred to the accumulation of sterol in the membrane as the "bulk" role. In support of a bulk role is the subcellular sequestering of sterol in the plasmalemma (32,49) and the sterol concentration dependence, ca 22-23 mol % relative to fatty acids, noted in studies designed to measure sterol-induced physicochemical and permeability properties of natural and artificial membrane systems (32,49). The question which we have posed for Oomycetes physiology is what is the developmental significance of the bulk role and what structural features of the sterol are essential for bulk activity. As we examined this problem, we found that sterols possessed additional non-metabolic roles each of which expressed itself with development (Fig. 6).

Our initial structure-activity studies were not designed to reveal bulk importance but to discriminate the specificity for molecular groups (3B -OH, length of side chain, etc) in sterol-controlled growth and reproduction. We found (Fig. 7) that a variety of sterols and "sterol-like" compounds, ie., triterpenoids, added to the cultures at 10ppm stimulate growth (1,6,7,24), although only specific sterols induced oospore production (1,50-53). The lack of activity was not due to uptake per se (2,6).

The specificity for  $\Delta^5$ -sterols in reproduction coupled with the well-described steroid hormones of Achlya, derived biosynthetically from a  $\Delta^5$ -sterol, seemingly indicated that P. cactorum metabolized the dietary sterols to analogous GIF. However, our recent sterol structure-oospore production studies (1,50,53) with P. cactorum fail to show structural homology for GIF of Achlya with those produced by Phytophthora. Had the hormones been similar then (indicating sine qua non, similar metabolites), for example, 20-epicholesterol should have failed to induce Phytophthora oogonia which it did not. While the sexual hormones for Achlya and Phytophthora appear now to be different, it remains unknown whether there is any similarity in sterol structural features governing growth. To answer this question we have recently repeated a series of experiments designed to study the influence of sterols on growth of P. cactorum cultured on a sterol-free media. The amount of sterol required to "spark" (a euphemism coined by Parks, 55) and stimulate maximal growth was found to be very small - 0.5 ppm, analogous to the sterol auxotrophs of yeast (54,55). This level, we

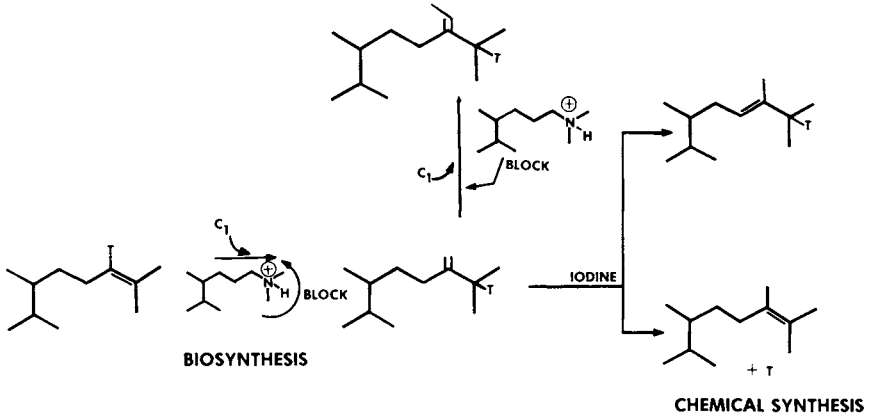


Figure 5. Mechanism of C-24 alkylation in *Saprolegnia ferax* and interference by 25-azacholesterol: isomerization of 24(28)-bond was induced in the presence of iodine to confirm biochemical 1,2-hydride transfer.

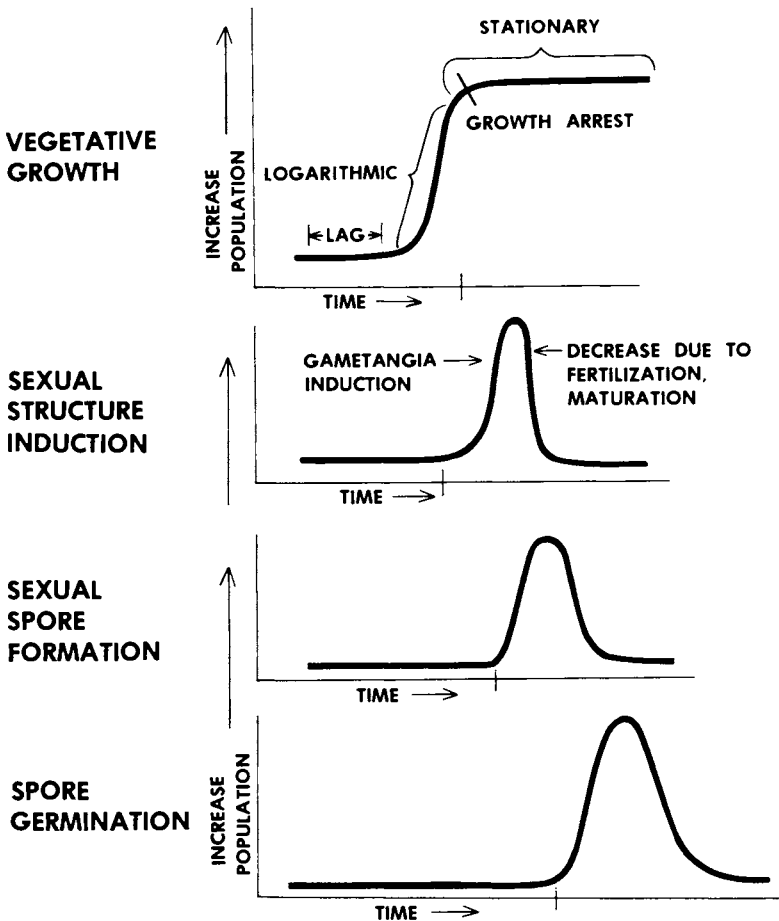
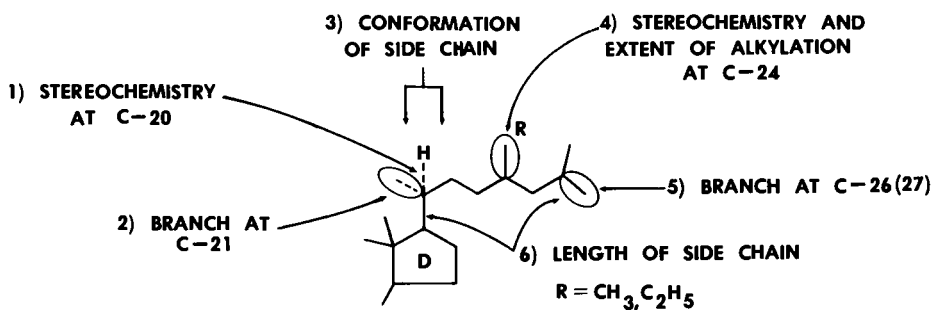


Figure 6. Sequence of developmental events in the fungal life cycle which may be controlled by sterols.



	DRY WT *	HYPHAL EXENSION	OOGONIA INDUCTION	OOSPORE PRODUCTION
1)	++	++	○	++
2)	○	○	○	○
3)	++	++	○	++
4)	○	++	○	++
5)	○	○	○	○
6)	++	++	○	++

++ VERY SIGNIFICANT; ○, NOT SIGNIFICANT

Figure 7. Effect of sterol structural features on biological properties of *P. cactorum*.

and others had previously found, fails to produce oogonia or oospores when the media is supplemented with agar (2,3,5 and ref. cited therein). In the more recent studies, we found that the fungus will respond to the sterol-mediated "spark" primarily at an early stage-specific phase in the growth cycle i.e., the lag to log transition period (Fig. 8), a cell cycle mediated process; addition of sterol prior or post to this developmentally predisposed event (note the growth characteristics of control) produced significantly less of a stimulatory influence, even though sterol (<sup>3</sup>H-labelled) is actively accumulated by the mycelia throughout logarithmic growth. That trace levels of sterols (in some organisms a structurally similar sterol may be functionally equivalent although the amount of the same sterol for induction of activity may be different at a later point in the growth cycle) and sterol-like compounds may regulate temporal changes in growth rate indicates gene regulation of developmental proteins and the cell cycle is mediated initially by something other than sterol-induced bulk alterations in physicochemical (fluidity) properties. Perhaps the mitotic index and growth-stimulation (a manifestation of increased nucleation, cell division, hyphal elongation and branching) is associated with sterol (and "sterol-like" compounds)-protein interactions (the so-called bridge model, proposed by Nes and Heftman 49, Fig. 9). The association of some bridged sterols might act to control vegetative development by signaling to the fungus that large masses of sterols will subsequently be made available for insertion into the membrane to play the bulk role. Other bridged sterols may be an essential binding factor which affects the topology of the protein strands and without it or a suitable replacement molecule produces a diminished response ultimately impacting on life cycle events.

The physiological importance of the bulk role appears to be linked to the development of the hyphal growth unit (56). More specifically, the bulk role appears to be a component of reproductive fitness which quantitatively controls gametangial induction. In support of this view, we have observed that, in contrast to the beneficial affects of cholesterol on the morphogenesis of *Phytophthora* mycelia (Fig. 10), 20-epicholesterol induced aberrant mycelia. The deleterious morphogenesis was evidenced in an altered volumetric and spatial distribution of the hyphal branches (Fig. 11), which presumably produced the hyphal tip fragmentation and aborted oospores observed in Fig. 11 (1,50,53). When the hyphal growth unit fails to assume the appropriate size for gametangial initiation, which has also been observed in the triterpenoid treatments, the mycelia remain in the vegetative mode. There are reasons to believe (57,58) that calcium (57), cyclic nucleotide metabolism (58) and perhaps fatty acid levels (58), in addition to sterols, affect the hyphal growth unit and reproduction indicating the potential for a physiological interdependency with minimally these four biochemical components.

*Achlya* and *Saprolegnia* may, like *P. cactorum*, utilize sterols in a sparking and bulk role during vegetative growth. As a result, each of the 4 end products may exhibit nonequivalent functions. When 25-azacholesterol is fed to *S. ferax*, C<sub>1</sub>-transferase activity is blocked, desmosterol accumulates and oosporogenesis is prevented. Presumably, cholesterol is a sparking agent,



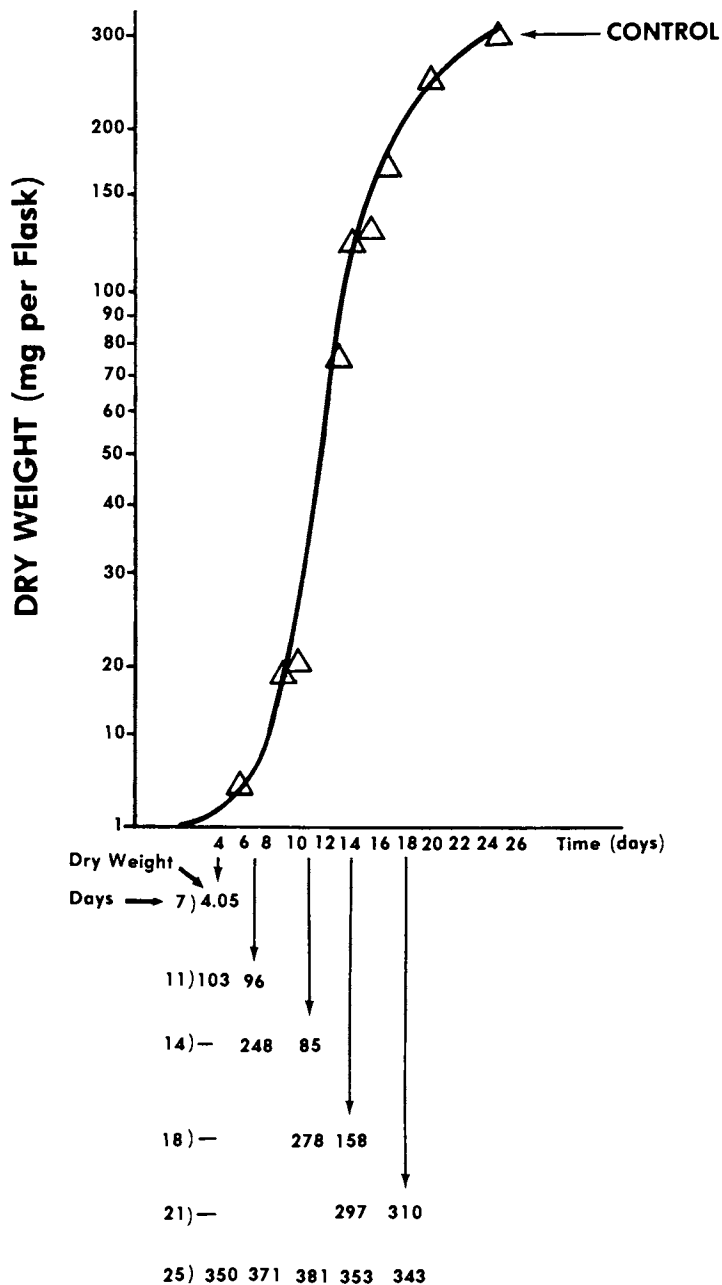


Figure 8. Growth curve (liquid media; 50 ml in 250 ml flasks) of *Phytophthora cactorum* supplemented with 0.5 ppm of cholesterol at different times following inoculation and a control experiment set-up together with the cholesterol treatment. Cultures were incubated at 20°C in the dark and seeded with a mycelial suspension previously grown in a sterol-free liquid media. The dry weights ranged by as much as 20 mg in mid to late log phase growth and 10 mg in early log and stationary phase growth.

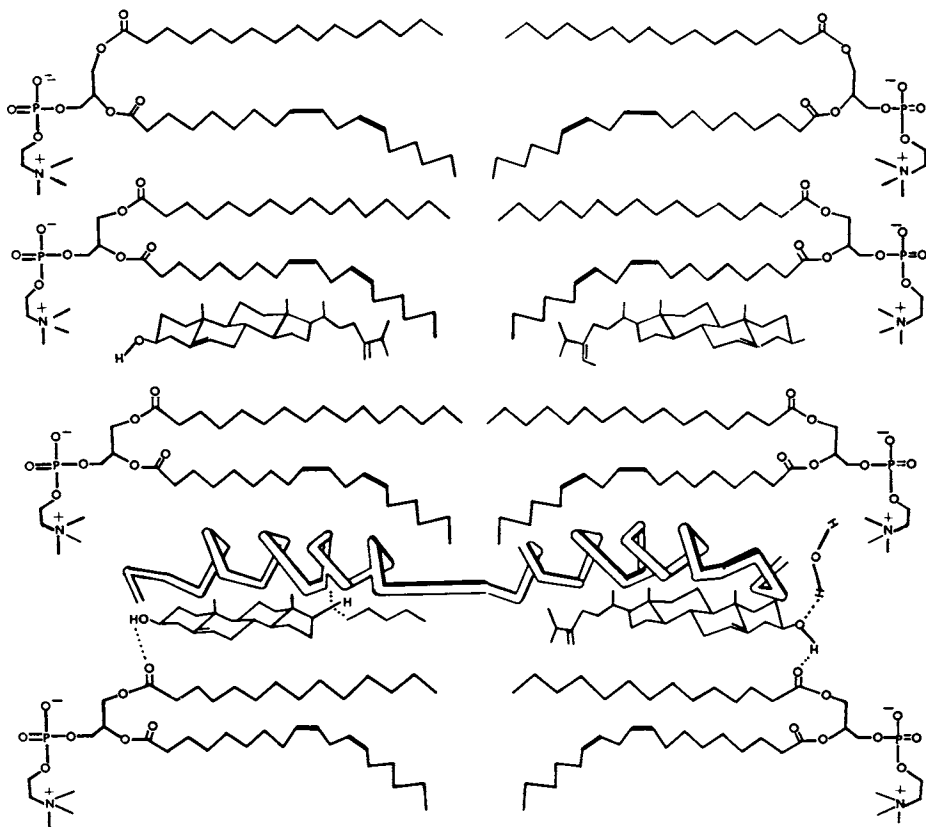


Figure 9. Bridge model of sterol-protein-lipid interactions.

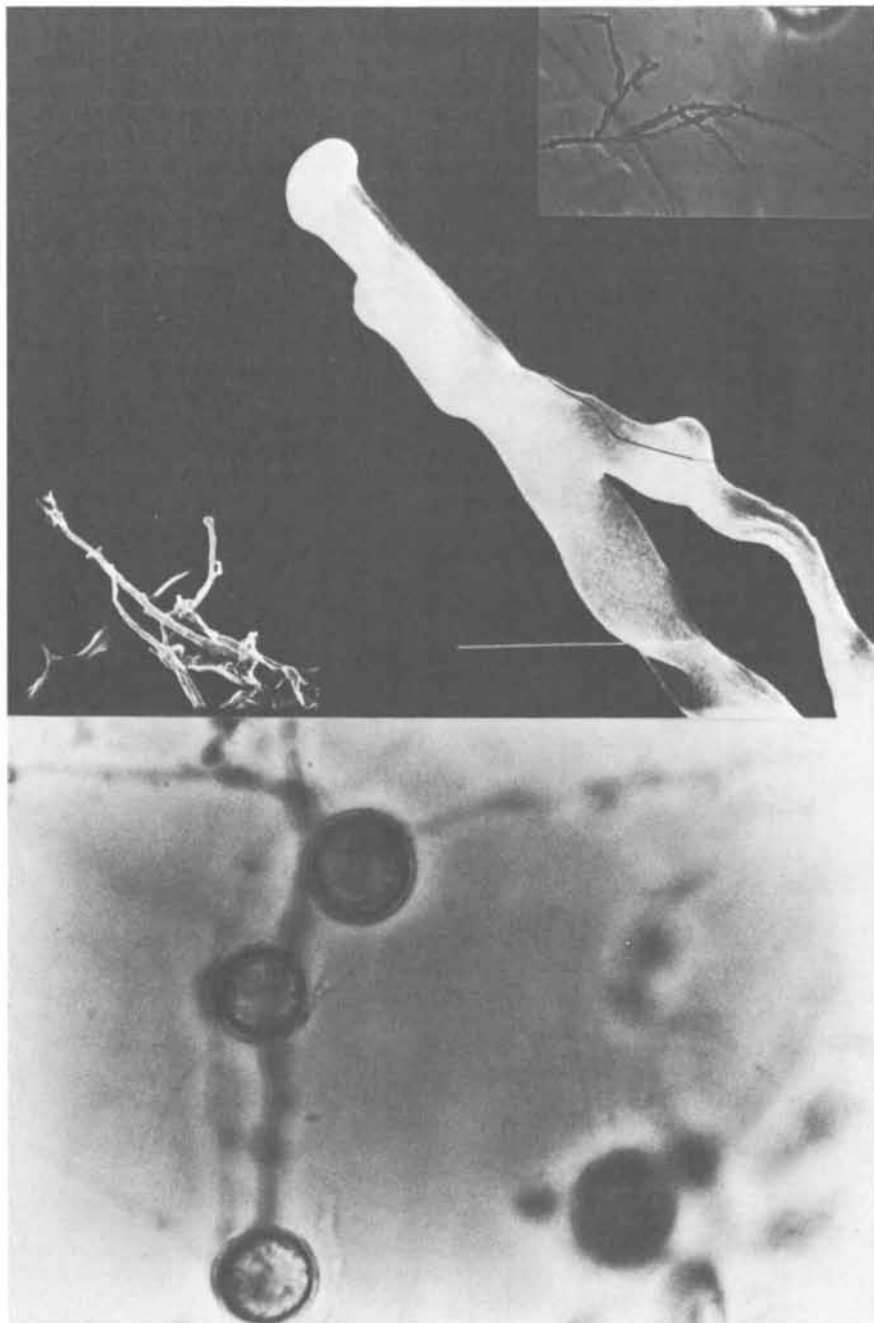


Figure 10. Micrographs of cultures of *P. cactorum* supplemented with 10 ppm cholesterol were obtained with a scanning electron (top; also note lower left-hand corner with a different magnification) and light (bottom and top right insert) microscope.

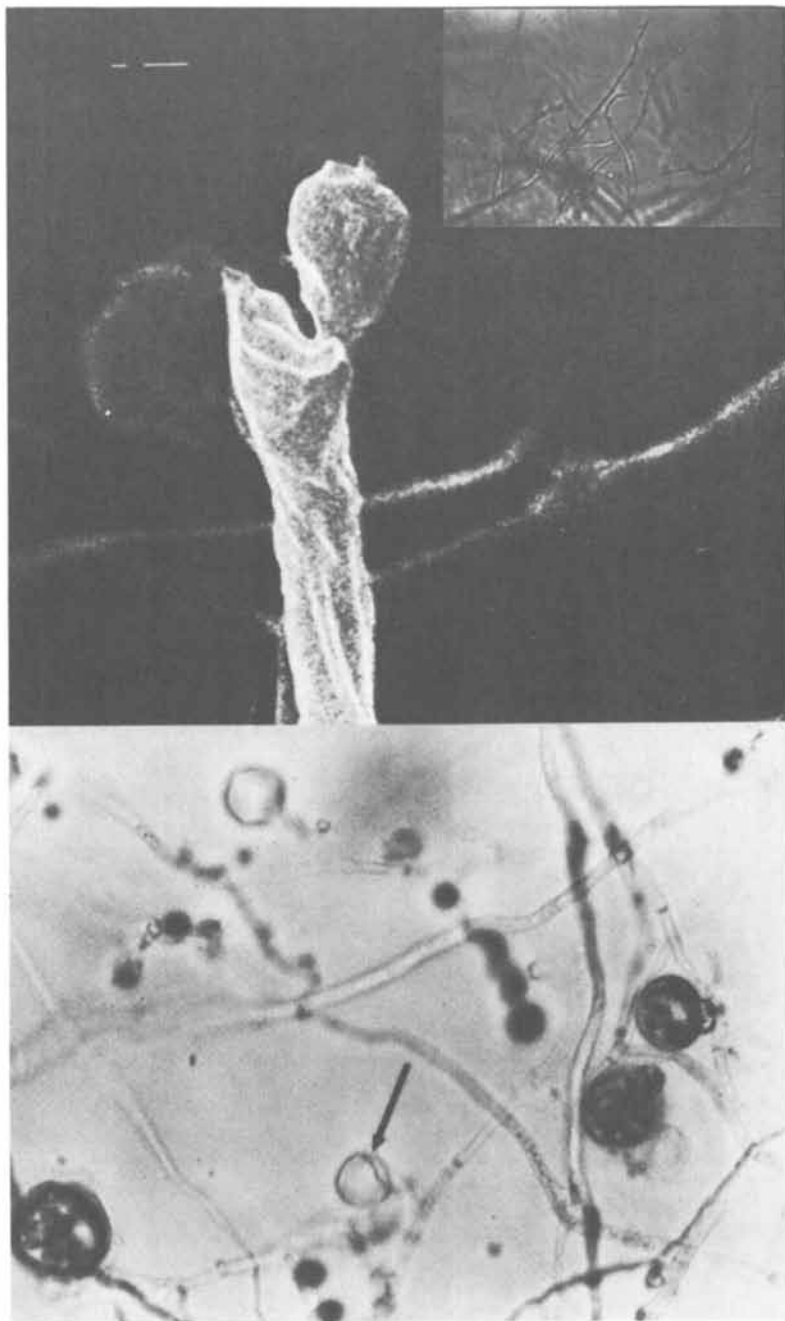


Figure 11. Micrographs of cultures of *P. cactorum* supplemented with 10 ppm 20-epicholesterol were obtained with a scanning electron (top) and light (bottom and top right insert) microscope.

24-methylenecholesterol and desmosterol play the bulk role and fucosterol has no apparent vegetative role, although it is synthesized to play another role i.e., as the metabolic precursor of the steroid hormones which regulate gametangia induction.

In conclusion, the hyphal growth unit and other vegetative characteristics i.e., growth rate and stationary phase population density, are influenced by sterols and "sterol-like" molecules. The structural features and levels of sterols for sparking may be more specific from those used for bulk activity. Apparently, for all the Oomycetes a critical mass of non-select sterols (bulk role) must accumulate prior to growth arrest in order that the hyphal growth unit assume a vegetative habit capable of producing and responding to GIF hormones. Cholesterol and fucosterol accumulated by *P. cactorum* appear to trigger sexual reproduction without additional metabolism. The selective advantage, however, for some but not all Oomycetes e.g. *Saprolegnia* and *Achlya* to synthesize and then metabolize fucosterol to steroid hormones in stationary phase growth is not clear. Nevertheless, all members have evolved to respond to sterol structure-function changes during the vegetative phase, thereby influencing reproduction. This implies that a physiological dependence exists for sterol-controlled developmental regulation. In terms of fungal evolution, the change in sterol profile induced by ecological, environmental or fungal host-mediated events may have been one of many interrelated forces which have acted via informational molecules in the vegetative phase to direct the gene(s) governing sporulation. If the latter is so, then physiological adaptation or the lack thereof to developmental modulation of sterol biosynthesis and function may be a significant contributing factor to evolutionary change and perhaps speciation.

#### Literature Cited

1. Nes, W.D.; Stafford, A.E. Proc. Natl. Acad. Sci. 1983, 80, 3227.
2. Nes, W.D. in "Isopentenoids in Plants: Biochemistry and Function"; Eds. Nes, W.D.; Fuller, G.; Tsai, L.; Marcel Dekker, 1984; pp. 267.
3. Elliott, C.G. Adv. Microbiol. Physiol. 1977, 15, 121.
4. McMorris, T.C. Lipids 1978, 13, 716.
5. Hendrix, J.W. Ann. Rev. Phytopathol. 1970, 8, 111.
6. Nes, W.D.; Saunders, G.A.; Heftmann, E. Lipids 1982, 17, 178.
7. Nes, W.D.; Patterson, G.W. J. Nat. Prod. 1981, 44, 215.
8. McCorkindale, N.J.; Hutchinson, S.A.; Pursey, B.A.; Scott, W.T.; Wheeler, R. Phytochemistry 1969, 8, 861.
9. Gottlieb, D.; Knaus, R.J.; Wood, S.G. Phytopathology 1978, 68, 1168.
10. Nes, W.D.; Le, P.H.; Berg, L.; Patterson, G.W.; Kerwin, J.K. Experientia 1985, in press.
11. Le, P.H.; Nes, W.D.; Parish, E.J. J. Amer. Oil Chem. Soc. 1984, 19, 544 (A).
12. Warner, S.; Sovocool, G.; Domnas, A. Mycologia 1983, 75, 285.
13. Warner, S.; Eirman, D.; Sovocool, G.; Domnas, A. Proc. Natl. Acad. Sci. 1982, 79, 3769.
14. Warner, S.; Domnas, A. Experimentl. Mycol. 1981, 5, 184.
15. Berg, L.R. Ph.D. Thesis 1982, Univ. Md.

16. Ludwig-Kohn, H.; Jahnke, K.D.; Bahnweg, G. Biochim. Biophys. Acta. 1982, 713, 463.
17. Popplestone, C.R.; Unrau, A.M. Phytochemistry 1973, 12, 1131.
18. Wood, S.G.; Gottlieb, D. Biochem. J. 1978, 170, 355.
19. Schlösser, E.; Shaw, P.D.; Gottlieb, D. Arch. Mikrobiol. 1969, 66, 147.
20. Richards, J.B. and Hemming, F.W. Biochem. J. 1972, 128, 1345.
21. Warner, S.A.; Sovocool, G.W.; Domnas, A. Phytochemistry 1982, 21, 2135.
22. Fryberg, M.; Oehlschlager, A.C.; Unrau, A.M. Arch. Biochem. Biophys. 1975, 173, 171.
23. Ko, W.H. J. Gen. Microbiol. 1985, 131, 2591.
24. Nes, W.D.; Patterson, G.W.; Bean, G.A. Lipids 1979, 14, 458.
25. Hendrix, J.W.; Apple, J.L. 1964 Phytopathology 54, 987.
26. Hendrix, J.W.; Norman, C.; Apple, J.L. Physiol. Plant 1966, 19, 159.
27. Hohl, H.R. Phytopathog. Zeitschrift 1975, 84, 18.
28. Bahnweg, G. Botanica Marina 1980, 23, 209.
29. Ko, W.; Ho, W. Ann Phytopathol. Soc. Japan 1983, 49, 316.
30. Zakai, A.I.; Zentmeyer, G.A.; Sims, J.J.; Keen, N.T. Phytopathology 1983, 73, 199.
31. Ricci, P.; Benveniste, P.; Bladocha, M. C.R. Acad. Sc. Paris 1985, 300, 119.
32. Bloch, K.E.; CRC Crit. Rev. Biochem. 1983, 14, 47.
33. Nes, W.R.; Varkey, T.E.; Krevitz, K. J. Amer. Chem Soc. 1977, 99, 260.
34. van Tamelen, E.E. J. Amer. Chem. Soc. 1982, 104, 6480.
35. Nes, W.D.; Benson, M. Le, P.H. J. Amer. Chem. Soc. Manuscript submitted.
36. Bu'Lock, J.D.; Osagie, A.U. Phytochemistry 1976, 15, 1249.
37. Ducruix, A.; Pasaard-Billy, C.; Devys, M.; Barbier, M.; Lederer, E.; J. Chem. Soc. Chem. Commun. 1973, 929.
38. Heintz, R.; Benveniste, P. J. Biol. Chem. 1974, 249, 4267.
39. Goad, L.J.; in "Lipids and Lipid Polymers in higher Plants"; Eds., Tevini, M.; Lichtenthaler, H.K.; Springer-Verlag, 1976, 146.
40. Rees, H.H.; Goad, L.J.; Goodwin, T.W. Biochem. J. 1968, 107, 417.
41. Silbert, D.F.; Ruch, F.; Vagelos, P.R. J. Bacteriol. 1968, 95, 1658.
42. Berg, L.R.; Patterson, G.W.; Lusby, W.R. Lipids 1983, 18, 448.
43. Fryberg, M.; Oehlschlager, A.C.; Unrau, A.M. J. Amer. Chem. Soc. 1973, 95, 5747.
44. Nes, W.D.; Heupel, R.C. Arch. Biochem. Biophys. 1986, 244, 211.
45. Nes, W.R.; Heupel, R.C.; Le, P.H. J. Chem. Soc. Chem. Commun. 1985, 1431.
46. Knights, B.A.; Elliott, C.G. Biochim. Biophys. Acta 1976, 441, 341.
47. Akhtar, M.; Hunt, P.F.; Parvez, M.A. Biochem. J. 1968, 103, 616.
48. Rahier, A.; Genot, J.; Schuber, F.; Benveniste, P.; Narula, A.S. J. Biol. Chem. 1984, 259, 15215.
49. Nes, W.D.; Stafford, A.E. Lipids 1984, 544.
50. Nes, W.D.; Heftmann, E. J. Nat. Prod. 1981, 44, 377.
51. Nes, W.D.; Patterson, G.W.; Bean, G.A. Lipids 1979, 14, 458.

52. Nes, W.D.; Hanners, P.K.; Bean, G.A.; Patterson, G.W. Phytopathology 1981, 72, 447.
53. Nes, W.D.; Nes, W.R. Experientia 1983, 39, 276.
54. Pinto, W.J.; Luzano, R.; Sekula, B.C.; Nes, W.R. Biochem. Biophys. Res. Commun. 1983, 112, 47.
55. Rodriguez, R.J.; Low, C.; Bottema, D.K.; Parks, L.W. Biochim. Biophys. Acta 1985, 837, 336.
56. Trinci, A.P.J. J. Gen. Microbiol. 1974, 81, 225.
57. Kerwin, J.L.; Washino, R.K. Experimentl Mycol. 1984, 8, 215.
58. Kerwin, J.L.; Washino, R.K. Can. J. Microbiol. in Press.

RECEIVED August 20, 1986

## Chapter 20

# Fatty Acids and Fungal Development: Structure-Activity Relationships

James L. Kerwin

Department of Entomology, University of California, Davis, CA 95616

Free and esterified fatty acids and their metabolites regulate fungal morphogenesis in diverse and poorly understood ways. Through use of structure-function relationships, the physiological bases for morphological development in fungi can be directly or indirectly investigated. Use of simple categories for classification, e.g. saturated vs. unsaturated or preferential partitioning in fluid or gel phases, can be misleading due to the presence of segregated lipid domains in cell membranes or to selective metabolism. Fatty acid composition has been shown to regulate a variety of membrane transport processes, oxidative phosphorylation, and a number of transferases and other enzymes. Fungal growth requirements for fatty acids can vary among genera, species or even isolates of the same species. Sporulation and spore germination are also affected by fatty acid composition as are the interactions between fungal parasites and their plant or insect hosts. Preliminary investigations support a role for oxygenated fatty acid derivatives in fungal morphogenesis.

Fatty acids, which are integral components of cellular and organelle membranes, primary storage products and substrates for secondary metabolism, are involved in the regulation of fungal morphogenesis. Changes in fatty acid composition and metabolism associated with fungal growth and differentiation (reviewed in 1-6) have been extensively documented; although alterations in fatty acid components have been associated with the development of fungi, specific structure-function relationships have rarely been established. Secondary metabolism of these compounds has also largely been ignored, especially concerning possible regulatory roles of e.g. oxidized fatty acids in fungi. The following review focuses on studies which attempt to more precisely relate fatty acid structure to physiological and morphological development in fungi, often in terms of their physical properties. Initial observations are also presented concerning potential regulatory roles for fatty acid cyclooxygenase and lipoxygenase products in fungal systems.

0097-6156/87/0325-0329\$06.00/0  
© 1987 American Chemical Society



### Fatty Acid Structure

Reviews of fungal fatty acid composition (5,7,8) reveal that their primary constituents are 12- to 20-carbon chain length unbranched compounds, with even-numbered chains predominant. Both saturated and unsaturated compounds occur, with palmitoleic (C-16:1), oleic (C-18:1), linoleic (C-18:2) and linolenic (C-18:3) acids the most common unsaturated moieties (5). As with most naturally occurring fatty acids (9), monounsaturated compounds usually contain a cis olefinic bond and polyunsaturated acids have methylene-interrupted cis double bonds. Although rare in occurrence and subjected to limited study, branched chains, hydroxy, oxo and epoxy acids are also synthesized (5). Lists of the structures of unusual fatty acids which can be employed in structure activity studies are presented in detail elsewhere (9-14).

When discussing diverse fatty acid structures, it is convenient to group related compounds into categories to facilitate the elucidation of structure-function relationships. The most obvious approach is to group compounds as being saturated or (poly) unsaturated; however, problems arise in instances where e.g. short chain saturated compounds and longer chain unsaturated acids have similar effects on membrane fluidity. An alternate approach involves grouping according to preferential partitioning into fluid or gel phases (15,16), which separates cis-unsaturated fatty acids from saturated and trans-unsaturated compounds. In some developmental systems categorization may not be possible due to preferential secondary metabolism of specific compounds, e.g. arachidonic acid, which subsequently regulates pivotal events in fungal morphogenesis. Quantitative structure-activity studies using linear free energy-related parameters such as partition coefficients, electronic substituent constants and steric properties (17,18) would precisely define relationships among diverse fatty acids, but this approach has not been exploited in fungal research.

### Physical Properties and Concepts

Acyl carbon chains in fully saturated phospholipids occur in extended all-trans conformations below the phase transition temperature (19, 20). The presence of a single cis double bond disrupts the close packing of the all-trans conformation and can profoundly affect physical properties. The position and configuration of double bonds affect basic characteristics such as melting point, with lower melting points found for compounds in cis rather than trans configurations and in fatty acids with double bonds near the center of the fatty acid chain (21-23). Carbon chain length, degree and position of unsaturation and the presence of cyclic or oxygenated moieties all affect membrane fluidity which in turn regulates a variety of cellular functions (24-26). Their effects are mediated in part by influencing the membrane lipid phase transition temperature (27-29) and related physical properties such as range and rate of acyl chain motion; however, extreme changes in levels of unsaturation often result in insignificant changes in fatty acyl motional parameters in both biological and model membrane systems (30). Recent observations on the properties of cyclopropane-containing membranes revealed that although the cyclic moiety perturbs membrane lipid packing, it actually stabilizes membrane structure by acting as a barrier to the

propagation of perturbations (31). Unexpected results such as these emphasize the complex nature of lipid-mediated events, and care should be taken in extrapolating properties exhibited by free fatty acids to effects on biological systems. An added complication when attempting to interpret membrane-mediated phenomena in terms of fatty acyl composition is the possible existence of laterally segregated lipid domains (32-35) which have been proposed to have both structural and functional significance.

Previous discussion has emphasized membrane-mediated (phospholipid) phenomena; free fatty acids, which can act on membranes or have completely different modes of action, have also been used to perturb fungal morphogenesis. Although the same physical properties outlined above may regulate their effects, selective metabolism of a limited number of specific compounds, e.g. by lipoxygenase enzymes, can often be of greater regulatory significance. This aspect of fatty acid metabolism is presented in greater detail in appropriate sections.

### Membrane Transport

Fungi have fatty acid carrier systems, often displaying some degree of specificity e.g. for 12- and 14-C compounds vs. 16- and 18-C saturated or unsaturated fatty acids in *Saccharomyces uvarum* and *Saccharomyces lipolytica* (36). Exogenous fatty acids can be incorporated or partitioned into membrane phospholipids and subsequently modify carrier systems for other classes of compounds. Short chain compounds, e.g. formate, acetate, butyrate, caprylic and caproic acids inhibit phosphate (37) and thiamine (38) uptake by *Saccharomyces cerevisiae*, perhaps by blocking the use of energy from adenosine triphosphate. The kinetics of arginine uptake by anaerobically grown *S. cerevisiae* is considerably altered following incorporation of linoleic acid into cells when compared to oleic acid-enriched cells (39). On the basis of comparative inhibition studies, it was concluded that the activity of membrane-bound transport proteins was probably altered by the nature of surrounding fatty acids.

Several related studies exploited differing fatty acid compositions of thermophilic, mesophilic and psychrophilic species of *Torulopsis* (40) and several other species of yeast (41). Glucose and leucine uptake occurred at measurable rates only at temperatures at which the various fungi grew. The fatty acid unsaturation index was high for psychrophilic species, intermediate for mesophiles and low for thermo-tolerant yeasts, and this was related to possible mediation of transport processes by membrane fluidity.

### Enzymic Regulation

Fatty acid auxotrophs of *S. cerevisiae* have been extensively utilized to probe relationships between fatty acid unsaturation and enzymic activity. A major area of investigation has been elucidation of the bases for loss of oxidative phosphorylation by yeast cultures characterized by less than ca. 20-30% unsaturated fatty acid (42-44). Much reduced or no activity in mitochondrial membranes with highly saturated fatty acyl compositions has been documented for cytochromes a, a<sub>3</sub>, b and c, mitochondrial ATPase, succinate oxidase and NADH oxidase (45-47). Fatty acids apparently mediate many mitochondrial functions

including the rate of proteolysis of mitochondrial translation products (48), dihydrolipoate-induced ATP synthesis in promitochondria (49), mitochondrial proliferation (50) and release from glucose repression (51). The  $\Delta$ -9 isomer was the most efficient of the *cis*-octadecenoic acid compounds evaluated in this latter study, with eicosaenoic acid supporting 15% of the release induced by oleic acid. All of the listed processes were inhibited by saturated fatty acids.

Linolenic acid generally accelerates the activity of a number of enzymes in anaerobic yeast cultures, including isocitrate lyase, malate synthase and malate dehydrogenase (52), and sterol synthesis (53). Linoleate and oleate had progressively less effect on synthesis of the three enzymes in the former study (52). Oleic and linoleic acid supplementation at 0.01% in anaerobic yeast cultures induced a 5- to 10-fold increase in sterol formation while elaidic acid inhibited induction (54). Stearic and palmitic acids suppressed anaerobic growth while lauric acid had no effect on either parameter. In a related investigation on sterol metabolism, oleic and lauric acids abolished growth inhibition by hypocholesteremic compounds, with saturated acids having no effect (55).

In other instances unsaturated fatty acids can strongly repress *S. cerevisiae* enzymic activity e.g. alcohol acetyltransferase (56), acyltransferases involved in glycerolipid synthesis (57), fatty acid biosynthesis (in both *S. cerevisiae* and *Candida lipolytica*, 58) and acetyl CoA carboxylase (59). Some enzymes are repressed by long chain fatty acids (57-59), while unsaturated compounds have specific effects in others (56). Defined mechanisms for these diverse responses are not clear, although nonspecific surfactant action is probably rare (57) and fatty acid metabolites mediate some repressive fatty acid effects (59).

### Temperature Adaptation and Fatty Acid Composition

The fatty acid composition of microorganism membranes can be altered by varying temperature during growth, with the usual pattern being an increase in short chain and/or unsaturated compounds with decreasing growth temperature (60-64). Membrane fluidity appears to be under regulatory control (65) since it must be maintained at a level compatible with cell growth and function (60, 66, 67).

A number of fungi produce more highly unsaturated fatty acids when grown or adapted to grow at low temperatures (68); however, temperature adaptation can involve increased synthesis of distinct 18-C monounsaturated or diunsaturated compounds by *Mucor mucedo* and *Aspergillus ochraceus*, respectively (69); significant shifts from stearic to palmitic acid by *S. cerevisiae* (70); increasing amounts of palmitoleic acid specifically without changing the total quantity of unsaturated fatty acids by the psychrophilic yeast *Candida utilis* (71); enhancement of linolenic acid at the expense of mono- and diunsaturated 18-C compounds by three species of *Leucosporidium* (72); or the synthesis of rare highly unsaturated compounds, e.g.  $\Delta$ -6,9,12,15-octadecetetraenoic acid by the Zygomycete *Thamnidium elegans* (73).

A related study documented greater amounts of unsaturated fatty acids in spores and mycelium of several species of thermophilic and thermotolerant mucoralean fungi when grown at 25°C rather than 48°C (74). These authors suggested that perhaps low dissolved oxygen in growth media at high temperatures would support only reduced levels

of fatty acid desaturase activity as had been proposed previously for other species of fungi (75,76). This may have occurred in their experimental system; however, considering the wide range of fungal species examined under diverse cultural conditions, it is unlikely that this hypothesis can serve generally to explain relationships between unsaturation and temperature acclimation.

Exceptions to cold temperature adaptation being accompanied by shorter chain or more unsaturated fatty acids include mycelial phospholipids with greater unsaturation when grown at 36°C vs 20°C (77), and the lack of correlation of cold temperature acclimation with increased lipid unsaturation or membrane fluidity by four fungi representative of four different fungal classes (78).

Many of these changes in membrane fatty acid composition probably act by modulating enzymic activity by regulating localized or bulk membrane fluidity; however, since most studies have not measured rates or ranges of lipid or protein motion as a function of changes in fatty acid composition, or even differentiated between total cell and phospholipid composition, there is little direct support for this hypothesis. An additional complication is the possible presence of laterally segregated lipid domains which could have profound effects on protein assembly or enzyme activity with minimal changes in bulk membrane fluidity.

#### Fatty Acids and Fungal Growth

There has been extensive use of anaerobic cultures or desaturase mutants of *S. cerevisiae* to probe the unsaturated fatty acid growth requirements of this yeast (79). A number of investigations have attempted to formulate empirical rules relating fatty acid structure to their ability to support growth. Using a desaturase mutant of *S. cerevisiae*, 23 fatty acids were evaluated and only those possessing a cis double bond at C-9 supported growth (80). This claim was disputed using a different desaturase mutant of this yeast in which 16C to 22C fatty acids with double bonds in positions  $\Delta 4$  through  $\Delta 9$  all supported growth (81). Arachidonic, eicosapentaenoic and docosahexaenoic acids had the highest efficiencies; it was concluded that there was a direct correlation between greater number of double bonds and increased growth. A third study using a different desaturase mutant examined a number of cis-octadecenoate isomers and found all isomers between  $\Delta 7$  and  $\Delta 12$  able to support extensive growth (82). The  $\Delta 6$  isomer was also effective, which is in contrast to results from other studies (79,80) which showed no activity using this compound.

A more recent study using anaerobic cultures of *S. cerevisiae* (79) concluded that those fatty acids which supported growth all had a maximum of 9 saturated C atoms before the occurrence of a cis double bond or a hydroxyl group, or 7 saturated C atoms before the occurrence of a trans ethylenic bond. These rules are in apparent general agreement with most published work although there are exceptions, e.g. the inhibitory action of cis- $\Delta 11$ -octadecenoate reported for one desaturase mutant (80) while supporting growth by *S. cerevisiae* under anaerobic conditions (75,79). Requirements for specific fatty acids for *S. cerevisiae* growth can vary with sterol structure (83) and it is probable that different desaturase mutants and different strains of this yeast grown anaerobically under different culture conditions could also have varying fatty acid require-

ments. There is no compelling reason to assume different isolates should have identical metabolic requirements; this alone is sufficient to explain occasional discrepancies in the literature concerning unsaturated fatty acid needs.

Saturated fatty acids are also required for growth by *S. cerevisiae* (84,85), with a negative correlation of growth demonstrated for high levels of dodecenoic and tridecenoic acids and a positive correlation with the presence of myristic, pentadecenoic and palmitic acids (86).

Fatty acid supplementation affects the growth of a number of fungi. Among the documented effects are the promotion of growth of the crustacean parasite *Haliphthoros milfordensis* by oleic acid, triolein and tripalmitolein. Saturated fatty acids were not effective while polyunsaturated fatty acids were inhibitory (87). When six thermophilic fungi were grown at 45°C on various fatty acids, optimum yields were obtained for all species on myristic, palmitic, stearic and oleic acids except for one isolate of *Chaetomium* which could not grow on myristic acid (88). Capric, caprylic, lauric, linoleic and arachidic acids were not utilized by any of the fungi. Comparative studies of several isolates of saprobic skin fungi provided direct evidence that different strains of the same species can have diverse growth requirements as speculated previously concerning *S. cerevisiae*. All three strains of *Pityrosporum ovale* utilized oleic acid, but in addition one used myristic acid only, one used lauric and myristic acids, and a third could use those two acids plus palmitic and stearic (89,90). A related species, *Pityrosporum orbiculare*, utilized only myristoleic and palmitoleic acids (90).

It is apparent from this brief outline that fungi have diverse fatty acid requirements to sustain growth, reflecting their adaptation to a variety of saprobic and parasitic habits. Elucidation of the precise role of specific fatty acids will probably reflect some of the enzymic activities outlined above, and requires research directed towards specific morphogenetic controls before more than the simplest of generalizations can be made.

### Sporulation

Changes in distribution and abundance of lipids have been documented in fungi undergoing asexual or sexual reproduction (91-93); however, rarely have specific structure-function relationships been established. Recently an exogenous requirement for specific fatty acids has been documented for the induction and maturation of the sexual oospore stage of *Lagenidium giganteum*, a facultative parasite of mosquito larvae (94,95). Free fatty acids and triacylglycerols containing saturated fatty acids from 14C to 20C in length induce limited numbers of viable oospores in liquid culture. Palmitoleic acid added to basal growth media containing appropriate sterols induced significant oosporogenesis, while oleic acid was less effective and linoleic and linolenic acids were toxic at relatively low concentrations; however, oleic and linoleic acids added as mono-, di- or triacylglycerols produced the maximum number of oospores. Trilinolenin, arachidonic and 11,14,17-eicosatrienoic acids induced no or few oospores (95). Analysis of whole cell fatty acids confirmed that all exogenous fatty acids were taken up from the media, although saturated fatty acids were incorporated more slowly. Metabolism of the

exogenous fatty acids occurred but to a limited extent. Delayed addition of triolein to developmentally synchronized cultures resulted in a gradual loss of oospore induction and maturation, which varied according to the concentration of added cholesterol (95).

Exogenous fatty acids are incorporated by *L. giganteum* into all major classes of lipids including phospholipids (unpubl. observ.). Membrane-mediated metabolism may partly explain the positive effect of certain unsaturated fatty acids on oosporogenesis, which would increase bilayer fluidity since the major fatty acid synthesized by *L. giganteum* on unsupplemented media is palmitic acid (95). A regulatory role in oospore induction has been documented for cyclic nucleotides (96) and bilayer fluidity is known to modify the activity of adenylate cyclase (30). *Neurospora crassa* mutants characterized by an aberrant colony morphology and a corresponding cyclic AMP deficiency reverted to normal cyclic nucleotide levels and colony morphology when linoleic,  $\alpha$ -linolenic or arachidonic acid was added to basal growth media (97). Palmitoleic, oleic or 16C to 20C saturated compounds had no effect. It is possible that a comparable process is operating in the induction of *L. giganteum* oosporogenesis. It is interesting to note that the Myxomycete *Dictyostelium discoideum*, whose morphogenesis is also regulated by cyclic AMP, dramatically increases its cellular unsaturated fatty acids, especially octadeca-5,11-dienoic acid, during its development from yeast to mature sporocarp (98). Arachidonic acid and its methyl, ethyl and propyl esters affect the movements of the multicellular migratory slug stage of this organism at micromolar concentrations (99). Methyl esters of oleic, linoleic and linolenic acids also disoriented slug phototaxis at 100-200  $\mu$ M concentrations. An unspecified prostaglandin at 10  $\mu$ M and 0.1  $\mu$ M leukotriene B<sub>4</sub> had no effect.

Two other isolates of *L. giganteum* (ATCC nos. 48336 and 48337) do not produce oospores *in vitro* using the protocol briefly described above for the California strain (ATCC no. 52675) of the fungus. The two former isolates also rarely produce oospores following infection of laboratory-reared *Culex tarsalis* mosquito larvae; however, large numbers of oospores are induced following infection of an alternate larval host, *Chaoborus astictopus*, the Clear Lake gnat. They also produce oospores in media supplemented with cod liver oil (unpubl. observ.). Preliminary investigation of this phenomenon has implicated 5,8,11,14,17-eicosapentaenoic acid (EPA) as the specific compound responsible for oospore induction. EPA is present in low quantities in laboratory-reared *C. tarsalis* larvae, which are incapable of synthesizing this fatty acid and must obtain it from their diet (R. Dadd, unpubl. observ.); however, it is present in relatively high concentrations in cod liver oil and fourth instar diapausing *C. astictopus* larvae (unpubl. observ.). This phenomenon is discussed in more detail below in relation to lipxygenase mediation of fungal morphogenesis.

### Spore Germination

Regulation of spore germination by specific fatty acids has been documented for several fungi. Macroconidial germination by *Microsporum gypseum* is stimulated by oleic, linoleic and arachidonic acids, while short chain saturated compounds were inhibitory and longer chain fatty acids had no effect (100). An even more specific

response has been demonstrated for asexual conidia produced by Erynia variabilis, a parasite of small dipteran insects (101). Conidia (asexual spores) are forcibly discharged by the fungus and subsequently develop in one of four ways: direct vegetative germination which leads to infection of a potential insect host; formation and discharge of a smaller secondary conidium with the same developmental possibilities as primary conidia; mycelial growth dependent solely on endogenous reserves; or death. In the presence of a basal agar medium containing yeast extract, chitin or chitosan, vegetative conidial germination is induced by oleic acid. Oleic acid on water agar alone resulted in secondary sporulation or death of discharged conidia. Palmitoleic, linoleic and linolenic acids were toxic to conidia over a wide range of concentrations, and an excess of oleic acid could mitigate these toxic effects. Palmitoleic acid was unique among the fatty acids which killed conidia in its promotion of mycelial growth subsequent to spore germination. Saturated fatty acids from 14C to 20C induced primarily secondary sporulation (101).

In a related study it was shown that E. variabilis conidia germinate vegetatively on the cuticular surface of adults of the lesser housefly, Fannia canicularis, and on basal media containing yeast extract plus lipid extracts of adult fly cuticles. In contrast, conidia developed primarily into secondary spores when discharged onto puparia of F. canicularis or basal media supplemented with puparial cuticular lipids. Although the relative free fatty acid compositions of the two different stages of housefly were nearly identical, adult cuticles contained nearly five times as much free fatty acid as puparial cuticular surfaces. It was concluded that the limitation of host range of E. variabilis to adult dipterans was due in part to characteristics of the fatty acids of this order of insects, i.e. sufficient oleic acid to induce spore germination; high levels of palmitoleic acid to enhance mycelial growth; and relatively low levels of inhibitory polyunsaturated 18C acids (102).

Subsequent research has shown that of several strains of a related entomophthoralean fungus, Erynia radicans, isolated from a diverse group of insects, only certain strains respond to oleic acid by vegetative conidial germination, and this can be related to host range (A. Uziel, R. Kenneth and I. Ben-Ze'ev, pers. communication). It appears, therefore, that cuticular fatty acid composition may have a definitive role in regulating host range by a number of entomopathogenic fungi.

#### Host-pathogen interactions

In addition to the entomophthoralean fungi discussed above, other laboratories have documented a role for fatty acids in the regulation of host-parasite interactions (103). Research on a number of physiological races of Phytophthora infestans, some of which infect cultivars of potato, Solanum tuberosum, has shown that both virulent and avirulent strains of the fungus contain chemical elicitors of hypersensitive resistance, including induction of sesquiterpenoid phytoalexin accumulation (104). After a number of inconclusive reports, the elicitors were identified as arachidonic (AA) and eicosapentaenoic (EPA) acids (105-107). Methyl esters of AA and EPA also elicited sesquiterpene accumulation (106) after a slight delay, while the propyl ester was much less active (103). All saturated fatty

acids, 18C compounds and arachidonyl cyanide failed to induce hypersensitive responses (103,108). Among the eicosatrienoic compounds tested, the  $\Delta 11,14$  and  $\Delta 11,14,17$  isomers were weakly active while the  $\Delta 5,8,11$  and  $\Delta 5,11,14$  compounds were highly active (103,108). The  $\Delta 5$  bond appears to be of importance for activity, perhaps due to enzymic substrate specificity, leading to a more metabolically active compound. Such enzymic modification could be due to lipoxygenase or cyclooxygenase activity, which is discussed in the final section of this review.

#### Regulation of fungal morphogenesis by lipoxygenase and cyclooxygenase products

Using the *P. infestans*-potato tuber system outlined above, elicitor-induced accumulation of phytoalexins has been inhibited by salicylhydroxamic acid and disulfiram (103,109,110); these compounds inhibit both cyanide-resistant respiration and lipoxygenase enzymes, these latter acting specifically on polyunsaturated fatty acids with a *cis*-1,4-pentadiene system to form hydroperoxides (111). It has been speculated without further data that lipoxygenase activity may be involved in eliciting the hypersensitive response in potato tubers (103).

Direct implication of cyclooxygenase enzymes in fungal morphogenesis has been obtained using the Oomycetes *Achlya caroliniana*, *Achlya ambisexualis* and *Saprolegnia parasitica*. Aspirin and indomethacin, both cyclooxygenase inhibitors, reduced or eliminated mycelial growth by these fungi, often inducing abnormal colony morphology in liquid culture (112). Inhibited colonies in the presence of 0.1 mM indomethacin, when allowed to grow for more than 10 days, assume normal colony morphology, but do not undergo sexual reproduction (oosporogenesis). Addition of prostaglandin  $F_{1\alpha}$  (PGF $_{1\alpha}$ ) at 2  $\mu$ g/ml partly overcame indomethacin induced growth inhibition, while PGF $_{2\alpha}$  and PGE had no effect. The authors suggested growth and oosporogenesis may be regulated by the interaction of a prostaglandin or PG-like substance produced via cyclooxygenase fatty acid metabolism with steroid or other hormones (112).

Using a developmentally synchronized *L. giganteum* culture system, our laboratory has documented that stage-specific addition of lipoxygenase inhibitors (nordihydroguaiaretic acid, esculetin,  $\alpha$ -naphthol, propyl gallate, salicylhydroxamic acid), at concentrations which do not affect asexual reproduction, blocks the induction and maturation (gametangial fusion, meiosis, spore cell wall formation, vesicular fusion within the immature spore) of oospores by this fungus. Cyclooxygenase inhibition using ibuprofen and to a lesser extent indomethacin had comparable specific effects on oosporogenesis. The cyclooxygenase inhibitors aspirin, salicylic acid and phenylbutazone, however, had minimal effect on *L. giganteum* oosporogenesis at concentrations less than those which had a deleterious effect on mycelium (unpubl. observ.). This does not preclude a role for cyclooxygenase metabolism in oosporogenesis by this fungus since the *L. giganteum* enzymes may be relatively insensitive to inhibition by these compounds. Also, the effects of these inhibitors on *L. giganteum* oxygenases, which may be different from those documented in mammalian systems, have not been investigated.

Tentative confirmation by thin layer chromatography (114) has



been made for the production of both cyclooxygenase and lipoxygenase products by L. giganteum, with more of both types of fatty acid metabolites isolated from the liquid culture medium than from the mycelium (unpubl. observ.). This implicates these compounds in some type of intercellular communication role. An intracellular regulatory role is also suggested for the lipoxygenase metabolites by the inhibitory effects on oospore maturation. The inhibitory effects of nordihydroguaiaretic acid on oospore induction could be reversed using partly purified eicosanoid extracts from growth media (113). Further elucidation of the nature and stage-specific synthesis of these products is in progress.

Arachidonic acid, which is synthesized by L. giganteum (95), and eicosapentaenoic acid, which is not, are suitable substrates for cyclooxygenase and lipoxygenase. Since two strains of this facultative parasite appear to initiate oosporogenesis only in the presence of exogenous EPA (unpubl. observ.), this compound is highly suspect as a potential regulatory molecule, perhaps after its lipoxygenase-mediated metabolism to a more active product. It is noteworthy that EPA is present, often as the principal fatty acid, in a number of Oomycetes (103, 107, 112, 114). Further evaluation of the role of EPA in sexual reproduction by this primitive class of fungi is warranted. Leukotrienes are a recently discovered class of lipoxygenase metabolites with highly potent biological effects (116-118). It is possible that these or related metabolites play a central regulatory role in fungal morphogenesis.

Lipoxygenase regulation of morphogenesis may not be limited to the oomycetous fungi described above. As an extension of a previous hypothesis regarding possible fatty acid hydroperoxide involvement in conidial germination by the Zygomycete Erynia variabilis (101), a strain of Erynia delphacis, a related parasite of small adult dipteran insects, was investigated. Conidia discharged by E. delphacis, which germinate vegetatively on yeast extract supplemented with triolein, undergo nearly 100% secondary sporulation when 150  $\mu$ M nordihydroguaiaretic acid is added to the germination medium (unpubl. observ.)

These initial observations suggest that lipoxygenase-mediated morphogenesis may be widespread among fungi. Research on fungal eicosanoids will provide insights into their basic developmental biology and serve as useful models for the role of these metabolites in mammalian systems.

#### Acknowledgments

Writing of this review was supported in part by USDA research grant CR 806771-02, RF-4148A, R. K. Washino, Principal Investigator. I thank Dr. R. Herman for the preprint of his cyclooxygenase article.

#### Literature Cited

1. Madelin, M. F., Ed. "The Fungus Spore"; Colston Papers: Butterworths, London, 1966; Vol. 18, 338 pp.
2. Hess, W. M.; Weber, D. J. In "Fungal Lipid Biochemistry"; Weete, J. D., Ed.; Plenum: New York, 1974; p. 358.
3. Reilsener, H. J. In "The Fungal Spore: Form and Function"; Weber, D. J.; Hess, W. M., Eds.; Wiley: New York, 1976; p. 165.

4. Brennan, P. J.; Lösel, D. M. Adv. Microbial. Physiol. 1978, 17, 48.
5. Weete, J. D. "Lipid Biochemistry of Fungi and Other Organisms"; Plenum: New York, 1980; 388 pp.
6. Weete, J. D. In "The Fungal Spore: Morphogenetic Controls"; Turian, G.; Hohl, H. R., Eds.; Academic: New York, 1981; p. 463.
7. Shaw, R. Adv. Lipid Research 1966, 4, 107.
8. Wassef, M. K. Adv. Lipid Research 1977, 15, 159.
9. Gunstone, F. D. "Comprehensive Organic Chemistry"; Haslam, E., Ed.; Pergamon: New York, 1979; Vol. 5, p. 587.
10. Hilditch, T. P.; Williams, D. N. "The Chemical Constitution of Natural Fats"; Chapman and Hall: London, 1964; 4th ed., 745 pp.
11. Gunstone, F. D. "An Introduction to the Chemistry and Biochemistry of Fatty Acids and their Glycerides"; Chapman and Hall: London, 1967.
12. Gunstone, F. D. Prog. Chem. Fats other Lipids 1979, 15, 75.
13. Pryde, E. H. "Fatty Acids"; American Oil Chemical Society, Champaign, Ill, 1979.
14. Badami, R. C.; Patil, K. B. Prog. Lipid Res. 1981, 19, 119.
15. Klausner, R. D.; Kleinfeld, A. M.; Hoover, R. L.; Karnovsky, M. J. J. Biol. Chem. 1980, 255, 1286.
16. Karnovsky, M. J.; Kleinfeld, A. M.; Hoover, R. L.; Klausner, R. D. J. Cell. Biol. 1982, 94, 1.
17. Martin, Y. C. "Quantitative Drug Design"; Marcel Dekker: New York, 1978.
18. Stuper, A. J.; Brugger, E.; Jurs, P. C. "Computer Assisted Studies of Chemical Structure and Function"; John Wiley: New York, 1979.
19. Tardieu, A.; Luzzati, V.; Reman, F. C. J. Mol. Biol. 1973, 75, 711.
20. Hauser, H.; Pascher, I.; Pearson, R. H.; Sundell, S. Biochim. Biophys. Acta 1981, 650, 21.
21. Kumau, W.-H. Angew. Chem. 1976, 15, 61.
22. Gunstone, F. D.; Ismail, I. A. Chem. Phys. Lipids 1967, 1, 264.
23. Christie, W. W.; Holman, R. T. Chem. Phys. Lipids 1967, 1, 407.
24. Singer, S. J. Annu. Rev. Biochem. 1974, 43, 805.
25. DeLisi, C.; Blumenthal, R., Eds. "Physical Chemical Aspects of Cell Surface Events in Cellular Regulation"; Elsevier: North Holland, New York, 1979.
26. Aloia, R. C., Ed. "Membrane Fluidity in Biology"; Academic: New York, 1983; 2 vol.
27. Chapman, D. Q. Rev. Biophys. 1975, 8, 185.
28. Melchior, D. L.; Steim, J. M. Annu. Rev. Biochem. Bioeng. 1976, 5, 205.
29. McElhaney, R. N. Chem. Phys. Lipids 1982, 30, 229.
30. Stubbs, C. D.; Smith, A. D. Biochim. Biophys. Acta 1984, 779, 89.
31. Dufourc, E. J.; Smith, I. C. P.; Jarrell, H. C. Biochem. 1984, 23, 2300.
32. Lee, A. G. Biochim. Biophys. Acta 1978, 507, 433.
33. Linden, C. D.; Wright, K. L.; McConnell, H. M.; Fox, C. F. Proc. Natl. Acad. Sci. U.S.A. 1973, 70, 2271.
34. Hoover, L.; Fujiwara, K.; Klausner, R. D.; Bhalla, D. K.; Tucker, R.; Karnovsky, M. J. Mol. Cell. Biol. 1981, 1, 939.
35. Karnovsky, M. J.; Kleinfeld, A. M.; Hoover, R. L.; Klausner, R. D. J. Cell. Biol. 1982, 94, 1.

36. Kohlwein, S. D.; Paltauf, F. Biochim. Biophys. Acta 1984, 792, 310.
37. Jager, S.; Borst-Pauwels, G. W. F. H. Acta Bot. Neer. 1970, 19, 147.
38. Iwashima, A.; Nose, Y. Biochim. Biophys. Acta 1975, 399, 375.
39. Keenan, M. A. J.; Rose, A. H. FEMS Microbiol. Lett. 1979, 6, 133.
40. Morton, H.; Watson, K.; Streamer, M. FEMS Microbiol. Lett. 1978, 4, 291.
41. Watson, K.; Morton, H.; Arthur, H.; Streamer, M. Biochem. Soc. Trans. 1978, 6, 380.
42. Proudlock, J. W.; Haslam, J. M.; Linnane, A. W. Biochem. Biophys. Res. Commun. 1969, 37, 847.
43. Marzuki, S.; Hall, R. M.; Linnane, A. W. Biochem. Biophys. Res. Commun. 1974, 57, 372.
44. Haslam, J. M.; Fellows, N. F. Biochem. J. 1977, 166, 565.
45. Watson, K.; Houghton, R. L.; Bertoli, E.; Griffiths, D. E. Biochem. J. 1975, 146, 409.
46. Marzuki, S.; Cobon, G. S.; Haslam, J. M.; Linnane, A. W. Arch. Biochem. Biophys. 1975, 169, 577.
47. Marzuki, S.; Cobon, G. S.; Crowfoot, P. D.; Linnane, A. W. Arch. Biochem. Biophys. 1975, 169, 591.
48. Luzikov, V. N.; Novikova, L. A.; Tikhonov, A. N.; Zubatov, A. S. Biochem. J. 1983, 214, 785.
49. Griffiths, D. E.; Hyams, R. L.; Bertoli, E. FEBS Lett. 1977, 74, 38.
50. Gurba, P. E. Thesis, Univ. Michigan, Ann Arbor, 1979.
51. Walenga, R. W.; Lands, W. E. M. J. Biol. Chem. 1975, 250, 9130.
52. Boll, M.; Loewel, M.; Berndt, J. Biochim. Biophys. Acta 1981, 677, 200.
53. Boll, M.; Loewel, M.; Berndt, J. Biochim. Biophys. Acta 1980, 620, 429.
54. Boll, M.; Loewel, M.; Berndt, J. Hoppe-Seyler's Z. Physiol. Chem. 1976, 357, 352.
55. Aaronson, S. Proc. Soc. Exp. Biol. Med. 1971, 136, 61.
56. Yoshioka, K.; Hashimoto, N. Agric. Biol. Chem. 1983, 47, 2287.
57. Morikawa, M.; Yamashita, S. Eur. J. Biochem. 1978, 84, 61.
58. Meyer, K. H.; Schweizer, E. Eur. J. Biochem. 1976, 65, 317.
59. Kamiryo, T.; Parthasarathy, S.; Numa, S. Proc. Natl. Acad. Sci. U.S.A. 1976, 73, 386.
60. Hunter, K.; Rose, A. H. Biochim. Biophys. Acta 1972, 260, 639.
61. Cronan, J. E. Jr.; Vagelos, P. R. Biochim. Biophys. Acta 1972, 265, 25.
62. Nozawa, Y.; Kasai, R. Biochim. Biophys. Acta 1978, 529, 54.
63. Martin, C. E.; Siegel, D.; Aaronson, L. R. Biochim. Biophys. Acta 1981, 665, 399.
64. Martin, C. E.; Johnston, A. M. Biochim. Biophys. Acta 1983, 730, 10.
65. Kates, M.; Kuksis, A., Eds. "Membrane Fluidity - Biophysical Techniques and Cellular Regulation"; Humana Inc.: Clifton, New Jersey, 1980.
66. DeLisi, C.; Blumenthal, R., Eds. "Physical Chemical Aspects of Cell Surface Events in Cellular Regulation"; Elsevier: North Holland, New York, 1979.

67. Mabrey, S.; Sturtevant, J. M. In "Methods in Membrane Biology"; Korn, E. D., Ed.; Plenum Press, New York, 1978; Vol. 9, p. 237.
68. Arthur, H.; Watson, K. J. Bacteriol. 1976, 128, 56.
69. Chavant, L.; Sancholle, M.; Montant, C. Phytochem. 1979, 18, 1471.
70. Kuyama, H.; Saito, M.; Joshi, V. C.; Gunsberg, S.; Wakil, S. J. J. Biol. Chem. 1979, 254, 12281.
71. Kerekes, R.; Nagy, G. Acta Aliment. 1980, 9, 93.
72. Watson, K.; Arthur, H.; Shipton, W. A. J. Gen. Microbiol. 1976, 97, 11.
73. Manocha, M. S.; Campbell, C. D. Can. J. Microbiol. 1978, 24, 670.
74. Sumner, J. L.; Morgan, E. D. J. Gen. Microbiol. 1969, 59, 215.
75. Meyer, F.; Bloch, K. Biochim. Biophys. Acta 1963, 77, 671.
76. Harris, P.; James, A. T. Biochem. J. 1969, 112, 325.
77. Parmegiani, R. M.; Pisano, M. A. Dev. Ind. Microbiol. 1974, 15, 318.
78. Chavant, L.; Wolf, C.; Fonvieille, J. L.; Dargent, R. Biochem. Biophys. Res. Commun. 1981, 101, 912.
79. Nes, W. D.; Adler, J. A.; Nes, W. R. Exp. Mycol. 1984, 8, 55.
80. Wisniewski, B. J.; Keith, A. D.; Resnick, M. R. J. Bacteriol. 1970, 101, 160.
81. Barber, E. D.; Lands, W. E. M. J. Bacteriol. 1973, 115, 543.
82. Ohlrogge, J. B.; Barber, E. D.; Lands, W. E.; Gunstone, F. D.; Ismail, I. A. Can. J. Biochem. 1976, 54, 736.
83. Buttke, T. M.; Jones, S. D.; Bloch, K. J. Bacteriol. 1980, 144, 124.
84. Adler, J. H.; Gealt, M. A.; Ness, W. D.; Nes, W. R. J. Gen. Microbiol. 1981, 122, 101.
85. Otoguru, K.; Awaya, J.; Tanaka, H.; Omura, S. J. Biochem. (Japan) 1981, 89, 523.
86. Esfahani, M.; Kucirka, E. M.; Timmons, F. X.; Tyagi, S.; Lord, A. E., Jr.; Henry, S. A. J. Supramol. Struct. Cell. Biochem. 1981, 15, 119.
87. Bahnweg, G. Bot. Mar. 1980, 23, 209.
88. Oso, B. A. Z. Allg. Mikrobiol. 1974, 14, 713.
89. Wilde, P. F.; Stewart, P. S. Biochem. J. 1968, 108, 225.
90. Meinhof, W.; Braun-Falco, O. Fette, Seifen, Anstrichm 1967, 69, 861.
91. Reisener, H. J. "The Fungal Spore"; Proc. 2nd Int. Fungal Spore Symp.; Weber, D. J.; Hess, W. M., Eds.; John Wiley: New York, 1976; p. 165.
92. Beilby, J. P.; Kidby, D. K. J. Lipid Res. 1980, 21, 739.
93. Weber, D. J.; Trione, E. J. Can. J. Bot. 1980, 58, 2263.
94. Kerwin, J. L.; Washino, R. K. Exp. Mycol. 1983, 7, 109.
95. Kerwin, J. L.; Washino, R. K. Submitted for publication, 1986.
96. Kerwin, J. L.; Washino, R. K. Exp. Mycol. 1984, 8, 215.
97. Scott, W. A. Proc. Nat. Acad. Sci. U.S.A. 1976, 73, 2995.
98. Long, B. H.; Coe, E. L. Lipids 1977, 12, 414.
99. Dohrmann, U.; Fisher, P. R.; Bröderlein, M.; Lackner, B.; Williams, K. L. J. Gen. Microbiol. 1984, 130, 2685.
100. Barash, I.; Conway, M. Loretto; Howard, D. H. J. Bacteriol. 1967, 93, 656.
101. Kerwin, J. L. J. Gen. Microbiol. 1982, 128, 2179.
102. Kerwin, J. L. Can. J. Microbiol. 1984, 30, 158.

103. Kúc, J.; Preisig, C. Mycologia 1984, 76, 767.
104. Varns, J. L.; Currier, W. W.; Kúc, J. Phytopathology 1971, 61, 968.
105. Bloch, C. B.; Kúc, J. Phytopathology 1983, 73, 828.
106. Bostock, R. M.; Kúc, J. A.; Laine, R. A. Science 1981, 212, 67.
107. Bostock, R. M.; Laine, R. A.; Kúc, J. A. Pl. Physiol. (Lancaster) 1982, 70, 1417.
108. Preisig, C. L.; Kúc, J. Phytopathology 1983, 73, 831.
109. Theologis, A. J.; Laties, G. G. Pl. Physiol. (Lancaster) 1978, 62, 232.
110. Stelzig, D. A.; Allen, R. O.; Bhatia, K. Pl. Physiol. (Lancaster) 1983, 72, 746.
111. Vliegenhart, J. F. G.; Veldink, G. A.; Verhagen, J.; Slappendel, S.; Vernooy-Gerritsen, J. In "Biochemistry and Metabolism of Plant Lipids"; Wintermans, J. F. G. M.; Kuiper, P. J. C., Eds.; Elsevier: Amsterdam, 1982; p. 265.
112. Herman, R. P.; Herman, C. A. Prostaglandins 1985, 29, 819.
113. Kerwin, J. L.; Simmons, C. A.; Washino, R. K. Prostaglandins Leukotrienes and Med. (in press).
114. Salmon, J. A.; Flower, R. J. Meth. Enz. 1982, 86, 477.
115. Gellerman, J. L.; Schlenk, H. Biochim. Biophys. Acta 1979, 573, 23.
116. Chakrin, L. W.; Bailey, D. M., Eds.; "The Leukotrienes - Chemistry and Biology"; Academic: New York, 1984.
117. Piper, P. J., Ed.; "Leukotrienes and other Lipoxygenase Products"; Research Studies Press (John Wiley): New York, 1983.
118. Piper, P. J. Physiol. Rev. 1984, 64, 744.

RECEIVED May 1, 1986

## Chapter 21

# The Involvement of Membrane-Degrading Enzymes During Infection of Potato Leaves by *Phytophthora infestans*

Robert A. Moreau

Eastern Regional Research Center, Agricultural Research Service, U.S. Department of  
Agriculture, Philadelphia, PA 19118

During the infection of potato leaves by *Phytophthora infestans* there is a rapid degradation of membrane components (chlorophyll, galactolipids, and phospholipids). We have identified an inducible phospholipase B activity which is secreted by the fungus in culture and also occurs during the infection of potato leaves. Under conditions which result in maximal induction of phospholipase activity in culture filtrates, comparable levels of galactolipase and triacylglycerol lipase are also observed. During the infection of potato leaves there was a large (>14-fold) increase in total phospholipase activity. Since potato leaves also contain very high levels of phospholipase and galactolipase activities, experiments were conducted to elucidate the possible involvement of the lipolytic enzymes of the pathogen and the host during infection and resistance.

*Phytophthora infestans* is the fungal pathogen that causes late blight of potatoes. The Irish "potato famine" of the 1840's was caused by *P. infestans*. Even today, late blight is still the single most important disease of potatoes worldwide (1). With modern farming techniques approximately 10% of the world potato crop and 4% of the U.S. potato crop are lost in the field to this disease each year (2,3). In addition to causing these large losses in the field, late blight and the secondary bacterial infections which often accompany it cause comparable losses during the storage of tubers.

Despite many years of intensive breeding research, no existing cultivars of European or North American potatoes allow commercial cultivation in humid regions without fungicide protection (1). At best, farmers can choose cultivars with a moderate level of general resistance (i.e., Sebago) which are protected by fewer applications

This chapter not subject to U.S. copyright.  
Published 1987, American Chemical Society

of fungicide than are required by other cultivars. Our laboratory is interested in studying disease resistance mechanisms at the subcellular level, especially at the membrane level. For our preliminary studies we have chosen to study the changes in membrane lipid composition that occur during a susceptible host-pathogen interaction. We have attempted to correlate changes in lipid composition with changes in the levels of lipolytic enzymes of the host and pathogen separately and during infection. This report will summarize our recent studies (4-7) of the *P. infestans*-potato leaf interaction and compare them with comparable studies of other host-pathogen interactions.

#### Changes in Host Ultrastructure and Lipid Composition during Infection

In the field, infection of potato plants by *P. infestans* is usually localized in the leaves. Encysted zoospores germinate on the leaf surface, form an appressorium, and penetrate the cuticular layers of the leaf. The recent work of Wilson and Coffey (8) indicates that direct penetration of epidermal cells adjacent to stomatal cells is the most common mode of entry. Hyphae spread both intra- and intercellularly forming haustoria when host cells are penetrated (9,10). In susceptible interactions, host cells contain "organelles the structure of which is disorganized" (10) or "very disintegrated organelles" (9). In contrast, fungal organelles remain intact long after the surrounding host tissue has been disrupted (10). In a resistant interaction the fungus actively infects the leaves for the first 9-12 h but is then subsequently killed by 24 h (9).

Unfortunately, no histochemical or cytochemical studies of the involvement of lipolytic enzymes during the infection of potato leaves by *P. infestans* have yet been reported. Histochemical and biochemical observations of potato tubers infected by *P. infestans* revealed elevated levels of esterase (measured with  $\alpha$ -naphthol acetate) in unidentified host organelles during infection (11). Cytochemical techniques were used to identify lipolytic activity in fungal and host cell walls during the infection of lettuce (*Lactuca sativa* L.) cotyledons by *Bremia lactucae* (12).

Very little is known about the changes that occur in the composition of membrane lipids during the infection of plants by fungal pathogens. Three such studies have been reported (13-15) and deal with the infection of susceptible leaves by rust species. In each case there was a rapid disappearance of chloroplast glycolipids and phosphatidyl glycerol, and evidence for the presence of fungal phospholipids during latter stages of infection. During the infection of bean plants (*Phaseolus vulgaris*) by *Uromyces phaseoli*, there was an increase in the proportion of unsaturated fatty acids and this change was attributed to fungal growth (16).

In our preliminary studies of the infection of susceptible potato leaves by *P. infestans* we also observed a similar rapid degradation of glycolipids and phosphatidyl glycerol. However, upon further investigation we realized that the unique composition of membrane lipids in *P. infestans* may provide a useful marker during infection. The polar lipid composition of healthy potato leaves and cultured *P. infestans* is shown in Table I. The lipid

Table I. Polar Lipid Composition of Potato Leaves and *P. infestans*. Fungal cultures were grown in liquid French bean media (4) for 14 days at 14° without shaking. Lipids were extracted (42) separated by 2-dimensional TLC (18). Individual spots were identified by comparison with standards, scraped from TLC plates, and analyzed for phosphorous (6) and hexose (43).

Lipid Class <sup>a</sup>	mole %	
	Healthy Potato Leaf	<i>P. infestans</i>
MGDG	53	0
DGDG	20	0
SQD	3	0
PC	14	46
PG	3	0
PE	6	39
PI	1	2
CAEP	0	13

<sup>a</sup> Abbreviations: MGDG = monogalactosyldiglyceride, DGDG = digalactosyldiglyceride, SQD = sulfoquinovasyldiglyceride, PC = phosphatidylcholine, PG = phosphatidylglycerol, PE = phosphatidylethanolamine, PI = phosphatidylinositol, CAEP = ceramide aminoethylphosphonate.

composition of potato leaves is comparable to that of the leaves of other species of angiosperms (17). *P. infestans* contains high levels of phosphatidylcholine and phosphatidylethanolamine, as is common for fungi (13), but it also contains an unusual sphingolipid, ceramide aminoethylphosphonate (CAEP) (Figure 1). This sphingolipid was previously identified in two closely related fungi *Pythium prolatum* and *Phytophthora parasitica* var. *nicotianae* (18). We have recently observed that after 6 days of infection CAEP is detectable in thin layer chromatograms of lipids from infected leaves. Since the fungal ceramide lacks carboxyl ester bonds it is resistant to hydrolysis by phospholipase B and its presence may render certain fungal membranes less vulnerable to degradation during infection. *P. infestans* also contains high levels of two unusual fatty acids, arachidonic acid (20:4) and eicosapentaenoic acid (20:5) (19) which are absent in the potato plant and could also be used as markers of fungal lipids during infection. We are currently studying the quantitative changes in these membrane lipid components during infection.

#### Properties of Phospholipases from Phytopathogens

Phospholipases are enzymes that catalyze the hydrolysis of membrane phospholipids. There are six types of phospholipases (A<sub>1</sub>, A<sub>2</sub>, B, C, D, and lysophospholipase). Each hydrolyses a different part of the phospholipid molecule and results in the formation of different



products (Figure 2). Although some assay techniques are capable of identifying the type of phospholipase activity, several of the common techniques only measure total breakdown of phospholipid. Phospholipase activity has been reported to occur in eleven phytopathogens (ten fungi and one bacteria) (4,16,20-27) (Table II). In six of these species, phospholipase B (which hydrolyzes two fatty acids per phospholipid molecule) was detected. Only three of these studies (20,21,22) have reported the effect of fungal phospholipases on plant tissue. In Table II, it is noted whether these enzyme activities were detected in fungal cultures (intracellular or extracellular), or in infected plant tissue. The pH optimum for each enzyme is also listed.

Table II. Occurrence of Phospholipase Activity in Phytopathogens

Species	Type of Phospholipase	Optimum pH	Localization <sup>a</sup> of Enzyme	Ref.
<u>Botrytis cinerea</u>	B	5.0	M	21
<u>Erwinia carotovora</u>	C	Assayed only at 8.0	E	22
<u>Erysiphe pisi</u>	A <sub>2</sub>	Assayed only at 8.9	I	20
<u>Fusarium solani</u>	?	4.0	E	23
<u>Phoma medicaginis</u>	B	?	M	24
<u>Phytophthora infestans</u>	B	9.0	E,I,P	4
<u>Rhizoctonia solani</u>	?	7.5 - 8.5	E	23
<u>Sclerotia sclerotiorum</u>	B	4.0	E,P	25
<u>Sclerotium rolfsii</u>	B	4.5	E,P	26
<u>Thielaviopsis basicola</u>	B	4.5 and 8.5	M,P	27
<u>Uromyces phaseoli</u>	A or B or C	4.0 - 5.0	P	16

<sup>a</sup> Abbreviations: E = from extracellular fungal culture; I = from intracellular fungal culture; M = from mixture of intracellular and extracellular; P = from infected plant tissue.

The properties of the phospholipase activities from cultures of P. infestans (4) are summarized in Table III. Of the five types of liquid media tested, the greatest phospholipase activity was obtained when P. infestans was grown in rye steep media. The phospholipase activity in rye culture filtrates was stimulated 15-fold by omitting glucose from the medium. The addition of 100 mg/liter of phosphatidylcholine (PC) to the rye medium caused an additional 35-fold stimulation in the activity of phospholipase. These experiments suggest that this enzyme activity is inducible. The addition of other lipids (sunflower oil, wax ester, and cholesterol oleate) to rye media also caused an apparent induction of phospholipase activity. We also detected extracellular phospholipase activity in lima bean agar cultures of P. infestans as shown in the second column of Table III. When the fungus was collected

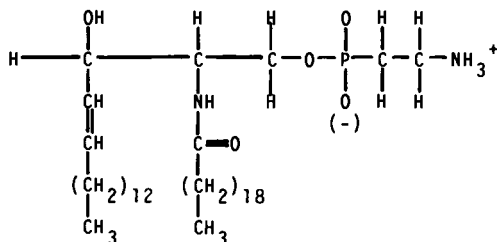


Figure 1. Ceramide aminoethylphosphonate (CAEP). When obtained from *P. infestans* the most common fatty acid is arachidonic acid and the most common long chain base is sphingosine.

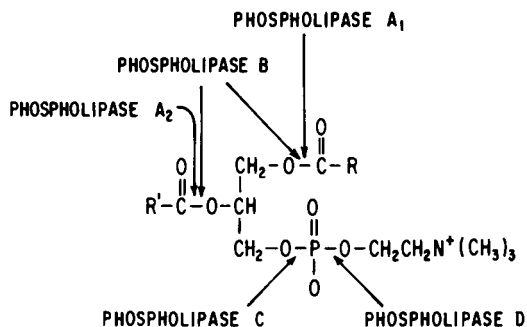


Figure 2. Sites of action of various types of phospholipases on phosphatidylcholine. Lysophospholipase hydrolyzes the carboxyl ester bond of a lysophospholipid (i.e., lysophosphatidylcholine).

**American Chemical Society**  
**Library**  
**1155 16th St., N.W.**  
**Washington, D.C. 20036**

Table III. A Summary of the Properties of the Phospholipase Activity from Cultures of *P. infestans* (4,7)

	Liquid Culture	Agar Culture
Media	Rye steep	Lima bean
Regulation	Repressed by glucose induced by PC	Not tested
Localization	Mostly extracellular	Extracellular
pH optimum	9.0	9.0
Effect of 5 mM DTT	96% Inhibition	94% Inhibition
Effect of 5 mM EDTA	None	55% Inhibition
Effect of 5 mM CaCl <sub>2</sub>	5% Inhibition	17% Inhibition
Km for PC	2.86 μM	Not tested
Substrate specificity	TG ~ PC ~ MGDG	PC > TG

<sup>a</sup> Abbreviations: PC = phosphatidylcholine, TG = triacylglycerol, MGDG = monogalactosyldiglyceride, DTT = dithiothreitol, EDTA = ethylenediaminetetraacetic acid.

from liquid rye cultures (with added PC to induce phospholipase activity) much more phospholipase activity (30-fold) was found extracellularly than intracellularly. The pH optimum of the enzyme activity from both sources was 9.0. Dithiothreitol severely inhibited both enzyme activities. EDTA (5 mM) had no effect on the enzyme from liquid rye culture, but inhibited the enzyme from agar cultures. Both enzymes were slightly inhibited by 5 mM CaCl<sub>2</sub>. A very low Km (2.86 μM) for phosphatidylcholine was measured using induced rye culture filtrate as a source of enzyme. When lipid substrates other than PC were tested the enzymes in the induced liquid culture were able to hydrolyze triacylglycerol (TG) and galactolipids (GL) at rates very similar to that for PC. In contrast, the enzymes from agar culture hydrolyzed PC at a rate about six times higher than for TG.

We have recently completed a study of the production of extracellular enzymes by germinating cysts of *P. infestans* (7). Although we were able to identify an esterase activity (p-nitrophenyl butyrate hydrolase) that appeared to be secreted during germination (0 to 20 h), phospholipase and lipase activities were apparently not secreted (Table IV). We are currently studying whether this esterase can hydrolyze any physiological substrates. This is an example of a case where the use of a nonphysiological substrate (PNP-butyrate) resulted in new and interesting information. However, extreme care must be exercised when trying to draw physiological conclusions from studies using nonphysiological substrates (i.e., p-nitrophenyl esters or 4-methylumbelliferyl esters). We are currently investigating a new phospholipase assay (28), which employs a fluorescent phospholipid substrate, 1-acyl-2-[6-[(7-nitro-2,1,3 benzoxadiazol-4-yl)amino]-caproyl] phosphatidylcholine(C<sub>6</sub>-NBD-PC). Our preliminary studies (Table IV) indicate

Table IV. Changes in the levels of extracellular enzyme activities during germination (0 to 20 h) of cysts of *P. infestans*. The levels of the first three enzymes were previously reported (7).  $C_6$ -NBD-PC hydrolysis was measured as described (28).

Enzyme	Enzyme Activity n mol/min/10 <sup>6</sup> Spores			
	0 h	5 h	10 h	20 h
p-nitrophenyl butyrate hydrolase	9.0	43.2	315.7	407.3
Lipase	.127	.132	.137	.138
Phospholipase ( <sup>14</sup> C-PC assay)	.590	.599	.604	.610
Phospholipase ( $C_6$ -NBD-PC assay)	.738	.735	.733	.732

that very similar values are obtained when phospholipase activity is measured with authentic phospholipid (<sup>14</sup>C-PC) or the fluorescent phospholipid ( $C_6$ -NBD-PC). If it proves reliable, this new fluorometric assay will be much more convenient (30 assays per hour verses 15 assays per day using <sup>14</sup>C-PC).

#### Properties of the Phospholipase Activity in Healthy Potato Leaves

Many types of plant tissue contain high levels of lipolytic acyl hydrolase (LAH) activity (29). These enzymes are capable of hydrolyzing phospholipids (phospholipase B), galactolipids (galactolipase), and acyl glycerols. The LAH's in potato tubers and bean leaves have been purified and extensively studied (29). In 1979 Matsuda and Hirayama (30) reported that the total activity of phospholipase in potato leaves was about 400-fold lower than in potato tubers. They subsequently purified a lipolytic acyl hydrolase from potato leaves (31). The enzyme had a molecular weight of about 110,000 and a pH optimum of 5.0. The rate of hydrolysis of galactolipids was 7-fold higher than for phospholipids.

The following experiments illustrate that when studying the involvement of phospholipase in the host-pathogen interaction, the total contribution of enzyme of host origin may be considerably higher than previously realized. Rodionov and Zakharova (32) recently reported very high rates of autolytic hydrolysis of membrane lipids in homogenates of potato leaves (26-37% of the phospholipids were hydrolyzed after 2 h at 0-1°). Our laboratory recently confirmed this observation and proceeded to study some of the properties of the lipolytic acyl hydrolase activity in potato leaves (6). Lipolytic acyl hydrolase activity is apparently inactivated by polyphenol oxidase or its toxic quinone products.

When polyphenol oxidase activity was controlled, phospholipase activities ranged from 1.04 to 11.60  $\mu$  mol/min/gfw in the leaves of 41 North American cultivars (6). These values are much higher than those previously reported for potato leaves (.009  $\mu$ mol/min/gfw) (30) and nearly as high as in potato tubers (2 to 30  $\mu$ mol/min/gfw) (5,30).

#### Change in the Levels of Lipolytic Enzymes during Infection

Hoppe and Heitefuss (16) reported a 2-3-fold increase in phospholipase activity during the infection of susceptible bean leaves by *U. phaseoli*. During the infection of potato leaves by *P. infestans* we observed a greater than 14-fold increase in total phospholipase activity (Figure 3) (4). The increase in phospholipase activity was roughly proportional to the amount of leaf surface area that was covered by the fungus. When phospholipase activity was measured with the fluorescent substrate ( $C_6$ -NBD-PC), as previously described (28), it closely paralleled the curve obtained with  $^{14}C$ -PC. However, when esterase activity was measured with another fluorometric substrate, 4-methylumbelliferyl laurate, it was highest at day 0 and decreased during infection. This indicates that 4-methylumbelliferyl laurate appears to be hydrolyzed by a lipolytic enzyme of the host.

To determine whether the increase in phospholipase activity during infection (Figure 3) was due to enzymes of fungal or host origin, the following experiments were performed. We observed that the pH optima of the phospholipase activity in fungal cultures was about 9.0 (Figure 4A,B) (4). Phospholipase activity was present in the uninfected leaves (Figure 4C), but it was much lower and exhibited optimum phospholipase activity at pH 6.0 as reported elsewhere (6). The infected leaf had a large peak of phospholipase activity at pH 8.0 to 9.0 and a smaller peak at pH 6.0. The two peaks of phospholipase activity in the infected leaves were further resolved by assaying in the presence of 5 mM DTT (Figure 4D) which is a potent inhibitor of fungal phospholipase. The peak of phospholipase activity at pH 9.0 was DTT-sensitive and likely to be of fungal origin. The peak of phospholipase activity at pH 6.0 was DTT-insensitive and is probably of host origin although further work is required to prove this beyond doubt.

#### Conclusions and Perspectives

These results indicate that during the infection of potato leaves by *P. infestans*, there is a rapid degradation of the host's membrane lipids (especially galactolipids) and a gradual increase in lipids of fungal origin (ceramide aminoethylphosphonate being the most unique). An analysis of phospholipase activity revealed that a DTT-sensitive phospholipase with an alkaline pH optima accumulated in infected leaves and was probably of fungal origin.

Although these preliminary studies indicate that lipolytic enzymes are produced by the fungus during infection, much more work is required to determine their actual role in infection and pathogenesis. Because the symptomology and biochemistry of infection are very similar to those events that occur during normal leaf

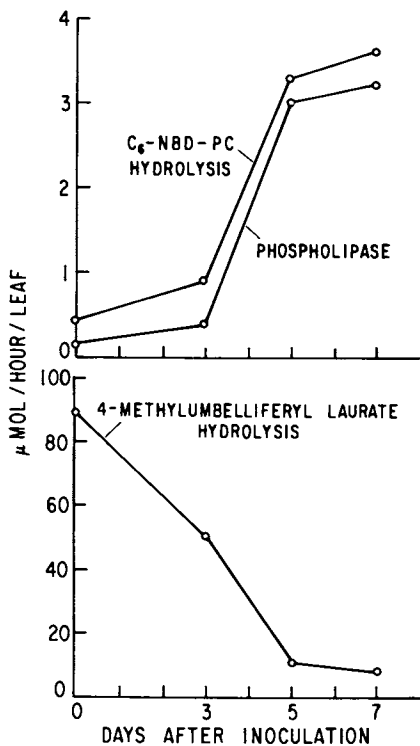


Figure 3. Time-course study of total phospholipase activity during infection of potato leaves by *P. infestans*. Phospholipase values were previously reported (4). C<sub>6</sub>-NBD-PC hydrolysis assays were performed at pH 9.0 as described (28). Hydrolysis of 4-methylumbelliferyl laurate was measured by a fluorometric technique (44).

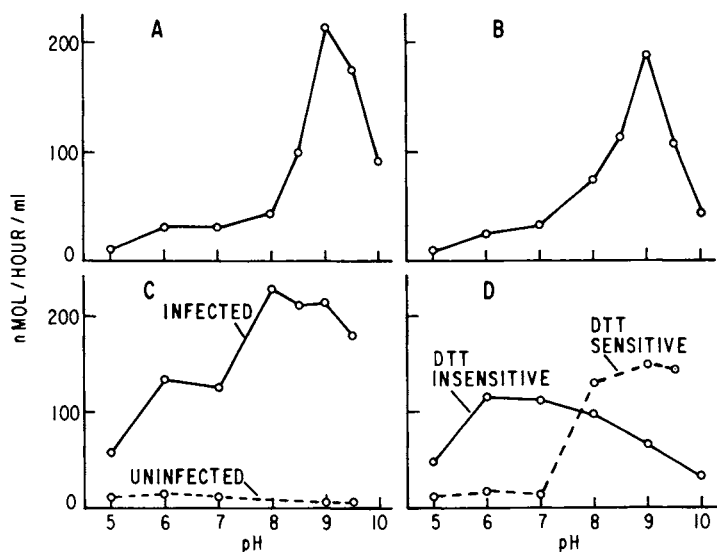


Figure 4. Effect of pH on total phospholipase activity in: A. culture filtrates of fungus grown on rye steep media + 10 mg PC for 7 days. B. Culture wash of fungus grown on lima bean agar for 14 days. C. Potato leaf, uninfected or infected for 7 days. D. Infected potato leaf (7 days) assayed in the presence of 5 mM dithioreitol (DTT-insensitive activity). DDT-sensitive activity was calculated by subtracting the phospholipase activity in the presence of DDT from that measured in the absence of DDT. Reproduced with permission from Ref. 4. Copyright 1984, Academic Press.

senescence (i.e., breakdown of chlorophyll, galactolipids, and protein), the two processes need to be very carefully compared (33). Both disease and senescence cause increases in membrane permeability (34,35). Recent work has shown that similar increases in the levels of free radicals, lipoxygenase activity, and lipid peroxidation occur during infection and senescence (36-38). Other studies indicate that under certain conditions phospholipid deesterification can be mediated by superoxide ( $O_2^-$ ) in the absence of phospholipases (39). The most recent studies from our laboratory suggest that some of the lipolytic enzymes in potato leaves are regulated by calmodulin (40). Even if produced, free fatty acids may not accumulate in healthy or infected leaves because peroxisomes are capable of metabolizing fatty acids via  $\beta$ -oxidation (41). These factors all indicate that membrane lipid metabolism in healthy and diseased leaves is a dynamic process and any change in membrane composition needs to be interpreted very carefully.

#### Literature Cited

1. Thurston, H. D.; Schultz, O. In "Compendium of Potato Diseases"; Hooker, W. J., Ed.; American Phytopathological Society: St. Paul, 1981; p. 40.
2. Currier W. W. Trends in Biochemical Science 1981, 6, 191.
3. Bills, D. D. In "Host Plant Resistance to Pests"; Hedin, P. A., Ed.; ACS SYMPOSIUM SERIES No. 62, American Chemical Society: Washington, DC, 1977, p. 47.
4. Moreau, R. A.; Rawa, D. Physiol. Plant Path. 1984, 24, 187.
5. Moreau, R. A. J. Ag. Fd. Chem. 1985, 33, 36.
6. Moreau, R. A. Phytochemistry 1985, 24, 411.
7. Moreau, R. A.; Seibles, T. S. Can. J. Bot. (in press).
8. Wilson, U. E.; Coffey, M. D. Ann. Bot. 1980, 45, 81.
9. Shimony, C.; Friend, J. New Phytol. 1975, 74, 59.
10. Coffey, M. D.; Wilson, U. A. Can. J. Bot. 1983, 61, 2669.
11. Pitt, D.; Coombes, C. J. Gen. Microbiol. 1969, 56, 321.
12. Duddridge, J. A.; Sargent, J. A. Physiol. Plant Path. 1978, 12, 289.
13. Hoppe, H. H.; Heitefuss, R. Physiol. Plant Path. 1974, 4, 11.
14. Lösel, D. M. New Phytol. 1978, 80, 167.
15. Lösel, D. M.; Lewis, D. H. New Phytol. 1974, 73, 1157.
16. Hoppe, H. H.; Heitefuss, R. Physiol. Plant Path. 1974, 4, 25.
17. Harwood, J. L. In "The Biochemistry of Plants: A Comprehensive Treatise"; Stumpf, P. K.; Conn, E. E., Eds.; Academic Press: New York, 1980; Vol. 4, p. 1.
18. Wassef, M. K.; Hendrix, J. W. Biochem. Biophys. Acta 1977, 486, 172.
19. Bostock, R. M.; Kuc, J. A.; Laine, R. A. Science 1982, 22, 67.
20. Faull, J. L.; Gay, J. L. Physiol. Plant Path. 1983, 22, 55.
21. Shepard, D. V.; Pitt, D. Phytochemistry 1976, 15, 1465.
22. Tseng, T. C.; Mount, M. S. Phytopathology 1974, 64, 229.
23. Tseng, T. C.; Bateman, D. F. Phytopathology 1968, 58, 1437.



24. Plumbley, R. A.; Pitt, D. Physiol. Plant Pathol. 1979, 14, 313.
25. Lumbsden, R. D. Phytopathology 1970, 60, 1106.
26. Tseng, T. C.; Bateman, D. F. Phytopathology 1969, 59, 359.
27. Lumbsden, R. D.; Bateman, D. F. Phytopathology 1968, 58, 219.
28. Wittenaur, L. A.; Shirai, K.; Jackson, R. L.; Johnson, J. D.; Biochem. Biophys. Res. Commun. 1984, 118, 894.
29. Galliard, T. In "The Biochemistry of Plants: A Comprehensive Treatise"; Stumpf, P. K.; Conn, E. E., Eds.; Academic Press: New York, 1980; Vol. 4, p. 85.
30. Matsuda, H.; Hirayama, O. Bull. Fac. Agric. Shimane Univ. 1979, 13, 105.
31. Matsuda, H.; Hirayama, O. Biochim. Biophys. Acta 1979, 573, 155.
32. Rodionov, V. S.; Zakharova, L. S. Soviet Plant Physiol. 1980, 27, 298.
33. Novacky, A. In "Biochemical Plant Pathology"; Callow J. A., Ed.; John Wiley and Sons: Birmingham, U.K., 1983; p. 347.
34. Wheeler, H. In "Plant Disease: An Advanced Treatise"; Horsfall, J. G.; Cowling, E. B., Eds.; Academic Press: New York, 1978; Vol. 3, p. 327.
35. Barber, R. F.; Thompson, J. E. J. Exp. Bot. 1980, 31, 1305.
36. Lupu, R.; Grossman, S.; Cohen, Y. Physiol. Plant Pathol. 1980, 16, 241.
37. Dhindsa, R. J.; Plumb-Dhindsa, P.; Thorpe, T. A. J. Exp. Bot. 1981, 32, 126.
38. Thompson, J. E.; Pauls, K. P.; Chia, L. S.; Sridhara, S. In "Biosynthesis and Function of Plant Lipids"; Thomson, W. W.; Mudd, J. B.; Gibbs, M., Eds. American Society of Plant Physiologists: Rockville, MD, 1983; p. 173.
39. Niehaus, W. J., Jr. Bioorg. Chem. 1978, 7, 77.
40. Moreau, R. A.; Isett, T. Plant Science (in press).
41. Gerhardt, B. FEBS Letts. 1981, 126, 71.
42. Hara, A.; Radin, N. S. Anal. Biochem. 1978, 90, 420.
43. Christie, W. W. "Lipid Analysis"; Pergamon Press: Oxford, U.K., 1982.
44. Hasson, E.P.; Laties, G. G. Plant Physiol. 1976, 57, 142.

RECEIVED May 1, 1986

## Chapter 22

# Use of Cerulenin and Butyrate in the Study of *Candida albicans* Germination

Ronald L. Cihlar and Kathryn A. Hoberg<sup>1</sup>

Department of Microbiology, Schools of Medicine and Dentistry, Georgetown University, Washington, DC 20007

The antilipogenic agent, cerulenin, has been shown to be an effective inhibitor of fatty acid biosynthesis in a variety of fungi. Exploitation of this property has led to investigations utilizing cerulenin to assess the role of phospholipids in the morphogenesis of these organisms. We have examined the utility of cerulenin and the fatty acid salt, sodium butyrate, in studies concerning vegetative germination and pathogenesis of *Candida albicans*. Both agents are effective as inhibitors of *C. albicans* germination at concentrations that do not significantly affect cell viability during the time course of experiments. As expected, cerulenin prevented germination by the inhibition of lipid biosynthesis, and such inhibition could be overcome by supplementation of cultures with palmitate. Butyrate had no effect on lipid biosynthesis, and presumably inhibits germination by an alternate mechanism. Since both agents inhibited germination by different routes, their effectiveness in identifying biochemical correlates of germination was assessed. The increase in chitin biosynthesis that normally accompanies *C. albicans* germination was inhibited by cerulenin and butyrate. Cerulenin-resistant mutants of *C. albicans* have also been isolated and partially characterized. Resistance was verified by unaltered germination capacities, growth kinetics, ultrastructural organization and continued lipid biosynthesis in the presence of the drug. In addition, the mutants have surface compositions which differ from the parental strain and from each other. These differences have been correlated with diminished capacity of the mutant strains to adhere *in vitro* to human buccal epithelial cells or to fibrin-platelet matrices.

<sup>1</sup>Current address: Surgery Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20205

0097-6156/87/0325-0355\$06.00/0  
© 1987 American Chemical Society

Dimorphism is a characteristic of a number of filamentous fungi, and may be defined as their ability to grow as either a yeast-like or mycelial morphology. Generally, the transition from one morphological form to the other occurs in response to environmental factors such as growth medium, incubation temperature or atmospheric composition (1,2). Since such conditions can easily be controlled and adjusted in the laboratory, several dimorphic fungi have been exploited experimentally to examine questions related to developmental processes (2,3). In addition, since many fungal pathogens of man are dimorphic, an array of investigations have attempted to assess the significance of dimorphism in relation to the invasiveness of these organisms. Studies concerning morphogenesis and pathogenesis of Candida albicans in particular, have appeared with increasing frequency. C. albicans is an indigenous member of the human microbial flora, and is a major contributor to the development of opportunistic disease (4). Investigations directed toward understanding dimorphism of C. albicans and the relation, if any, to its virulence have examined primarily changes in cell wall structure and composition (5,6), as well as concomitant adjustments in the activity of enzymes involved in cell wall biosynthesis that accompany vegetative germination (yeast to hyphal transition; referred to hereafter as germination) (7,8). In contrast, little information is available concerning the function of the plasma membrane in the dimorphic response and in wall assembly; however, the association of a number of cell wall biosynthetic enzymes with the membrane, and the participation of membrane components in transport and secretion processes, implicate the membrane in morphogenesis and cell wall assembly.

We have approached this issue by employing agents that inhibit germination of C. albicans, with the aim of identifying key biochemical and molecular events that may be membrane-associated, and that serve to regulate the yeast to hyphal transition. Specifically, we have investigated the suitability of the antibiotic, cerulenin, and the fatty acid salt, sodium butyrate, for use in these types of investigations. Cerulenin, an antilipogenic agent produced by Cephalosporium caerulens, specifically inhibits fatty acid biosynthesis in a variety of bacteria, yeast and fungi (9). It has been utilized to study the role of membrane biosynthesis and fatty acid synthesis in differentiation of several fungal systems including Rhizopus stolonifer (10), Ceratocystis ulmi (11), and Botryodiplodia theobromae (12). Sodium butyrate, on the other hand, has been found to influence diverse processes, including cellular differentiation (13), regulation of enzyme activity (14), modification of histones (15) and cell cycle events (16), and has been utilized largely in studies with higher eukaryotic cells. The direct mechanism whereby butyrate potentiates such effects has not been defined; however, the agent has not been reported to directly inhibit lipid synthesis. The results of our investigations utilizing cerulenin and butyrate are discussed below.

### Materials and Methods.

Culture conditions. *C. albicans*, strain 4918 (17), was the wild-type strain employed in all experiments. Unless otherwise noted, all cultures were grown for 18 hr at 28°C in Phytone-peptone broth supplemented with 0.1% glucose. Cells were then collected by centrifugation, washed and resuspended in PBS (0.12M sodium chloride, 0.037M sodium phosphate, pH 7.2), to final concentration of  $5 \times 10^7$  cells/ml. At the onset of experiments, appropriate amounts of a standardized culture were resuspended in Phytone-peptone broth. Induction of germination was mediated by shifting cultures to an incubation temperature of 37°C. Germination was monitored by light microscopy. The agents cerulenin and butyrate were utilized at final concentrations of 1  $\mu$ g/ml and 20 mM, respectively.

Biochemical Determinations. Protein biosynthesis, lipid biosynthesis and chitin biosynthesis were quantitated as described earlier (18).

Ultrastructural Analysis. Samples to be examined by electron microscopy were prepared as described by Persi and Burnham (19). All samples were fixed using the following schedule: glutaraldehyde (4% (w/v), 6h), osmium tetroxide (20% (w/v), 6h) tannic acid (10% (w/v), 3h), and osmium tetroxide (20% (w/v), 2h). Each fixative was prepared in 0.2M cacodylate buffer, pH 7.2, and samples were washed between fixations with cacodylate buffer. Subsequently, all cells were treated with uranyl acetate, washed, dehydrated through a graded ethanol series, and finally embedded in Maraglas (Polysciences, Inc.). Ultrathin sections were cut, post-stained with lead citrate and examined in a Phillips 300 transmission electron microscope operating at 60 Kv.

### Results and Discussion.

Effect of cerulenin and butyrate on germination of *C. albicans*. We have previously demonstrated that cerulenin and butyrate effectively inhibit germination of *C. albicans*, strain 4918 (18). At cerulenin concentrations between 1.0 and 5.0  $\mu$ g/ml, germination is inhibited 90 to 95%. In cells in which germination does occur, germ tubes are significantly stunted when compared to untreated controls (Figure 1a and b). A small percentage of cells also germinate (1-3%) in the presence of 20 mM butyrate and these levels can be reduced to virtually zero at concentration of 50 mM or greater. Butyrate-treated cells remained in chains and obtained a larger size than those in untreated or cerulenin-treated cultures. In all other experiments cerulenin and butyrate were used at concentrations of 1  $\mu$ g/ml and 20 mM, respectively.

The effect of cerulenin and butyrate upon cell architecture was examined further by electron microscopy. No differences were apparent between untreated controls (Figure 2) and butyrate-treated cells (data not shown). In both cases membrane bound organelles (e.g., mitochondria, nuclei etc.) appeared normal,

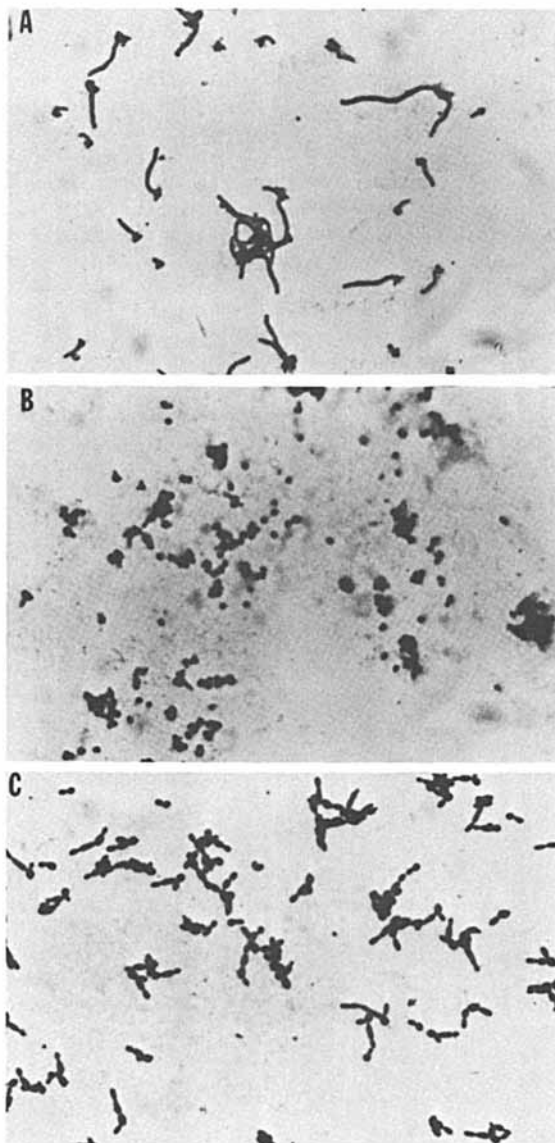


Figure 1. Photomicrographs of *C. albicans* cells after 3.5 h of incubation at 37°C. (a) Untreated control. (b) Cultures supplemented with 1 µg/ml of cerulenin 30 min before incubation. (c) Cultures supplemented with 20 mM butyrate 30 min before incubation. Magnification = 360X. (Reprinted with permission of the authors (18), and the American Society for Microbiology).

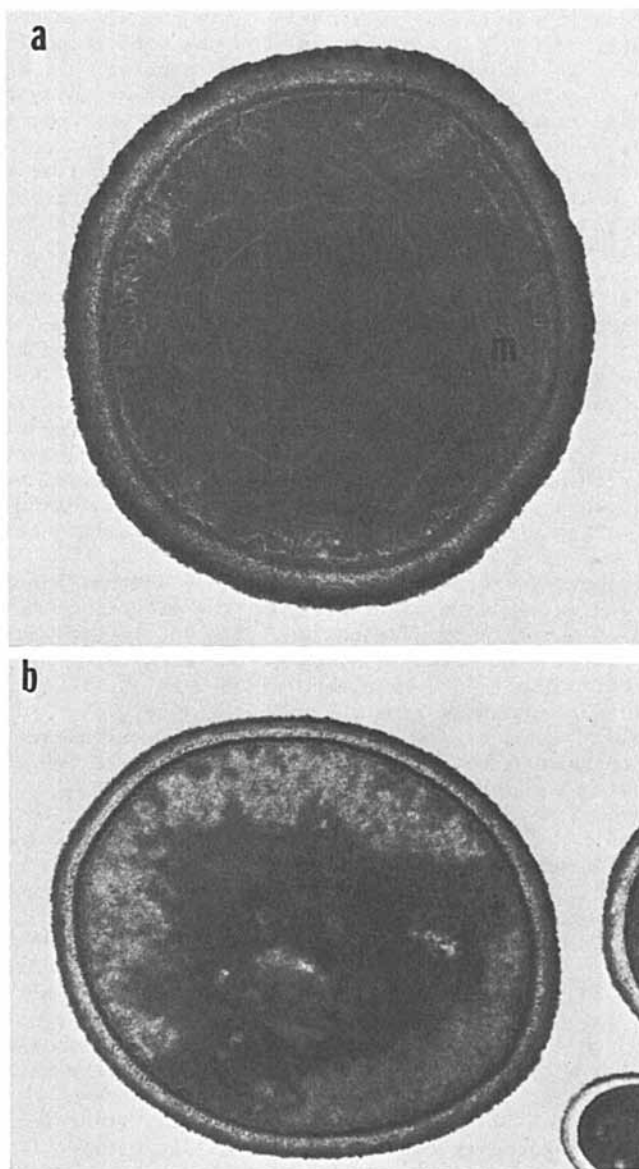


Figure 2. Ultrastructure of cerulenin-treated and untreated *C. albicans* cells. Electron micrographs of *C. albicans*, 4918 grown in phytone peptone broth for 3 hr at 28 °C. Panel a: Untreated cells; Magnification = 28,400X. Panel b: Cerulenin treated cells; Magnification = 22,135X; m designates mitochondria.

and numerous invaginations were observed in the plasma membrane. In contrast, several ultrastructural changes were noted in cerulenin-treated cells. These include an apparent disorganization of mitochondria and an absence of plasmalemma invaginations. These observations are consistent with the idea that cerulenin interferes with the biogenesis of the plasma membrane and membrane organelles in *C. albicans*, by virtue of inhibition of lipid synthesis. On the other hand, butyrate treatment does not result in ultrastructural changes in membranous cellular structures, suggesting that the agent may not directly affect lipid synthesis.

The effect of cerulenin and butyrate on lipid synthesis was examined directly by determining their influence on incorporation of [<sup>3</sup>H]acetate into hot methanol/chloroform-extractable material after induction of *C. albicans* germination. The kinetics of [<sup>3</sup>H]acetate incorporation into lipid containing material were essentially identical in butyrate treated and untreated-controls for a period of at least 180 min. In contrast, supplementation of cultures with cerulenin resulted in approximately a 90% reduction in such synthesis. Both butyrate and cerulenin inhibited germination during these experiments, while untreated cells undergo germination.

Experiments were performed to determine whether the addition of exogenous fatty acids might overcome the effects of either cerulenin or butyrate on germination. The addition of as little as .01% palmitate to cerulenin-treated cultures restores germinative capacity "Table I." Supplementation with oleate increased germination of similarly treated cultures by only 5%. Addition of either palmitate or oleate to butyrate treated cultures or to cultures containing both cerulenin and butyrate was not effective in overcoming the inhibitory action of the agents.

The results of this group of experiments suggest that:

- (1) Cerulenin and butyrate inhibit *C. albicans* germination.
- (2) Cerulenin most likely interferes with morphogenesis by inhibiting lipid synthesis. The mechanism whereby butyrate blocks morphogenesis is unknown; however the data suggest that inhibition is not directly related to reduced lipid synthesis. This might be expected since ACP-butyrate is an intermediate in long chain fatty acid synthesis, and is unlikely to inhibit synthesis, although auto-inhibition by free butyrate cannot be excluded. In any event it is most likely that cerulenin and butyrate inhibit *C. albicans* germination by different mechanisms.
- (3) The fact that lipid synthesis continues in butyrate-treated cells despite the fact that germination is inhibited, implies that although such synthesis may be required during germination, this is not sufficient to insure that morphogenesis will occur.

Cerulenin and butyrate in investigations concerning biochemical correlates of germination. Since butyrate does not inhibit germination by interfering directly with lipid biosynthesis, it can be used to help distinguish between biochemical events inhibited by cerulenin that are correlates of morphogenesis from

those events merely coupled to inhibition of lipid biosynthesis. In this regard, we have examined the effects of the agents on chitin synthase activity. The enzyme was chosen for analysis since it is known to be differentially expressed during *C. albicans* germination (8,20), is associated with the plasma membrane (21), and has been reported to require phosphatidyl serine for maximum activity. In these experiments cultures containing cerulenin or butyrate were shifted to 37°C, aliquots were removed at 20 min intervals (e.g., +20 min, +40 min, +60 min), and were then pulsed for an additional 20 min with [<sup>3</sup>H]N-acetyl-glucosamine. Chitin was extracted and quantiated as described previously. The results indicated that chitin deposition was inhibited by both

Table I. Fatty Acid Supplementation of Cerulenin-and/or Butyrate Treated Cultures

Cerulenin <sup>a</sup>	Butyrate <sup>b</sup>	Palmitate <sup>c</sup> (%)	Oleate <sup>c</sup> (%)	Germination <sup>d</sup>
-	-	-	-	++++
+	-	-	-	-
+	-	-	.02	+
+	-	.02	-	++++
+	-	.02	.02	++++
+	-	.01	.01	++++
+	-	-	.01	+
+	-	.01	-	++++
-	+	-	-	-
-	+	-	.02	-
-	+	.02	-	-
-	+	.02	.02	-
-	+	.01	.01	-
-	+	-	.01	-
-	+	.01	-	-
+	+	.02	-	-
+	+	-	.02	-
+	+	.02	.02	-
+	+	.01	.01	-
+	+	-	.01	-
+	+	.01	-	-

<sup>a</sup>Cerulenin was added to a final concentration of 1 ug/ml.

<sup>b</sup>Sodium butyrate was employed at a final concentration of 20 mM.

<sup>c</sup>Palmitate and oleate were maintained as 1% stock solutions in 10% BRIJ 35. BRIJ 35 controls were negative.

<sup>d</sup>Germination was determined microscopically, 3 hr following a shift to an incubation temperature of 37°C. (++++) = >90% of germination, (+) = 5-10%, (-) = no germination.

Overnight cultures of *C. albicans* strain 4918 were resuspended in phytone-peptone broth ( $5 \times 10^7$ /ml) under the conditions indicated above. All cultures were pre-incubated for 30 min at 28°C prior to the shift up in temperature (37°C).



agents. During each successive pulse cerulenin inhibited chitin synthesis 46%, 73% and 80%, respectively, while butyrate inhibited chitin synthesis 46%, 73% and 80%, respectively, when compared to untreated controls. Overall protein synthesis was not inhibited during these time intervals as judged by incorporation of [<sup>3</sup>H]leucine into TCA precipitable counts. These results suggest that the apparent increase in chitin synthesis that accompanies *C. albicans* germination (8,20), is a valid biochemical correlate of development. In addition the combined use of cerulenin and sodium butyrate may provide a general approach to assess the importance of membrane biogenesis, as well as adjustments in the activity of membrane associated enzymes, to morphogenesis.

Cerulenin resistant mutants of *C. albicans*. In order to extend our investigations concerning the molecular regulation of dimorphism of *C. albicans*, cerulenin-resistant mutants have been isolated and partially characterized. The mutants, designated 4918-2 and 4918-10, have apparent differences in their cell surface structure that, when fully understood, may provide insights concerning the virulence of *C. albicans*.

Spontaneous cerulenin-resistant mutants were isolated as described elsewhere (22). The mutants were conclusively identified as *C. albicans* strains on the basis of sugar assimilation patterns, and by their ability to form germ tubes and chlamydospores. The only consistent difference noted among strains 4918, 4918-2 and 4918-10 was in the ability of strain 4918-10 to utilize glycerol as its sole carbon source; the other two strains were unable to grow with glycerol as the carbon source. Variability was also observed in the assimilation by the mutant strains of the sugar alcohols, adonitol, xylitol and sorbitol.

The nature of the cerulenin resistance of the mutant strains was first investigated by constructing growth curves of both strains in the presence of cerulenin. In these experiments samples were removed hourly from newly initiated cultures in YEPD (2.0% yeast extract, 1% peptone, 1% glucose) incubated at 37°C, and the optical density at 540 nm for each aliquot was determined. The growth kinetics of untreated cultures of strains 4918, 4918-2 and 4918-10 and cerulenin-treated cultures of 4918-2 and 4918-10 were identical. In each case logarithmic growth was entered at approximately 4 hr after the onset of the experiments, while the stationary growth phase was reached at 10-12 hr time points. The final cell density (optical density of 1.6) was the same in all cases. In contrast, wild-type cultures treated with cerulenin reached a cell density of only 12-15% of the other cultures.

The resistance of 4918-2 and 4918-10 to the inhibitory effects of cerulenin was substantiated by determining whether the agent affected the rate of lipid synthesis in each strain. As before, the rate of lipid synthesis was quantitated by determining incorporation of [<sup>3</sup>H]acetate into hot methanol/chloroform extractable-material. The results demonstrated that the kinetics of lipid synthesis was not significantly affected by cerulenin.

Whether or not qualitative differences in lipid composition exist between wild type and mutant strains remains under investigation.

It was reasoned that cerulenin resistant strains of *C. albicans* might arise in at least two ways: 1) A mutation in the gene sequences encoding  $\beta$ -Ketoacyl-ACP synthase, the enzyme inhibited by the action of cerulenin (9), or 2) Mutation(s) resulting in cell surface changes rendering the organism impermeable to the agent. Several observations suggest that cerulenin resistance of 4918-2 and 4918-10 may fall into this latter category.

Ultrastructural analysis was first performed to determine whether obvious surface differences existed between wild-type and mutant strains. The notion that such differences might exist stemmed from observations that growth of the mutant strains in liquid media resulted in flocculant appearing cultures in comparison to the wild type, and that such cells were difficult to resuspend after collection by filtration or centrifugation. However, no differences were noted by electron microscopy in surface architecture of mutant strains concerning the number or thickness of the layers of the cell wall.

Although cell surface ultrastructure was identical in mutant and wild type strains, differences have been observed in the composition of cell wall material of the mutant strains in comparison to wild type. One dimensional polyacrylamide gel analysis of proteins extracted from cell walls of each strain showed that strain 4918-2 cell walls contained a protein of an apparent molecular of 26,000 daltons that was absent in wall preparations of 4918 and 4918-10. In addition, a quantitative difference in a protein with a molecular weight of approximately 46,000 daltons was detected. This protein was present in lower amounts in strain 4918-2 than in the other strains.

A significant difference was also observed in cell wall polysaccharide composition. Specifically, strain 4918-10 and 4918-2 had 15.8% and 18.4% more reducing sugar, respectively, associated with their walls when compared with the parental strain. Direct quantitation of glucose, however, revealed that the amount of glucose present in the walls of mutant strains was nearly 11% less than that present in wild-type controls. It appears that the difference in total reducing sugar content was due to significantly higher mannose content in cell walls isolated from mutant strains. The wall of each mutant strain contains at least 27% more mannose than does that of the wild-type strain (23).

The differences observed prompted experiments to determine if functions associated with the cell surface were affected by such alterations. In particular, the adherence capabilities of the strains was assessed by measuring adherence to buccal epithelial cells and to fibrin-platelet matrices (24,25). Adherence of strains 4918-2 and 4918-10 was greatly reduced in both systems when compared to control values. Adherence of the mutant strains was reduced to approximately 60% and 85% in comparison to results obtained with strain 4918 in the buccal cell and fibrin-platelet systems, respectively.

Since the ability of C. albicans to adhere to cells in vivo may be a factor contributing to virulence of the organism, investigations with the mutant strains have turned toward examining their relative virulence and their antigenic composition as opposed to the wild type. In this respect recent investigations have shown that the mutant strains 4918-2 and 4918-10 are significantly less virulent in the rabbit model of Candida albicans (26). Work is in progress to ascertain the molecular mechanisms for the reduced virulence.

#### Acknowledgments

This work was supported in part by Public Health Service Grants DE07168 and HL21370 from the National Institutes of Health. We thank Dr. Richard Calderone for his help and advice, and Adriana Cabrales for manuscript preparation.

#### Literature Cited

1. Stewart, P.R., and P.J. Rogers. 1978. In: "The Filamentous Fungi." (J.E. Smith and D.R. Berry, eds.) pp. 164-196. Wiley and Sons, New York, New York.
2. Szanizlo, P.J., C.W. Jacobs, and P.A. Geis. 1983. In: Fungi Pathogenic for Humans and Animals." (D.H. Howard, ed.) pp.323-436. Marcel Dekker Inc., New York, New York.
3. Sypberd, P.S., P.T. Borgia, and J. Paznokas. 1978. Adv. Microb. Physiol. 18:67-104.
4. Odds, F.C. 1979. "Candida and Candidosis." University Park Press. Baltimore, Maryland.
5. Schewitz, C.R., R. Martin, and H. Verberberg. 1978. Sabouraudia. 16:115-124.
6. Cassone, A., N. Simonetti, and V. Strippoli. 1973. J. Gen. Microbiol. 77:417-526.
7. Chattaway, F.W., R. Bishop, M.R. Holmes, F.C. Odds, and A.J.E. Barlow. 1973. J. Genl. Microbiol. 75:97-109.
8. Braun, P.C., and R.A. Calderone. 1979. J. Bacteriol. 140:666-670.
9. Omura, S. 1981. Meth. Enzymol. 72:520-532.
10. Nickerson, K.W., and E. Leastman. 1978. Exp. Mycol. 2:26-31.
11. Brambl, R., M. Wenzler, and M. Josephson. 1978. J. Bacteriol. 128:21-27.
12. Nickerson, K.W., D.J. McNeel, and R.K. Kulkarni. 1982. FEMS Microbiol. Lett. 13:21-25.
13. Leder, A., and P. Leder. 1975. Cell 5:319-322.
14. Littlefield, B.A., N.B. Cidlowski, and J.A. Sidlowski. Arch. Biochem. Biophys. 201:174-184.
15. Kruh, J. 1982. Mol. Cell. Biochem. 42:65-82.
16. van Wijk, R., L. Tichonicky and J. Kruh. 1983. Eur. J. Biochem. 129:456-460.
17. Manning, M. and T.G. Mitchell. 1980. J. Bacteriol. 142:714-719.

18. Hoberg, K.A., R.L. Cihlar, and R.A. Calderone. 1983. *Anti-microb. Agents and Chemother.* 24:401-408.
19. Persi, M.A., and J.C. Burnham. 1981. *Sabouraudia.* 19:1-8.
20. Chiew, Y.Y., M.G. Shepherd, and P.A. Sullivan. 1980. *Arch. Microbiol.* 125:97-104.
21. Duran, A., B. Bowers, and E. Cabib. 1975. *Proc. Nat. Acad. Sci. U.S.A.* 72:3952-3955.
22. Cihlar, R.L., K.A. Hoberg, and R.A. Calderone. 1984. *In: "Microbiology, 1984"*. (L. Leive and D. Schlessinger, eds.) pp. 148-149. American Society of Microbiology, Washington, D.C.
23. Hoberg, K.A., R.L. Cihlar, and R.A. Calderone. 1986. *Inf. Immun.* 51:102-109.
24. King, R.D., J.C. Lee, and A.L. Morris. 1980. *Infec. Immun.* 27:667-674.
25. Maisch, P.A., and R.A. Calderone. 1981. *Infec. Immun.* 32:92-97.
26. Calderone, R.A., R.L. Cihlar, D.O.-S. Lee, K. Hoberg, and W.M. Scheld. 1985. *J. Inf. Dis.* 152:710-715.

RECEIVED May 1, 1986

## Author Index

- Andrews, J., 10  
Bach, Thomas J., 109  
Bishop, D. G., 10  
Bradford, Susan, 140  
Chitwood, David J., 200  
Cihlar, Ronald L., 355  
Crawford, Mark S., 152  
Croteau, Rodney, 76  
Elakovich, Stella D., 93  
Ettinger, William F., 152  
Feldlaufer, Mark F., 187  
Fuller, Glenn, 2, 44  
Geisler, Victoria J., 140  
Hanners, Patrick K., 140  
Heupel, Rick C., 140  
Hoberg, Kathryn A., 355  
Johnson, Mark A., 76  
Kabara, Jon J., 220  
Kannenber, Elmar, 239  
Kerwin, James L., 329  
Kleppinger-Sparace, 10  
Kolattukudy, P. E., 152  
Le, Phu H., 140  
Lichtenthaler, Hartmut K., 109  
Lozano, Ruben, 187, 200  
Lusby, William R., 200  
Meudt, Werner J., 53  
Moreau, Robert A., 343  
Mudd, J. B., 10  
Nes, W. David, 2, 140, 304  
Nes, William R., 252  
Parish, Edward J., 140  
Phinney, B. O., 25  
Poralla, Karl, 239  
Roddick, James G., 286  
Sanchez, J., 10  
Soliday, Charles L., 152  
Sparace, S. A., 10  
Spray, C. R., 25  
Stumpf, P. K., 44  
Svoboda, James A., 176, 187, 200  
Thomas, S., 10  
Thompson, Malcolm J., 176, 187, 200  
Weete, John D., 268  
Weirich, Gunter F., 187  
Woloshuk, Charles P., 152

## Subject Index

### A

- Abies grandis, interactions with the fir engraver beetle, 79  
Absidia coerulea, phosphorylation of compactin, 115  
Absidia cylindrospora, phosphorylation of compactin, 115  
Absidia glauca, phosphorylation of compactin, 115  
Acetyl coenzyme A, formation, 45  
Achlya, sterol utilization, 318,321  
Achlya ambisexualis, growth inhibition, 337  
Achlya caroliniana, growth inhibition, 337  
Acyl carrier protein, fatty acid synthase sequence, 45-46  
Acyl carrier protein derivatives, desaturation, 13,14f  
Acyl lipids, formation, 47-48  
Aglycone skeletons  
  saponins, 288f  
  Solanum glycoalkaloids, 288f  
Allelopathic agents,  
  structure, 98f  
Alternaria solani, removal of mono-saccharide units of glycoalkaloids, 297,299  
Ambrosia psilostachya, production of sesquiterpene lactones, 104  
Antifungal activity, plant steroids, 286-301  
Antifungal azoles, names, 270-271t  
Aphelenchus avenae, ecdysteroids, 214  
Apis mellifera, effect of sterols on brood production, 183  
Artemisia absinthium, sesquiterpene inhibitor production, 104  
Ascaris, sterol biosynthesis, 202-203  
Ascaris suum, ecdysteroids, 214  
Aspergillus nidulans, studies of sterol inhibitors, 276-277  
Aspergillus niger, studies of fatty acids, 222,223  
Aspergillus ochraceus, temperature adaptation, 332  
Aspergillus terreus, production of mevicolin, 110

## Author Index

- Andrews, J., 10  
Bach, Thomas J., 109  
Bishop, D. G., 10  
Bradford, Susan, 140  
Chitwood, David J., 200  
Cihlar, Ronald L., 355  
Crawford, Mark S., 152  
Croteau, Rodney, 76  
Elakovich, Stella D., 93  
Ettinger, William F., 152  
Feldlaufer, Mark F., 187  
Fuller, Glenn, 2, 44  
Geisler, Victoria J., 140  
Hanners, Patrick K., 140  
Heupel, Rick C., 140  
Hoberg, Kathryn A., 355  
Johnson, Mark A., 76  
Kabara, Jon J., 220  
Kannenberg, Elmar, 239  
Kerwin, James L., 329  
Kleppinger-Sparace, 10  
Kolattukudy, P. E., 152  
Le, Phu H., 140  
Lichtenthaler, Hartmut K., 109  
Lozano, Ruben, 187, 200  
Lusby, William R., 200  
Meudt, Werner J., 53  
Moreau, Robert A., 343  
Mudd, J. B., 10  
Nes, W. David, 2, 140, 304  
Nes, William R., 252  
Parish, Edward J., 140  
Phinney, B. O., 25  
Poralla, Karl, 239  
Roddick, James G., 286  
Sanchez, J., 10  
Soliday, Charles L., 152  
Sparace, S. A., 10  
Spray, C. R., 25  
Stumpf, P. K., 44  
Svoboda, James A., 176, 187, 200  
Thomas, S., 10  
Thompson, Malcolm J., 176, 187, 200  
Weete, John D., 268  
Weirich, Gunter F., 187  
Woloshuk, Charles P., 152

## Subject Index

### A

- Abies grandis, interactions with the fir engraver beetle, 79  
Absidia coerulea, phosphorylation of compactin, 115  
Absidia cylindrospora, phosphorylation of compactin, 115  
Absidia glauca, phosphorylation of compactin, 115  
Acetyl coenzyme A, formation, 45  
Achlya, sterol utilization, 318,321  
Achlya ambisexualis, growth inhibition, 337  
Achlya caroliniana, growth inhibition, 337  
Acyl carrier protein, fatty acid synthase sequence, 45-46  
Acyl carrier protein derivatives, desaturation, 13,14f  
Acyl lipids, formation, 47-48  
Aglycone skeletons  
  saponins, 288f  
  Solanum glycoalkaloids, 288f  
Allelopathic agents,  
  structure, 98f  
Alternaria solani, removal of mono-saccharide units of glycoalkaloids, 297,299  
Ambrosia psilostachya, production of sesquiterpene lactones, 104  
Antifungal activity, plant steroids, 286-301  
Antifungal azoles, names, 270-271t  
Aphelenchus avenae, ecdysteroids, 214  
Apis mellifera, effect of sterols on brood production, 183  
Artemisia absinthium, sesquiterpene inhibitor production, 104  
Ascaris, sterol biosynthesis, 202-203  
Ascaris suum, ecdysteroids, 214  
Aspergillus nidulans, studies of sterol inhibitors, 276-277  
Aspergillus niger, studies of fatty acids, 222,223  
Aspergillus ochraceus, temperature adaptation, 332  
Aspergillus terreus, production of mevinolin, 110

Aubergenone, structure, 95f  
 Auxin-brassinolide  
   interaction, 64-67  
 Azole sterol inhibitors, 276,277t

## B

Bacillus acidocaldarius, hopanoid and lipid content, 244-247  
Bacillus subtilis, reproduction of  
   Turbatrix aceti, 201  
 Bark beetle attack, biochemistry of conifer resistance, 78-79  
 Bioassays, quantitative, brassinosteroids, 61-64  
 Bisabolene, structure, 98f  
Bombyx mori, metabolism of dietary sterols, 177  
Botryodiplodia theobromae, use of cerulenin to distinguish, 356  
Botrytis cinerea, toxicity of glycosides, 290,295-299  
Brassica campestris, presence of 28-norbrassinone, 59  
Brassica napus, biologically active agent, 53  
 Brassinolide  
   biological activity, 59  
   chemical and biological aspects, 53-73  
   effect on crop production, 70-73  
   effect on geotropism, 70,71f  
   effect on growth of pulvini, 70,72f  
   effect on proton secretion, 67  
   structure, 55,56f  
 Brassinolide-auxin  
   interaction, 64-67  
 Brassinolide-ethylene  
   interaction, 67-70,71f  
 Brassinolide-gibberellic acid  
   interaction, 67-69  
 Brassinosteroids  
   bean first internode  
     bioassay, 62-64  
   bean second internode  
     bioassay, 61-62  
   natural occurrence in plants, 55  
   quantitative bioassays, 61-64  
   rice-lamina inclination test, 62  
   structure, 55,58f  
Bremia lactucae, infection of lettuce, 344  
Brugia pahangi, sterol biosynthesis, 202  
Bursaphelenchus xylophilus, amine toxicity, 205  
 Butyrate, investigations concerning biochemical correlates of germination, 360-362

## C

Caenorhabditis, nutritional requirement for sterols, 201-203  
Caenorhabditis elegans  
   effects of inhibitors on sterol metabolic pathways, 208-213  
   general discussion, 202-205,208  
Calliphora vicina, ecdysone conversions, 189  
 Campesterol, structure, 179f  
Candida albicans  
   cerulenin-resistant mutants, 362-364  
   effect of cerulenin and butyrate on germination, 357-360  
   growth inhibition, 276,278  
   morphogenesis and pathogenesis, 356  
   photomicrographs, 358f  
   ultrastructure, 359f  
Candida lipolytica, fatty acid biosynthesis, 332  
Candida utilis, temperature adaptation, 332  
 Capsenone, structure, 95f  
Capsicum annuum, as a source of sesquiterpenoid phytoalexin, 100  
Capsicum frutescens, use in host-parasite studies, 101  
 Capsidiol, structure, 95f  
 Caryophyllene, structure, 98f  
Castanea crenata, as a source of 6-deoxocasterone, 59-60  
 Castasterone  
   biological activity, 60  
   IUPAC equivalent name, 57  
Cephalosporum caerulens  
   growth regulation, 115  
   inhibition of fatty acid synthesis, 356  
 Ceramide aminoethylphosphonate, 347f  
Ceratocystis, growth inhibition, 81  
Ceratocystis fimbriata, infection of sweet potatoes, 102  
Ceratocystis ulmi  
   infection of Wych elm, 103  
   use of cerulenin to distinguish, 356  
Cercospora arachidicola, sterol content, 274  
 Cerulenin, investigations concerning biochemical correlates of germination, 356,360-362  
Chaetomium, growth of isolated compounds on acids, 334  
Chaoborus astictopus, infection, 335  
 Chloroplast glycerolipids, biosynthesis, 10-23  
 5,22,24-Cholestatrienol, structure, 179f  
 Cholesterol, structure, 179f  
Chondrostereum purpureum, infection of elm, 103  
Circinella muscae, phosphorylation of compactin, 115

- Cladosporium fulvum, growth inhibition, 287
- Coleoptera  
phytophagous insects that convert phytosterols to cholesterol, 180-182  
phytophagous insects unable to convert phytosterols to cholesterol, 183
- Colletotrichum, catalysis of ester hydrolysis, 157
- Colletotrichum capsici, spore suspensions, 163
- Colletotrichum gloeosporioides, cutinase secretion, 163,166
- Colletotrichum graminicola, spore suspensions, 163
- Colletotrichum orbiculare, growth inhibition, 290,295
- Colletotrichum pomoides, resistance of tomato fruits, 299
- Compactin, structure, 112
- Conifer resistance to bark beetles, biochemistry, 76-89
- Conifer resistance to their fungal symbionts, biochemistry, 76-89
- Coriulus versicolor, infection of elm, 103
- Corticium rolfsii, fruit rot, 299
- Corynebacterium sepedonicum, bacterial ring rot of potatoes, 298
- Cotton--See Gossypium species
- Cucurbita maxima,  
4-desmethylsterols, 253,254t
- Culex tarsalis, infection, 335
- Cutin, fungal interaction with plants, 152-173
- Cutinase  
comparison of homologous peptides, 159,162f  
isolation and  
characterization, 154-158  
mechanism of catalysis, 157,158f  
mRNA content, 170,171f  
nucleotide sequence, 159-161  
role in pathogenesis, 163-170  
structure, 159
- Cutinase activity  
extracellular fluid of Fusarium solani pisi, 170,171f  
released into the extracellular fluid during spore germination, 166,168t
- Cyclooxygenase, regulation of fungal morphogenesis, 337-338
- Cyperus serotinus, isolated inhibitory fraction, 104
- D
- Dacrydium cupressinum, isolation of podocarpic acid, 140
- Datura stramonium, production of antifungal compounds, 102
- Dehydroipomeamarone, structure, 96f
- Dendroctonus brevicomis, aggregation pheromone precursor, 79-80
- Dendroctonus ponderosae, resistance of pine, 80
- 6-Deoxocastasterone  
biological activity, 59  
IUPAC equivalent name, 57
- Deoxodolichlosterone  
biological activity, 59  
IUPAC equivalent name, 57
- 4-Desmethylsterols, Cucurbita maxima, 253,254t
- Diacylglycerol, biosynthesis, 18
- Diacylglycerol moiety of chloroplast glycerolipids, 15-16
- Dictyostelium discoideum, increases in fatty acids during development, 335
- Digalactosyldiacylglycerol,  
biosynthesis, 18
- 4a,5-Dihydrocompactin, structure, 112
- 4a,5-Dihydromevinolin, structure, 112
- Diplodea pinea, inhibition of spore germination, 81
- Diriofilaria immitis, sterol biosynthesis, 202,214
- Dolicholide  
biological activity, 60  
IUPAC equivalent name, 57
- Dolichos lablab, isolation of brassinosteroid, 59
- Dolichosterone  
biological activity, 59-60  
IUPAC equivalent name, 57
- Dolico lablab, isolation of dolicholide, 60
- Dysdercus fasciatus, sterol metabolism, 183
- E
- Ecdysone  
IUPAC equivalent name, 57t  
structure, 178f
- Ecdysteroid(s)  
content in meconium fluid, 191-194  
embryonated eggs of Manduca sexta, 194-198  
isolation from Manduca pupae, 192f  
isolation from Manduca sexta, 190f  
structure, 178f
- Eggplant--See Solanum melongena
- Electron micrographs, pea-stem cuticle, 164f
- Elm--See Ulmus glabra
- 3-Epiecdysome, metabolic scheme, 189,190f
- Epilachna varivestis, sterol content, 180



*Ergosterol* biosynthesis,  
inhibitors, 268-282  
*Erynia delphacis*, conidial  
germination, 338  
*Erynia radicans*, response to oleic  
acid, 336  
*Erynia variabilis*, conidial  
germination, 336,338  
*Escherichia coli*  
acyl carrier protein, 45-46  
effect on *Caenorhabditis*, 201  
Esterase activity, released into the  
extracellular fluid during spore  
germination, 166,168t  
Esters, antimicrobial insecticidal  
agents, 220-236  
Ethylene-brassinolide  
interaction, 67-70,71f  
*Europhium clavigerum*, resistance of  
conifers, 80,82

## F

*Fannia canicularis*, germination on  
the cuticular surface, 336  
Farnesol, structure, 96f,98f  
Farnesyl acetate, structure, 98f  
Fatty acids  
and fungal developments, 329-338  
antimicrobial activity, 221-227  
antimicrobial insecticidal  
agents, 220-236  
composition, temperature  
adaptation, 332-333  
enzymic regulation, 331-332  
esterification, 224  
fungi spore germination, 334-336  
general discussion, 4-5  
host-pathogen interactions, 336-337  
membrane transport, 331  
physical properties, 330-331  
plants, a model system, 44-51  
structure, discussion, 330  
synthase sequence, 45-47  
synthesis in plants, 11-13,44-45  
*Foeniculum vulgare*, monoterpene  
cyclization, 87  
*Fomes annosus*, growth inhibition, 81  
Fucosterol, structure, 179f  
Fungal elicitors and conifer  
resistance, 81-82  
Fungal growth  
fatty acids, 333-334  
inhibition by inhibitors of  
ergosterol  
biosynthesis, 268-282  
Fungal life cycle, sequence of  
development controlled by  
sterols, 319f  
Fungitoxicity  
glycoalkaloids, 287-291

Fungitoxicity--Continued  
saponins, 287-291  
Furanosequiterpenoid stress  
metabolites from sweet potato,  
structure, 96f  
*Fusarium caeruleum*, disruption of  
spores, 290,298  
*Fusarium oxysporum*,  
inhibition, 287,290,297-298  
*Fusarium solani pisi*  
bioassays on pea epicotyl, 163-167  
cutinase mRNA content, 170,171f  
degradation by fungal pathogens, 154  
isolated enzyme, 159  
nystatin-resistant mutants, 291  
pectinase production, 172  
penetration of pea-stem  
cuticle, 163,164f  
resin-flow induction, 81

## G

Galactolipids  
biosynthesis, 18-19  
general discussion, 5,6t  
*Geotrichum flavobrunneum*  
antibiotic isolation, 129  
azasterol production, 269  
Geotropism, effect of  
brassinolide, 70,71f  
*Gibberella fujikuroi*  
early 3-hydroxylation  
pathway, 32,34f  
isolation of gibberellins, 25  
Gibberellic acid-brassinolide  
interaction, 67-69  
Gibberellins  
biosynthesis, 29-41  
purification steps, 26,28f  
*Gloeosporium fructigenum*, fruit  
rot, 299  
Glutinosone, structure, 95f  
Glycerol-3-phosphate, acylation, 16-17  
Glycoalkaloids  
as resistance factors, 297-300  
fungitoxicity, 287-291  
Gossypium species, as a source of sesqui-  
terpenoid phytoalexins, 103  
Gossypol, structure, 97f  
Growth inhibitors, in radish  
seedlings, 131,132f

## H

*Haemonchus contortus*, ecdysteroids, 214  
*Haliphthoros milfordensis*, growth  
promotion by oleic acid, 334  
*Heliothis zea*  
growth prevention of larvae, 260  
metabolism of phytosterols, 177

- Helminthosporium avenae, infection of saponins, 297
- Helminthosporium turcicum, growth inhibition, 290,295
- Hemiptera, phytophagous insects unable to convert phytosterols to cholesterol, 180-181
- 28-Homobrassinolide  
biological activity, 60  
IUPAC equivalent name, 57
- 28-Homodolicholide  
biological activity, 60  
IUPAC equivalent name, 57
- 28-Homodolichosterone  
biological activity, 60  
IUPAC equivalent name, 57
- Hopanoids
- Bacillus acidocaldarius  
content, 244-245,247f  
function in model membrane systems, 246-249  
occurrence, 242-244,245f  
sterol equivalents in bacteria, 239-249  
structures, 242,243f
- Zymomonas mobilis content, 246,247f
- 7-Hydroxycostal, structure, 96f
- 7-Hydroxycostol, structure, 96f
- 11-Hydroxy-9,10-dehydronerolidol, structure, 95f
- 20-Hydroxyecdysone, structure, 178f
- 9-Hydroxynerolidol, structure, 95f
- Hymenoptera, phytophagous insects unable to convert phytosterols to cholesterol, 183-184
- I
- Ipomeabisfuran, structure, 96f
- Ipomeamarone, structure, 96f
- Ipomoea batatas, as a source of sesquiterpenoid phytoalexins, 102
- Ips typographis, production of aggregation pheromone, 80
- ent-Isokaurene, 37,40f
- Isopentenoid compounds, 7
- J
- Jimsonweed--See Datura stramonium
- K
- ent-Kaurene, 37,40f
- L
- Lactuca sativa, changes during infection, 344
- Lagenidium giganteum, sporulation, 334-335,337-338
- Lanosterol, inhibition of demethylation, 269-276
- Lauric acid, structure, 4t
- Lemma minor  
as a plant model system, 48-50  
distribution of fatty acids, 49t  
labeled acetate incorporation, 49-51
- Lepidoptera, phytophagous insects that convert phytosterols, 177,179f
- Leucosporidium, enhancement of linoleic acid, 332
- Linoleic acid, structure, 4t
- Lipids  
biosynthesis in chloroplasts, 10-23  
classes, 4-7  
derived from fatty acids, 5  
description, 3  
interactions, 8,9f  
not derived from fatty acids, 7  
overview, 2-9  
polar, 5
- Lipolytic enzymes  
change in levels, 350  
during infection, 350
- Lipophilic antimicrobial agents, 221-227
- Lipoxygenase, regulation of fungal morphogenesis, 337-338
- Lippia nodiflora, as a source of sesquiterpenoid phytoalexins, 104
- Locusta migratoria, ecdysteroids, 189
- Lycopersicon esculentum, as a source of sesquiterpenoid phytoalexins, 102
- Lycopersicon pimpinellifolium, resistance to bacterial wilt, 298
- M
- Makisterone A, structure, 178f
- Malonyl coenzyme A, formation, 45
- Manduca sexta  
free and conjugated ecdysteroids, 187-198  
metabolism of sterols, 177  
toxicity of  
29-fluorostigmasterol, 202
- Meconium fluid, ecdysteroid content, 191-194
- Megachile rotundata, use of dietary phytosterols, 183
- Meloidogyne incognita, inhibition of sterol metabolism, 205
- Meterodera zaeae, sterol content, 204
- 24-Methylene-cholesterol, structure, 179f
- Methylococcus capsulatus  
synthesis of hopanoids, 249  
synthesis of sterols, 240,249

- Mevalonic acid  
   chemical structure, 7  
   early pathway to  
     gibberellins-12-aldehyde, 29,30f
- Mevinolin  
   effect on growth and chemical  
     composition of cell suspension  
     cultures of *Silybum*  
     marianum, 126,127t  
   effect on isoprenoid accumulation in  
     radish seedlings, 118-124  
   effect on isoprenoid synthesis in  
     primary leaves of  
     wheat, 124,125t  
   effect on plastidic prenylipids and  
     prenylquinones, 122,123t  
   plant-growth regulation, 109-134  
   secondary physiological  
     responses, 126-129  
   structure, 112f
- Mevinolin-type metabolites, comparison  
   of inhibition  
   constants, 110,113-114t
- Microsporium gypseum*, macroconidial  
   germination, 335
- Monacolin, structure, 112f
- Monascus ruber*, mevinolin  
   production, 110
- Monilinia fructicola*, inoculation of  
   peppers, 101-102
- Monilinia fructigena*  
   fruit rot, 299  
   sterol inhibitor studies, 276,278
- Monogalactosyldiacylglyceride,  
   substrate, 47
- Monogalactosyldiacylglycerol  
   biosynthesis, 18  
   positional distribution in leaf  
     glycerolipids, 15-16,17t
- Monoterpene in conifers  
   alterations, 76  
   biosynthesis, 82-84  
   components, 77f  
   quantitative analysis, 83
- Monoterpene cyclization,  
   mechanism, 84-88
- Mucor hiemalis*, compactin  
   hydroxylation, 110
- Mucor mucedo*, temperature  
   adaptation, 332
- Mycoplasma*, dependence on sterols, 240
- Mycosphaerella*, infection of papaya  
   fruits, 166
- Myristic acid, structure, 4t
- Myrothecium cerrucaria*, studies of  
   fatty acids, 223
- N
- Nannocystis exedens*, sterol  
   content, 240
- Nematodes  
   function of sterols, 213-215  
   inhibition of sterol  
     metabolism, 205-208  
   lack of de novo sterol  
     biosynthesis, 202-203  
   parasitic, sterol composition, 203
- Nematospiroides dubius*, growth  
   inhibition, 208
- Neurospora crassa*  
   mutants, reversion to normal cyclic  
     nucleotide levels, 335  
   slime mutant, sensitivity to  
     propiconazole, 281
- Nicotiana* species, as a source of sesqui-  
   terpenoid phytoalexins, 101
- Nippostrongylus brasiliensis*, sterol  
   requirements, 202,208
- Nocardia autotrophica*, hydroxylation  
   of compounds, 115
- Nonhydroxylation pathway, for *Pisum*  
   sativum, 31-33f
- 28-Norbrassinolide  
   biological activity, 60  
   IUPAC equivalent name, 57
- 28-Norbrassinone  
   biological activity, 59  
   IUPAC equivalent name, 57
- O
- Octahydrophenanthrene lactones,  
   structure, 141f
- Oleic acid, structure, 4t
- Oncopeltus fasciatus*, sterol  
   conversions, 180
- Oomycetes  
   sterol biosynthesis, 304-326  
   sterol composition, 308-309t  
   sterol occurrence, 304-318  
   sterol requirements, 304-326
- Oryza sativa*, as a source of  
   dolichosterone, 60,62
- Ostertagia ostertagi*, toxicity of  
   alkylamines and amides, 205
- 9-Oxonerolidol, structure, 95f
- 6-Oxydendrolasin, structure, 96f
- P
- Palmitic acid, structure, 4t
- Panagrellus redivivus*, 203-205  
   ecdysteroids, 214  
   sterol biosynthesis, 203
- Pectinaceous barrier, enzymatic  
   penetration, 172,173f
- Penicillium*, compactin production, 110
- Penicillium italicum*, growth  
   inhibition, 279
- Pepper--See *Capsicum annuum*

- Phaseolus vulgaris  
 castasterone content, 60,62  
 changes during infection, 344  
 presence of  
   6-deoxocastasterone, 59-61
- Phocanema decipiens, ecdysis, 214
- Phosphatidylcholine, positional distribution in leaf  
 glycerolipids, 16,17t
- Phosphatidylglycerol  
 in chloroplasts,  
   biosynthesis, 19,21t  
 positional distribution in leaf  
 glycerolipids, 16,17t
- Phospholipase activity  
 during infection of potato leaves, 351f  
 effect of pH, 352f  
 from phytopathogens, 345-349  
 in healthy potato leaves, 349-350  
 on phosphatidylcholine, 347f
- Phytoalexins, discussion, 99-100
- Phytopathogens, phospholipase activity, 346
- Phytophagous insects, steroid metabolism, 176-185
- Phytophthora cactorum  
 growth curve, 322f  
 metabolism of sterols, 260  
 micrographs, 324-325f  
 sporulation, 295  
 sterol occurrence, 306-309,315-321  
 toxicity of glycosides, 290
- Phytophthora cinnamomi, growth inhibition, 278,280
- Phytophthora infestans  
 changes in host ultrastructure during infection, 344-345  
 changes in levels of extracellular enzyme activities during germination, 349t  
 changes in lipid composition during infection, 344-345  
 disintegration of zoospores, 291  
 host-pathogen interactions, 336-337  
 membrane-degrading enzymes during infection of potato leaves, 343-353  
 polar lipid composition, 345t  
 potato resistance, 100,299
- Phytophthora parasitica, sphingolipid content, 345
- Phytosterol synthesis, effect of inhibitors, 129-133
- Pieris brassicae, ecdysteroids, 189
- Pinus pinaster, formation of acyclic hydrocarbons, 84
- Pinus radiata  
 monoterpenes, 81  
 seedlings, 84
- Pinus virginiana, resin flow from wounds, 81
- Pisum sativum  
 nonhydroxylation pathway, 31-33  
 pathogen, 154
- Pityrosporium orbiculare, utilization of acids, 334
- Pityrosporium ovale, utilization of acids, 334
- Plant growth regulation  
 by mevinolin, 109-134  
 by sterol biosynthesis inhibitors, 109-134
- Plant lipids--See Lipids
- Plant steroids  
 antifungal activity, 286-301  
 See also Steroids
- Plodia interpunctella, sterol metabolism, 177
- Podisus maculiventris, ecdysteroid synthesis, 184
- Podocarpic acid  
 chemical synthesis  
   scheme, 142-144,145f  
 structure, 141f  
 synthesis and fungistatic activity, 140-147
- Podocarpic acid derivatives  
 chemical synthesis  
   scheme, 142-144,145f  
 structure, 141f
- Podocarpus cupressinus, isolation of podocarpic acid, 140
- Podocarpus dactyloides, isolation of podocarpic acid, 140
- Potato--See Solanum tuberosum
- Potato leaves, polar lipid composition, 345t
- Pseudomonas lachrymans, infection of tobacco, 101
- Pseudomonas solanacearum, resistance in plant roots, 298
- Pythium prolatum, sphingolipid content, 345
- Pythium ultimum, release of amino acids, 291

## R

- Raphanus sativus, cultivation of seedlings, 116
- Resin composition and conifer resistance, 79-81
- Rhabditis maupasi, nutritional requirement for sterols, 201
- Rhizoctonia solani, infection of tubers, 295,298
- Rhizopus nigricans, inoculation of cotton tissues, 103
- Rhizopus stolonifer  
 growth inhibition, 278  
 use of cerulenin to distinguish, 356

- Rhodospirillaceae, hopanoid content, 244
- S
- Saccharomyces cerevisiae  
anaerobic growth, 269  
entry of protons into cells, 281  
growth inhibition, 279-280  
growth response to sterols, 262t  
phosphate and thiamine uptake, 331-334  
primary sterol, 255-256  
sparing activity of cholesterol for ergosterol, 264t  
sterol content, 274,276  
structure-function relationship of sterols, 252-265
- Saccharomyces lipolytica, fatty acid membrane transport, 331
- Saccharomyces uvarum, fatty acid membrane transport, 331
- Salvia officinalis, use in synthesis of monoterpene olefins, 86-87
- Saponins  
as resistance factors, 297-300  
fungitoxicity, 287-291  
steroids, 292-293f
- Saprolegnia ferax  
mechanism of C-24 alkylation, 319f  
radiolabeling feeds, 316-317t
- Saprolegnia parasitica, growth inhibition, 337
- Schizophyllum commune, transformations of compactin, 115
- Scolytus ventralis, interactions with grand fir, 79
- Septoria linicola, growth inhibition, 290,295
- Septoria lycopersici, tomatine inactivation, 297
- Sesquiterpenes  
allelopathic agents, 93-104  
as phytoalexins, 93  
structures, 93-98f
- Sesquiterpenoid stress metabolites  
from cotton, structure, 97f  
from eggplant, structure, 95f  
from elm, structure, 97f  
from potato, structure, 94f  
from sweet pepper, structure, 95f  
from sweet potato, structure, 96f  
from tobacco, structure, 95f
- Silybum marianum, cell suspension cultures, 116,126,127t
- Sitosterol, structure, 179f
- Solanum melongena, as a source of sesquiterpenoid phytoalexins, 101
- Solanum steroidal glycoalkaloids, 289f
- Solanum tuberosum  
infection by Phytophthora infestans, 336
- Solanum tuberosum--Continued  
phytoalexin research, 100
- Sordaria fimicola, growth inhibition, 280
- Spirostane skeleton, 288f
- Spodoptera frugiperda, sterol metabolism, 177
- Staphylococcus aureus, sterol synthesis, 240
- Stearic acid, structure, 4t
- Steinernema feltiae, nutritional requirement for sterols, 201
- Steroids  
found in plants, major groups, 287t  
variability of metabolism among phytophagous insects, 176-185
- Sterols  
biosynthesis, 240,241f,264-265  
configuration at C-20, 258,259f  
evidence for a dual role, 263-264  
function, 256-257,318  
general discussion, 7  
in membranes, function, 240-242  
in nematodes  
metabolism and function, 200-215  
nutritional requirement, 201-202  
in Saccharomyces cerevisiae, structure-function relationships, 252-265  
inhibitors  
names, 270-271t  
structures, 272-273f  
isolated from commercial sources, 310-311t  
metabolism in Caenorhabditis elegans, 208-213  
metabolism in free-living nematodes, 203-207  
occurrence, 239-240  
structural variability, 252-255  
synthesis inhibitors, 129,130f
- Stigmasterol, structure, 179f
- Streptomyces scabies, effect of tuber alkylolids, 298
- Sulfoquinovosyldiacylglycerol  
biosynthesis, 20-22  
positional distribution in leaf glycerolipids, 15-16,17t
- Sweet potato--See Ipomoea batatas
- Synecephalastrum nigricans, hydroxylation of compactin, 110
- T
- Tanacetum vulgare, cyclizations of geranyl pyrophosphate, 87
- Taphrina deformans  
growth inhibition, 280,281  
sterol inhibitor studies, 276
- Teasterone, IUPAC equivalent name, 57
- Tenebrio molitor, sterol levels, 180
- Tetrahymena, sterol metabolism, 249

- Tetrahymina pyriformis, sterol metabolism, 260
- Thamnidium elegans, synthesis of unsaturated compounds, 332
- Thea sinensis, presence of 28-norbrassinone, 59
- Tobacco--See Nicotiana species
- Tobacco hornworm--See Manduca sexta
- Tomatine  
effect of pH on binding to cholesterol, 294f  
effect on release of peroxidase from liposomes, 296t
- Tomato--See Lycopersicon esculentum
- Torulopsis glabrata  
depositions of chitin, 278  
oxidative removal of methyl groups, 269  
sterol accumulation, 274  
sterol inhibition, 277t
- Tribolium castaneum, sterol metabolism, 180
- Tribolium confusum, sterol metabolism, 180
- Trichoderma viride, studies of fatty acids, 223
- Trichophyton interdigitale, studies of fatty acids, 222
- Trichophyton mentagrophytes, studies of fatty acids, 223
- Trichophyton purpureum, studies of fatty acids, 222
- Trichosporium, resin production in conifers, 81
- Trichosporium symbioticum, interactions with grand fir, 80
- Trichostrongylus colubriformis, isolation of hormones, 214
- Triticum aestivum, seedling cultivation, 116
- Trogoderma granarium, sterol metabolism, 183-184
- Turbatrix aceti, nutritional studies, 201, 203-205
- Typhasterol  
biological activity, 60  
IUPAC equivalent name, 57
- U
- Ulmus glabra, isolation of sesquiterpenes, 103
- Uridinediphosphate-galactose, 18
- Uromyces phaseoli, infection of bean plants, 344
- Ustilago maydis  
sterol content, 280  
sterol inhibitor studies, 276
- V
- Venturia inequalis, spore suspensions, 163
- Verticillium albo-atrum, inoculation of cotton tissues, 103
- Vertrum steroidal alkaloids, 294f
- Vibrio parahaemolyticus, fatty acid studies, 226
- W
- Waxes, as lipids in organisms, 7
- Y
- Yeast  
general discussion, 255-256  
importance of methyl groups, 261-263
- Z
- Zea mays, dwarf mutants, 35-39
- Zymomonas mobilis, hopanoid content, 246, 247f

*Production by Cara Aldridge Young  
Jacket design by Pamela Lewis*

*Elements typeset by Hot Type Ltd., Washington, DC  
Printed and bound by Maple Press Co., York, PA*