Pheromone Biosynthesis by Selected Species of Grain and Bark Beetles

by

Désirée Vanderwel

B. Sc. (Honors), University of Victoria, 1981

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR IN PHILOSOPHY

in the

Department of Chemistry

SIMON FRASER UNIVERSITY

MARCH 1991

APPROVAL

Name: Désirée Vanderwel

Degree: DOCTOR OF PHILOSOPHY

Title of Thesis: Pheromone Biosynthesis in Selected Species of Grain and Bark Beetles

Examining Committee:

Chairman: Dr. F.W.B. Einstein

Dr. A. C. Oehlschlager, Senior Supervisor

Dr. J. H. Borden, Supervisory Committee

Dr. K. N. Slessor, Supervisory Committee

Dr. B. L. Funt, Supervisory Committee

Dr. T. J. Borgford, Internal Examiner

Dr. W. L. Roelofs, External Examiner
Department of Entomology, Cornell University

Date Approved: 11 April 1991

PARTIAL COPYRIGHT LICENSE

I hereby grant to Simon Fraser University the right to lend my thesis, project or extended essay (the title of which is shown below) to users of the Simon Fraser University Library, and to make partial or single copies only for such users or in response to a request from the library of any other university, or other educational institution, on its own behalf or for one of its users. I further agree that permission for multiple copying of this work for scholarly purposes may be granted by me or the Dean of Graduate Studies. It is understood that copying or publication of this work for financial gain shall not be allowed without my written permission.

Title of Thesis/Project/Extended Essay

Pheromone Biosynthesis by Selected Species of Grain and Bark Beetles

Author:

(signature)

Desiree Vanderwel

(name)

April 26, 1991

(date)

ABSTRACT

The overall goal of this thesis was to investigate pheromone biosynthesis in grain and bark beetles. Insects were exposed to either radio- or stable isotope-labelled precursors. Incorporation of label into pheromones was determined by scintillation counting or gas chromatography/selected ion monitoring-mass spectroscopy, respectively.

Most known aggregation pheromones of cucujid grain beetles are macrolides, called "cucujolides". Objectives of this project were to determine the biosynthetic origin of these cucujolides and to study the mechanism of cyclization. Studies were performed on *Oryzaephilus mercator* (Fauvel) and/or *Cryptolestes ferrugineus* (Stephens). In *C. ferrugineus*, cucujolide **I** (numbered according to *J. Chem. Ecol.*, 1988, 14:2069-2096) was of terpenoid origin. Cucujolides **II**, **III**, **IV** and **V** were found to be of fatty acid origin. The *de novo* biosynthesis of all cucujolides (**I** through **V**) was detected. The cyclizations of the hydroxy acid precursors to form **I** in *C. ferrugineus*, and **II** in *C. ferrugineus* and *O. mercator*, proceeded with retention of the hydroxyl oxygens.

The cyclic ketals *exo*- and *endo*-brevicomin are pheromones of many economically important scolytids. The objective of this project was to study brevicomin biosynthesis in the mountain pine beetle (MPB), *Dendroctonus ponderosae* Hopkins and the western balsam bark beetle, *Dryocoetes confusus* Swaine. Insects exposed to (*Z*)- or (*E*)-D_2-nonen-2-one produced *exo*- or *endo*-brevicomin, respectively, that were clearly enriched in two deuterium atoms. The D_2-*exo*-brevicomin produced by male MPB was found to be of the natural (+)-chirality by complexation chromatography. The biosynthetic transformation apparently proceeds through a keto-epoxide intermediate. Both oxygens of brevicomin are derived from molecular oxygen.

Ipsdienol is a common bark beetle pheromone. The chirality of ipsdienol varies widely between different species, populations and even individuals. The objective of this project was to test the hypothesis

that increased activity of the oxidation/reduction equilibrium between ipsdienol and ipsdienone effects a larger proportion of *S*-(+)-ipsdienol. The study insects were *Dendroctonus ponderosae* Hopkins, two populations of *Ips pini* (Say), and *Ips paraconfusus* Lanier. The hypothesis was disproved.

ACKNOWLEDGEMENTS

First and foremost I would like to thank my senior supervisor, Professor Cam Oehlschlager, for his enthusiasm, exciting ideas, and for providing an excellent work environment. I would also like to thank Professor John Borden for his guidance and encouragement, and Professor Mario Pinto for the constructive criticism of this thesis and for the many helpful discussions held during the course of this work. Additionally I owe a debt of gratitude to my family for their support.

Many individuals have persistantly and generously shared both their time and technical expertise with me. I would like to thank Dr. Harold Pierce, Jr. for many hours of patient instruction and stimulating discussions. I also enjoyed fruitful discussions with Regina Gries, Dr. Gerhard Gries and Dr. Dan Miller, and would additionally like to thank them for supplying many of the insects used in this study. I greatly appreciate helpful discussions with Dr. Blair Johnston and Dr. Mohan Singh, and would like to thank them for supplying some of the chemicals used in this study. I would also like to thank Dr. Rob Currie for the constructive editing of this thesis and for assisting with the statistical analyses. Helpful input by Dr. Sowmianarayanan Ramaswamy, Dr. Brian Morgan and Jean-Pierre Lafontaine was also much appreciated.

I would also like to thank the many people who helped to make my experience as a graduate student invigorating and fun as well as educational. These especially include the individuals in the laboratories of Professors Oehlschlager, Borden and Winston.

I would like to thank Art Stock, Leslie Chong and Steve Seybold for generously supplying the many of the insects used in this study; Marcy Tracy for assisting with the NMR analyses; Greg Owen for assisting with the GC/MS analyses; Denyse Zenner and Penny Snell for administrative assistance; and Professor Robert Cushley and Pherotech Inc. for the gifts of some of the chemicals used in this study.

I would also like to thank the Natural Sciences and Engineering

Research Council of Canada, Simon Fraser University, and the R. H. Wright Foundation for the financial support of this research.

TABLE OF CONTENTS

CHAPTER IV

IPSDIENOL BIOSYNTHESIS IN *DENDROCTONUS PONDEROSAE*, *IPS PARACONFUSUS*, AND TWO POPULATIONS OF *IPS PINI*

List of Tables

List of Figures

List of Synthetic Schemes

GENERAL INTRODUCTION

Interest in insect pheromones has been aroused by the potential for these behaviour-modifying chemicals to be used for the detection and manipulation of insect pest populations. As a result much research has been directed toward the identification, synthesis and commercial use of pheromones. More recently, effort has been directed at elucidation of the pathways of pheromone biosynthesis.

A detailed knowledge of pheromone biosynthesis is desirable for several reasons. Control of a specific insect pest could be effected through interruption of its pheromone production (Jones and Berger, 1978). Unique pheromone biosynthetic pathways could be harnessed to activate "biorationally designed" pesticides (Prestwich *et al.*, 1984). An understanding of these pathways could be used to predict the existence of logical candidate pheromones. Factors that regulate pheromone biosynthesis (such as hormones, presence of suitable precursors, *etc.*) could be exploited to induce elevated levels of pheromone production.

The preceding is not merely a hypothetical list. An understanding of pheromone biosynthesis in a lepidopteran species enabled the investigators to predict the existence of a previously overlooked pheromone (Bjostad *et al.*, 1984). Hormonal stimulation of pheromone production by a bark beetle allowed the accumulation of enough pheromone to permit its identification (Francke *et al.*, 1977). It is likely that a greater understanding of pheromone biosynthesis would lead to further benefits.

Why study pheromone biosynthesis in beetles?

To date more than 300,000 species of coleoptera have been described: one animal in every four is a beetle (Evans, 1977). As a group, beetles effect a staggering economic and social impact on humankind through the destruction of field crops, stored products and forests. Conversely, many beetles are beneficial, as they prey upon insect pests. Knowledge gained

in the quest to understand coleopteran biochemistry could be exploited to control the pests among the group, and to aid the beneficial members.

In this context, coleopteran pheromone biosynthesis is a target worthy of study. There is little doubt that effective communication between conspecific individuals during activities such as mate-finding and host-colonization contributes to the reproductive success of many beetles. Beetles can use visual and vibrational cues for communication, but olfactory signals (pheromones) are very important.

However, the identification of coleopteran pheromones is often a difficult task. Those that have been identified (over a hundred to date) indicate a chemically diverse group (Vanderwel and Oehlschlager, 1987); *a priori* predictions of the chemical nature of suspected pheromones are therefore limited. Many pheromones are emitted at exceedingly low levels, and/or may be produced (or be detected in bioassays) only under special environmental or physiological conditions. Some pheromones exhibit activity only when present in combination with other pheromones. The meaning of some messages can be drastically altered through variation of the quantity, relative proportion, and/or chirality of each component. All of these factors hamper the elucidation of coleopteran pheromones. An understanding of the biochemistry of pheromone production, in conjunction with the biology and behavior of the organisms, might help to explain why beetles use the pheromones that they do, when they do. This might allow the prediction of the identity of unknown pheromones.

Organization of this thesis

This thesis summarizes my efforts to further the understanding of pheromone biosynthesis in beetles. It contains four chapters:

Chapter I is a review of the literature.

Chapter II summarizes my studies of the biosynthesis of the macrolide aggregation pheromones of the grain beetles *Cryptolestes ferrugineus* (Stephens) and *Oryzaephilus mercator* (Fauvel). These

pheromones, known as "cucujolides", are lactones of medium ring size (eleven to fourteen members). The goals of this project ranged from determining the biosynthetic origins of these cucujolides, to determining the direction of the lactonization reactions.

Chapter III summarizes my investigation of the biosynthesis of the cyclic ketals *endo*- and *exo*-brevicomin. These and similar compounds are widely used as pheromones by bark and ambrosia beetles. Prior to this study virtually nothing was known about their biosynthesis (Vanderwel and Oehlshlager, 1987). In this thesis, biosynthetic precursors of the brevicomins were identified and the mechanism of the transformation of these precursors to the cyclic ketals was examined.

Chapter IV summarizes my study of ipsdienol biosynthesis in bark beetles. Other investigators have realized that ipsdienol is produced by many different species of bark beetles, and that the chirality of the ipsdienol used varies widely. This variation occurs not only among species, but among populations of the same species, and even among individuals of the same species (references summarized in Vanderwel and Oehlshlager, 1987). The biochemical mechanism for this variation is not understood. The present study explores the validity of the suggestion by Fish *et al.* (1984) that an oxidation-reduction equilibrium between ipsdienol and its ketone, ipsdienone, might be involved in the biochemical manipulation of the chirality of ipsdienol.

CHAPTER I

LITERATURE REVIEW

This review summarizes major advances to the study of coleopteran pheromone biochemistry. A more exhaustive review is presented in Vanderwel and Oehlschlager (1987).

Almost all studies of pheromone biosynthesis in beetles have concerned the boll weevil, *Anthonomus grandis*, and the scolytid bark beetles. Therefore the first two sections of this review are devoted to studies of pheromone biosynthesis in these organisms. The third section is devoted to an overview of the involvement of microorganisms in "pheromone" biosynthesis.

A) Pheromone biosynthesis in the boll weevil

Grandlure, the sex pheromones of the male boll weevil, *Anthonomus grandis*, can be produced either through the derivatization of dietary components or through *de novo* synthesis. It was observed that pheromone production is enhanced in weevils feeding on their natural food source, cotton buds (Hardee, 1970; Hedin *et al.*, 1975). This led to the hypothesis that plant-derived constituents are utilized for pheromone production. Tumlinson *et al.* (1970) proposed a biosynthetic route from monoterpene alcohols to the four monoterpenoid components of grandlure (**1** to **4**, Figure 1), through an intermediate such as γ-isogeraniol (**5**). Subsequent studies demonstrated that nerol and geraniol, which are present in the host (Hedin *et al.*, 1971), could serve as pheromone precursors (Thompson and Mitlin, 1979). Mitlin and Hedin (1974) demonstrated that boll weevils could also synthesize their pheromones *de novo*. Radiolabelled acetate, mevalonate and D-glucose were incorporated into all four components of grandlure. The relative proportion of pheromone contributed by the *de novo* pathway when host precursors are available is not known.

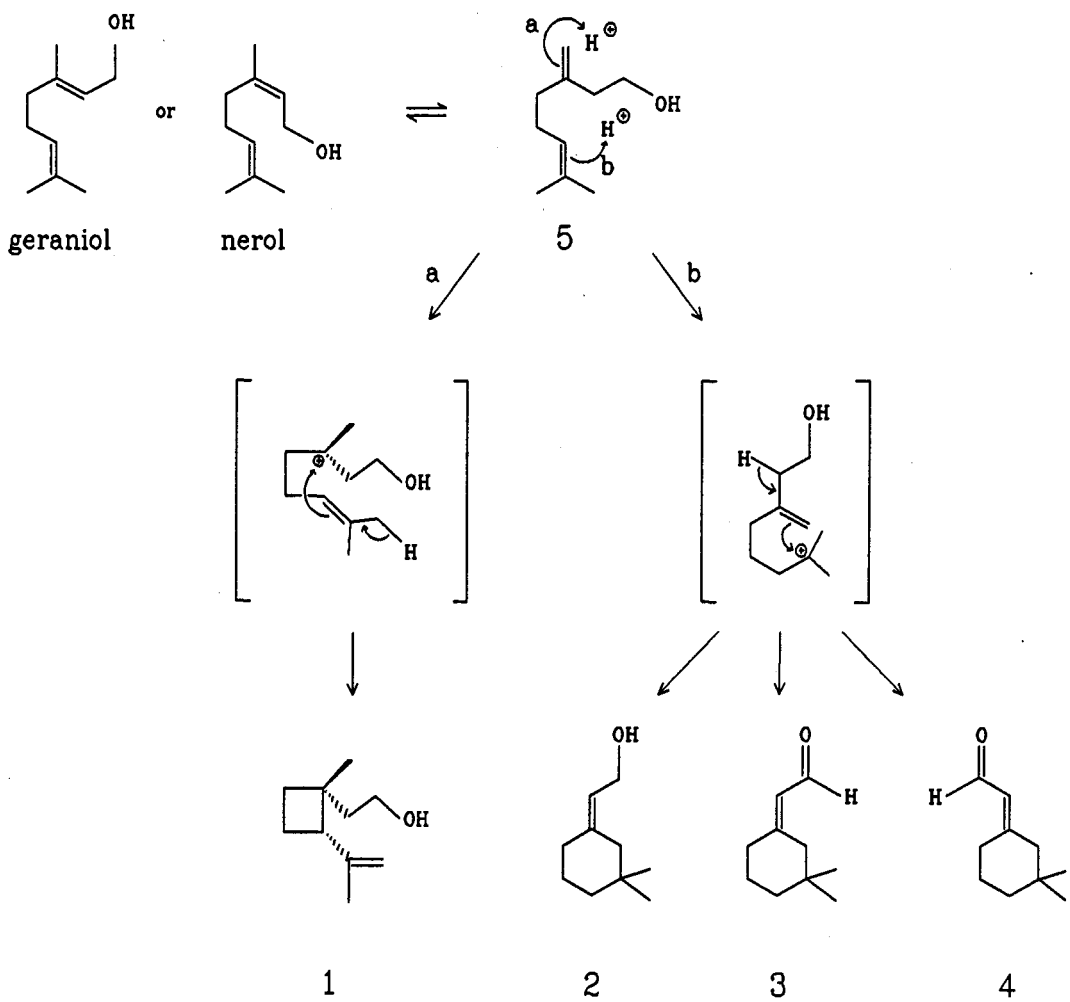


Figure 1: Biosynthesis of boll weevil sex pheromones (**1** through **4**) from geraniol or nerol.

B) Pheromone biosynthesis in bark beetles

Bark beetles are capable of a remarkable diversity of chemical signals: their repertoire is reputed to include long-range and short-range aggregation pheromones, sex pheromones, epideictic ("spacing") pheromones, "switching" pheromones, and antiaggregation pheromones

(Borden, 1985, and references cited therein; Dr. Gerhard Gries*, personal communication). To date, most of the pheromones identified are either terpenoid alcohols, the corresponding ketones, or bicyclic ketals and acetals (Borden, 1985; Vanderwel and Oehlschlager, 1987). Most terpenoid pheromones appear to be derived from host monoterpenes, but the ketals and acetals are of uncertain biosynthetic origin (Vanderwel and Oehlschlager, 1987).

1. Biosynthesis of terpenoid alcohol and ketone pheromones

The resin exuded by trees in response to insect boring contains monoterpenes. These monoterpenes are toxic to beetles (Smith, 1961, 1965a,b; Coyne and Lott, 1976; Raffa *et al.*, 1985). Scolytids must possess effective mechanisms for detoxification in order to survive extensive exposure to resin (Hughes, 1973a; White *et al.*, 1980; Francke and Vité, 1983). The prevailing biochemical strategy appears to involve oxidation of the hydrophobic toxicants to products that, by virtue of their hydrophilic "handles", are more easily mobilized for excretion or storage (Dowd *et al.*, 1983; Ahmad, 1986).

It is widely believed that the oxidation of host monoterpenes is performed by polysubstrate monooxygenases (PSMOs, also called mixed-function oxidases), since these enzymes are largely responsible for the metabolism of dietary toxins (Dowd *et al.*, 1983). To test this hypothesis *in vivo*, Hunt and Smirle (1988) topically treated male and female mountain pine beetles, *Dendroctonus ponderosae*, with two known inhibitors of PSMO activity (piperonyl butoxide and sesame oil) before exposing the beetles to vapors of the host monoterpenes α-pinene and myrcene. Reduced levels of monoterpene oxidation products and an accumulation of monoterpene precursors were observed. Scolytid PSMOs may have become specialized to deal with host tree resin. The microsomal cytochrome P-450 isolated from

---

* Department of Biological Sciences, Simon Fraser University. Burnaby, BC, Canada. V5A 1S6.

Dendroctonus terebrans exhibits an unusually high specificity for the *in vitro* oxidation of the host monoterpene α-pinene (White *et al.*, 1979).

The metabolic products of most host monoterpenes were determined in detailed studies of the volatiles of female *Dendroctonus ponderosae* feeding on its natural hosts, lodgepole and ponderosa pine (Libbey *et al.*, 1985; Pierce *et al.*, 1987). Three metabolic pathways were proposed to account for the alcohol derivatives encountered (Pierce *et al.*, 1987). One metabolic pathway, called MP-I, apparently facilitates the oxidation of any vinyl methyl group which is *E* to an allylic methylene (Figure 2). This system does not discriminate between substrates, as judged from the relative proportions of monoterpene precursors in the host oleoresin and the corresponding alcohol products.

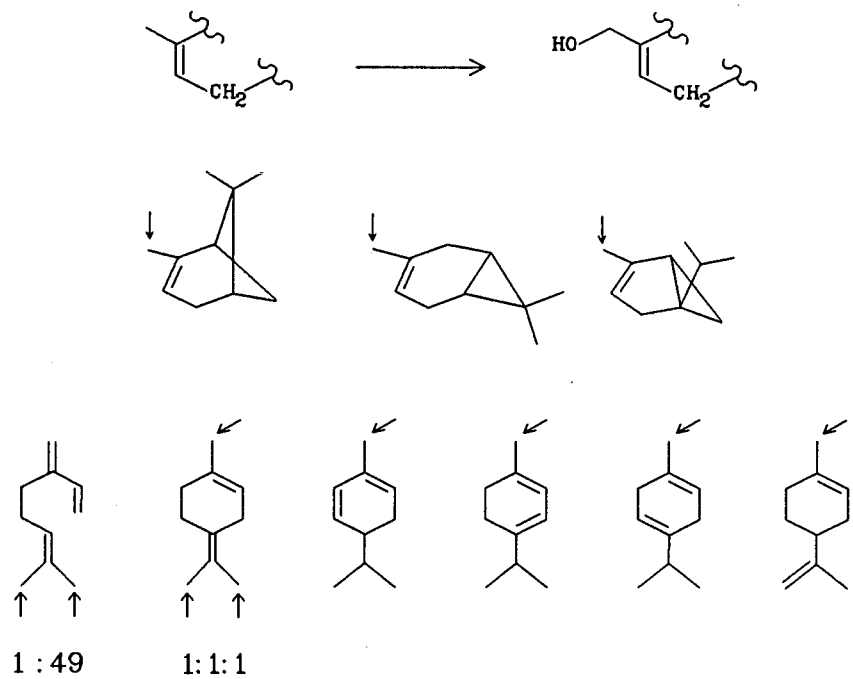


Figure 2: Regiospecific oxidation of host monoterpenes by MP-I in female *D. ponderosae* (Pierce *et al.*, 1987). The arrows denote sites oxidized by MP-I.

The second oxidation system (MP-II) is apparently specific for the hydroxylation of allylic methylenes *E* to vinyl methyl groups in bicyclic monoterpenes such as α-pinene and 3-carene (Figure 3).

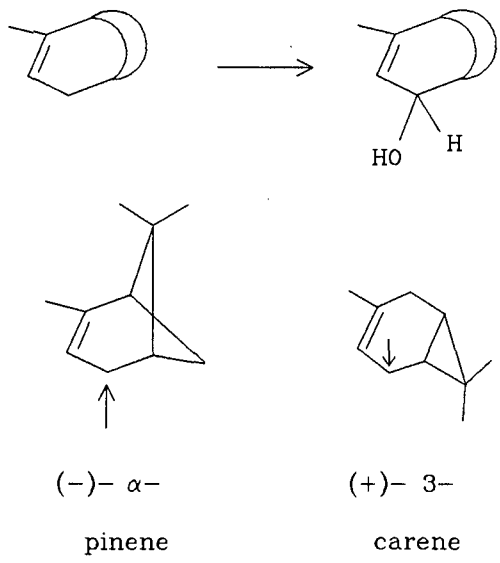
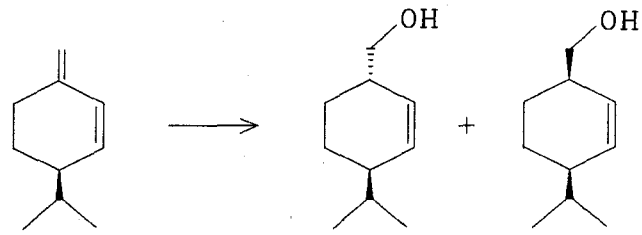


Figure 3: MP-II-mediated oxidation of bicyclic monoterpenes to secondary alcohols in female *D. ponderosae* (Pierce *et al.*, 1987). The arrows denote the sites oxidized by MP-II.

The third enzymatic system (MP-III) is not an allylic oxidase; rather it mediates the anti-Markovnikov hydration of the major host monoterpene, (-)-ß-phellandrene (Figure 4). This system is apparently specific for (-)-ß-phellandrene, since hydration products of other host monoterpenes (such as ß-pinene) were not detected in *D. ponderosae* volatiles (Pierce *et al.*, 1987).



(–) –β–
phellandrene

8 : 1

Figure 4: MP-III mediated anti-Markovnikov hydration of ß-phellandrene in female *D. ponderosae* (Pierce *et al.*, 1987).

Pathways analogous to MP-I, II and III are widespread through the scolytidae (Vanderwel and Oehlschlager, 1987). Some volatile products of these detoxification processes appear to have been adopted for use as pheromones (Hughes, 1973a; White *et al.*, 1980; Francke and Vité, 1983). The biosynthesis of these pheromones from host monoterpenes primarily involves allylic oxidation or hydration, but may be accompanied by secondary reactions such as further oxidation, hydrogenation, or rearrangement of the carbon skeleton (Francke and Vité, 1983).

To summarize thus far, scolytids convert host monoterpenes to the corresponding alcohols, ostensibly as a detoxification strategy. This conversion occurs through allylic oxidation and, less commonly, through hydration. The alcohols produced often undergo subsequent reaction, including elimination, rearrangement, or oxidation. Some volatile derivatives of some host monoterpenes, most notably α-pinene and myrcene, are used as pheromones. It is curious that the volatile oxidation products of other host monoterpenes are apparently rarely used as pheromones (see summary in Table 1). The reason(s) for this phenomenon are not understood.

Table 1: Scolytid metabolites of host monoterpenes (monoterpenes arranged in decreasing order of abundance in Lodgepole Pine sapwood[1]). Those known to function as scolytid pheromones are indicated as such.

Host monoterpene	Scolytid-produced metabolites[2]	Known pheromone?[3]
ß-phellandrene	2-*p*-menthen-7-ol	no
3-carene	3-caren-9-ol	no
	3-caren-10-ol	**yes**
	3-caren-5-one	no
	3-caren-10-one	no
	m-mentha-4,6-dien-8-ol	no
ß-pinene	myrtenol	**yes**
	trans-pinocarveol	no
α-pinene	*o*-cymene[4]	no
	p-cymene[4]	no
	p-mentha-1,5-dien-8-ol	**yes**
	p-mentha-1,5,8-triene[4]	no
	4-methyl-2-pentanol	no
	myrtenol	**yes**
	cis-pinen-2-ol	**yes**
	toluene[4]	no
	verbenene[4]	no
	cis-verbenol	**yes**
	trans-verbenol	**yes**
	verbenone	**yes**
terpinolene	*p*-mentha-1,4(8)-dien-7-ol	no
	p-mentha-1,4(8)-dien-9-ol	no
	p-mentha-1,4(8)-dien-10-ol	no
γ-terpinene	cuminyl alcohol	no
	p-mentha-1,3-dien-7-ol	no
	p-mentha-1,4-dien-7-ol	no
	p-mentha-1,5-dien-7-ol	no
myrcene	amitinol	**yes**
	ipsdienol	**yes**
	ipsdienone	no
	ipsenol	**yes**
	E-myrcenol	**yes**[5]
	Z-myrcenol	no

Table 1: continued next page

Table 1: continued....

Host monoterpene	Scolytid-produced metabolites[2]	Known pheromone?[3]
camphene	camphenol	no
α-terpinene	cuminyl alcohol	no
	p-mentha-1,3-dien-7-ol	no
	p-mentha-1,4-dien-7-ol	no
	p-mentha-1,5-dien-7-ol	no
limonene	*p*-mentha-1,8-dien-10-ol	no
	perilla alcohol	no
	perilla aldehyde	no

[1]According to Shrimpton, 1972, 1973; [2]Unless otherwise stated, data taken from Pierce *et al.*, 1987 and/or from Vanderwel and Oehlshlager, 1987; [3]Unless otherwise stated, data taken from Borden, 1985; [4]Gries, *et al.*, 1990b; [5]Miller, *et al.*, 1990.

The derivatization of myrcene and α-pinene have been most extensively investigated. The results of these studies are summarized in the following sections.

a) Pheromones derived from myrcene

Myrcene, the only prominent acyclic monoterpene in pine oleoresin, is oxidized at three different allylic positions by *Dendroctonus* and *Ips* beetles. Both allylic methyl groups are oxidized, producing (*E*)- or (*Z*)-myrcenol, while only one of the allylic methylenes is oxidized, producing (-)-(*R*)- and (+)-(*S*)-ipsdienol (Figure 5).

Some species of beetles are capable of further derivatizing ipsdienol. For example, (*S*)-ipsdienol is enantioselectively rearranged to amitinol by *I. amitinus* (Francke and Vité, 1983). (*R*)-Ipsdienol does not undergo a similar rearrangement but, in some *Ips* species, this enantiomer is selectively reduced to ipsenol (Fish *et al.*, 1979; Renwick and Dickens, 1979). (*R*)-Ipsdienol can also be oxidized to the corresponding ketone,

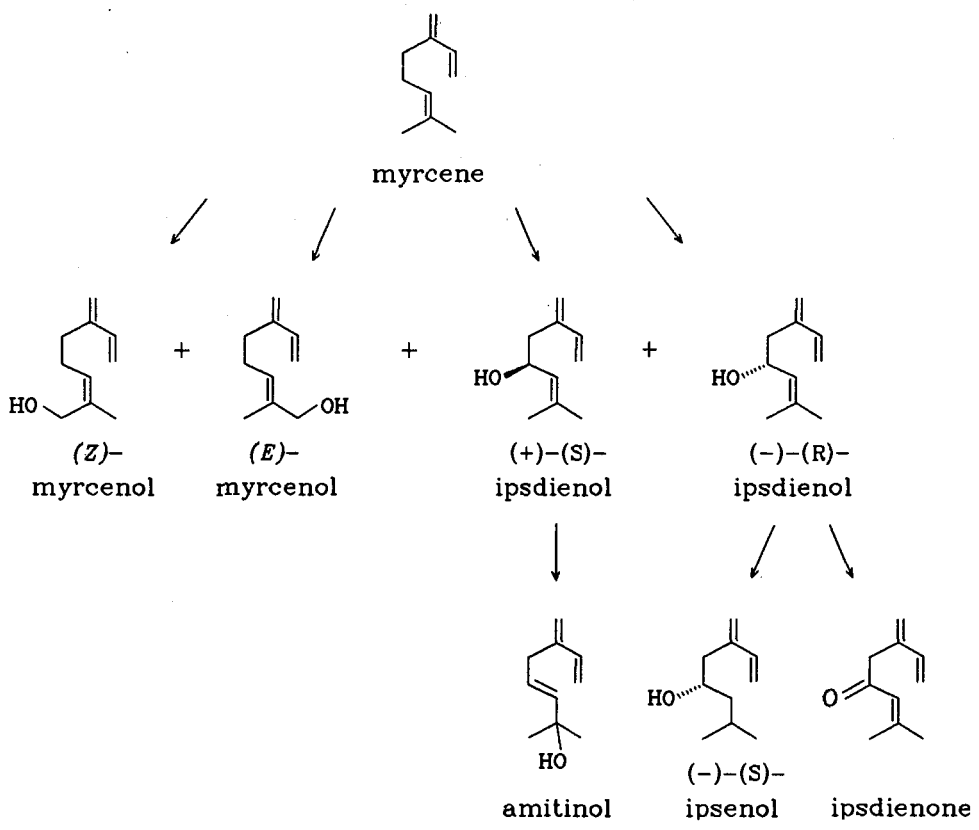


Figure 5: Metabolites of the host monoterpene myrcene produced by *Dendroctonus* and *Ips* beetles.

ipsdienone (Fish *et al.*, 1979, 1984; also discussed in *Chapter IV*). These transformations are summarized in Figure 5.

Initial evidence for the conversion of myrcene to ipsdienol and ipsenol was provided by Hughes (1974) and by Hughes and Renwick (1977a), who showed that *I. paraconfusus* males could produce ipsdienol and ipsenol when exposed to myrcene vapors. Ipsenol was produced when these insects were treated topically with ipsdienol. More conclusive evidence was supplied by Hendry *et al.* (1980), who demonstrated the conversion of deuterium-labelled myrcene to labelled ipsdienol and ipsenol by male *I. paraconfusus*. Fish *et al.* (1979) demonstrated the *in vivo* conversion of deuterated ipsdienol to deuterated ipsenol.

Myrcene derivatives are commonly used as pheromones by bark beetles (Borden, 1985, and references cited therein). The widespread use of pheromones would seem to preclude their effectiveness for species-specific communication. Attraction is partially limited to conspecifics through variation in the number of pheromones involved, as well as variation in pheromone ratios and chiralities. The latter aspect is discussed more thoroughly in *Chapter IV*.

b) Pheromones derived from α-pinene

α-Pinene is oxidized at both allylic positions by *Ips* and *Dendroctonus* beetles, forming myrtenol and *cis*- and *trans*-verbenol (Figure 6) (references summarized in Vanderwel and Oehlschlager, 1987). These alcohols are also subject to further reaction to form a variety of products, as shown in Figure 7 for *trans*-verbenol (Gries *et al.*, 1990b).

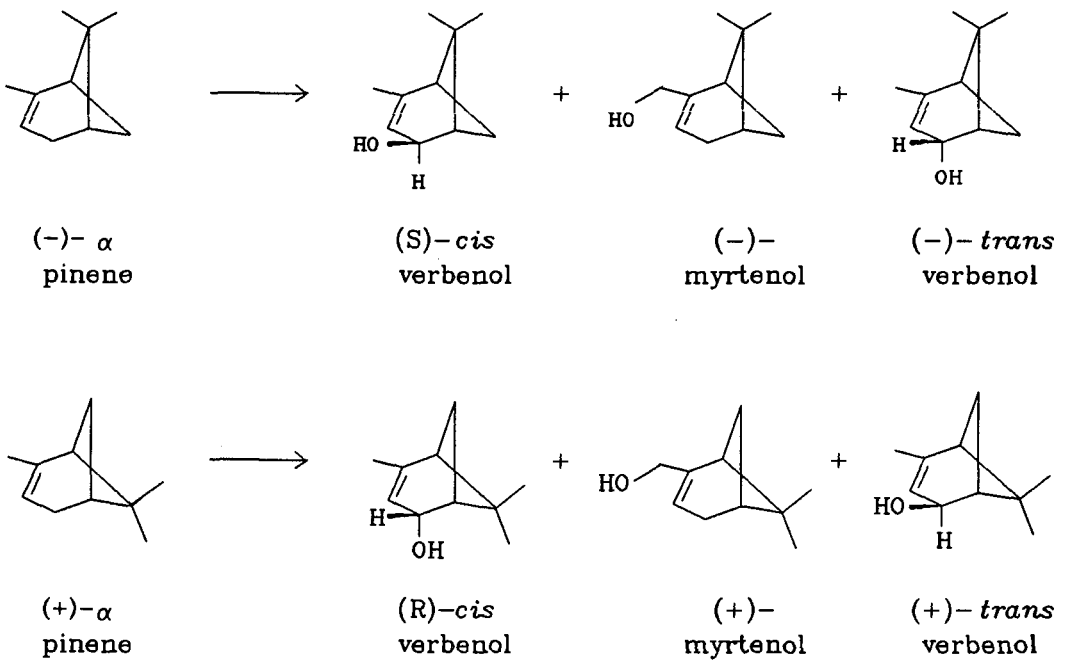


Figure 6: Metabolites of the host monoterpene α-pinene produced by scolytid bark beetles. Products of both isomers of α-pinene are shown.

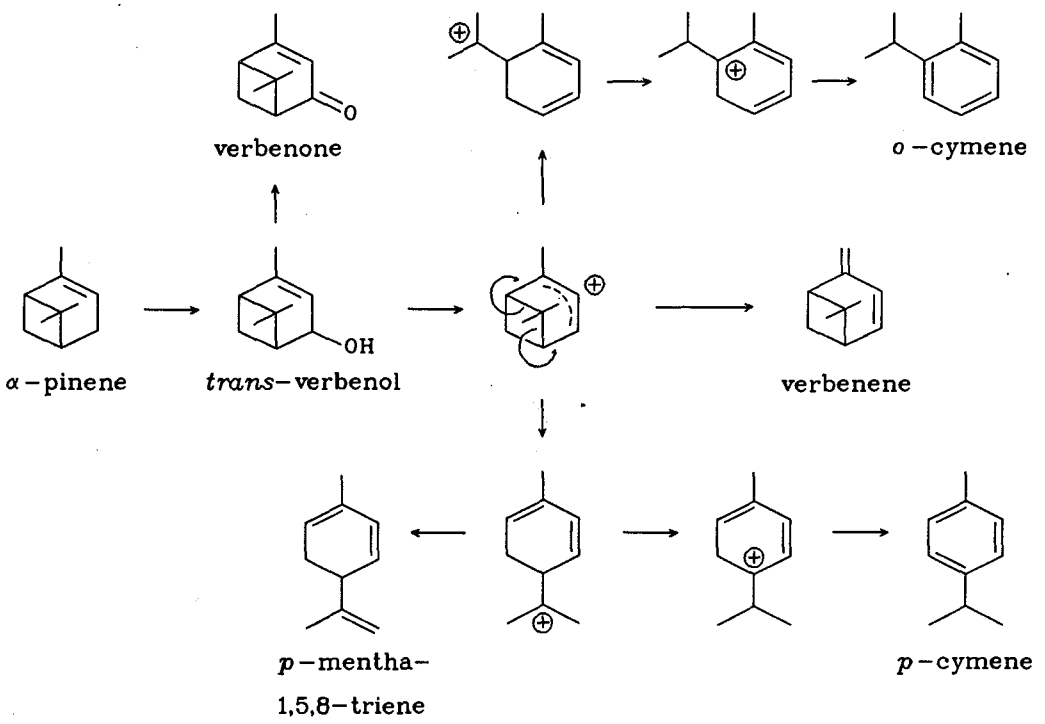


Figure 7: Metabolites of the host monoterpene α-pinene produced by *Dendroctonus* and *Ips* beetles (adapted from Gries *et al.*, 1990b).

Until recently, evidence that α-pinene is metabolized to pheromones was largely circumstantial. Renwick *et al.* (1973) noted that *D. frontalis* produced myrtenol (in addition to other oxidation products) upon exposure to α-pinene. Similarly, *I. paraconfusus* produced (+)- and (-)-myrtenol after exposure to the corresponding enantiomers of α-pinene (Renwick *et al.*, 1976b). Such studies are inconclusive if, as some workers have suggested (Hughes, 1975; Conn *et al.*, 1984), exposure to any monoterpene will trigger pheromone production from sequestered precursors. There is some evidence that *Dendroctonus* beetles are able to sequester host monoterpenes (Hughes, 1975; Rudinsky *et al.*, 1977). Conn *et al.* (1984) demonstrated that *trans*-verbenol production by female *D. ponderosae* was triggered by exposure to 3-carene, which is not generally believed to be a precursor of *trans*-verbenol.

More definitive evidence that α-pinene is metabolized to pheromones

such as *trans*-verbenol was provided by Gries *et al.* (1990b). These investigators demonstrated that female *D. ponderosae* exposed to deuterated α-pinene produced labelled *trans*-verbenol, toluene, verbenene, *p*-mentha-1,5,8-triene, o- and *p*-cymene. They suggested that *trans*-verbenol rearranges to form the latter products (Figure 7). These metabolites were produced in a dose dependent manner when exposed to *trans*-verbenol, and beetles exposed to labelled *trans*-verbenol produced labelled verbenene, *p*-mentha-1,5,8-triene, o- and *p*-cymenes.

Verbenone, a multifunctional pheromone utilized by several scolytid species (Borden, 1985), is considered to be derived from α-pinene through oxidation of the verbenols. Although Hughes (1975) provided circumstantial evidence for this route, with the demonstration that *D. frontalis* males exposed to α-pinene contained about twice as much verbenone as unexposed males, other studies have failed to corroborate this relationship (Renwick *et al.*, 1973; Byers, 1983a, 1983b). Attempts to correlate verbenone production by adult beetles with α-pinene exposure are complicated by the possibility that the precursor may be carried over from earlier developmental stages. Hughes (1975) demonstrated that *D. frontalis* pupae do not complete the metabolism of α-pinene to the known oxidation products, but likely store the terpene as a conjugated intermediate. This could be metabolized to yield the pheromones *trans*-verbenol and verbenone once the insect becomes a mature adult. Another complication of such studies is that symbiotic microorgansms may actually be responsible for verbenone biosynthesis. This aspect is discussed in more detail later.

There is evidence that, at least in those cases where the oxidation products have been adopted for use as pheromones, the metabolism of host monoterpenes can be highly stereo- and enantioselective (Renwick *et al.*, 1976b; Harring, 1978; Fish *et al.*, 1979; Renwick and Dickens, 1979; Klimetzek and Francke, 1980; Byers, 1983a). Due to the pheromonal activity of *cis*- and *trans*-verbenols in several *Dendroctonus* and *Ips*

Table 2: Comparison of isomers of verbenol produced from (+)- and (-)-α-pinene by different species of bark beetles.

Species	Verbenol produced from α-pinene enantiomers[1]			
	(+)-α-pinene		(-)-α-pinene	
	(+)-*trans*	(*S*)-*cis*	(-)-*trans*	(*R*)-*cis*
***Ips* species**[2]				
I. amitinus	-	P[3]	-	-
I. typographus	-	P[3]	-	-
I. paraconfusus	-	P[3]	-	-
***Dendroctonus* species**				
D. brevicomis[4]	+	-	P[5]	-
D. ponderosae[6]	+	-	+	-
***Pityokteines* species**[7]				
P. spinidens	+	+	+	+
P. curvidens	+	+	+	+
P. vorontzovi	+	+	+	+

[1] "+" = present; "-" = absent; "P" = present and is a known pheromone of the species; [2]Renwick *et al.*, 1976b; Klimetzek and Francke, 1980; [3]Borden, 1985, and references cited therein; [4]Byers, 1983a; [5]Bedard *et al.*, 1980; Byers, 1983a; [6]Gries *et al.*, 1990b; [7]Harring, 1978.

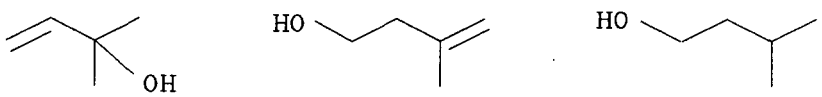
species, the oxidation of α-pinene to these allylic alcohols has been investigated in some depth. As summarized in Table 2, the varying selectivities of the oxidase systems of different species of beetles give rise to different isomeric mixtures of *trans*- and *cis*-verbenol. This may be one way to ensure species specificty of the pheromone message.

An additional source of α-pinene-derived semiochemicals that should be considered is from the auto-oxidation of α-pinene. Upon exposure to air, α-pinene auto-oxidizes to a variety of compounds (Moore *et al.*, 1956), which include *cis*- and *trans*-verbenol and, in turn, verbenone (Borden *et al.*, 1986). It is possible that biologically significant

quantities of these behavior-modifying chemicals are formed when the host tree exudes resin in response to attack (Borden *et al.*, 1986). The possibility that α-pinene-derived semiochemicals are produced independently of insect metabolism has interesting implications on the dynamics of host colonization (Borden *et al.*, 1986).

c) Pheromones synthesized de novo

Bark and ambrosia beetles also use several short chain alcohols as pheromones. These include 2-methyl-3-buten-2-ol (**6**), 3-methyl-3-buten-1-ol (**7**) and 3-methyl-1-butanol (**8**), utilized by *Ips typographus*, *Ips cembrae*, and *Platypus flavicornis*, respectively (Bakke *et al.*, 1977; Stoakley *et al.*, 1978; and Renwick *et al.*, 1977) (Figure 8). These five-carbon, branched-chain alcohols are obviously isoprenoid, but their origin is unknown. They could arise either as degradation products of host monoterpenes, or from *de novo* biosynthesis. Lanne *et al.* (1989) have recently shown that radiolabelled mevalonate is incorporated into **6** by *I. typographus*, indicating that this alcohol can be synthesized *de novo*. For reasons which are not clear, incorporation of radioactivity from glucose or acetate into **6** was not detectable.



6 7 8

Figure 8: Short chain isoprenoid alcohol pheromones.

It is unknown if compounds **6**, **7** and **8** are produced by the insect or by associated microorganisms. The production of **6** and **7** is hormonally controlled in *I. typographus* (Hackstein and Vité, 1978) and *I. cembrae* (Renwick and Dickens, 1979), respectively. It is difficult to envision

insect-regulation of production by microorganisms. Yeasts isolated from *I. typographus* (Leufvén *et al.*, 1984) and a yeast (Brand *et al.*, 1977) and a basidiomycete (Brand and Barras, 1977) associated with *D. frontalis*, produce **8** *in vitro*. However, neither **6** nor **7** were produced by these microorganisms. Moreover, methylbutenol production by *I. typographus* ceases when the tree has been overcome, even though microorganism populations are still increasing (Lanne *et al.*, 1989). This circumstantial evidence supports suggestions that **6** and **7**, at least, are synthesized *de novo* by the insects themselves.

2. Biosynthesis of 2-phenylethanol

2-Phenylethanol is believed to be an aggregation pheromone of *I. paraconfusus* (Renwick *et al.*, 1976a; Borden, 1985). The male noctuid moth *Mamestra configurata*, which also uses 2-phenylethanol as a pheromone, converts the amino acid phenylalanine to the pheromone in a multistep pathway believed to proceed as shown in Figure 9 (Clearwater, 1975; Weatherston and Percy, 1976).

It seems likely that 2-phenylethanol is similarly derived in bark beetles. High levels of phenylalanine (the precursor of lignin) are believed to be present in the phloem (Gries *et al.*, 1990c). Gries *et al.* (1990c) demonstrated that male *Ips pini* exposed to deuterium-labelled phenylalanine produced deuterium-labelled 2-phenylethanol. Interestingly, labelled toluene was also produced. The significance of this is not clear: toluene is itself highly toxic and did not appear to have pheromonal activity in field tests (Gries *et al.*, 1990c).

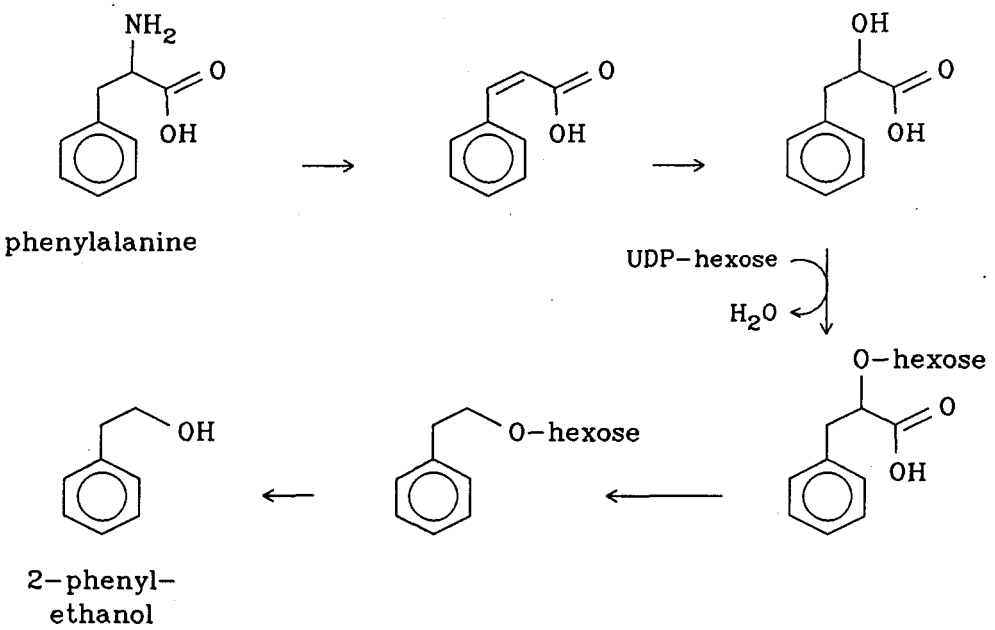
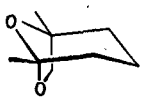


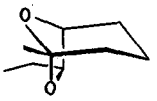
Figure 9: Biosynthesis of 2-phenylethanol from phenylalanine by male *Mamestra configurata*.

3. Biosynthesis of cyclic ketal and acetal pheromones

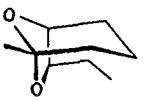
Despite advances in our knowledge of coleopteran pheromone biosynthesis on several fronts, virtually nothing is known about the biosynthesis of bicyclic ketal and tricyclic acetal pheromones. These compounds include frontalin, *exo*- and *endo*-brevicomin, multistriatin, lineatin, chalcogran, and (*E*)-7-methyl-1,6-dioxaspiro[4.5]decane, **9** (Figure 10). It has been suggested that multistriatin, chalcogran, and **9** are derived *in vivo* through the cyclization of acetogenins (Bradshaw, 1985; Philips *et al.*, 1985; Vanderwel and Oehlschlager, 1987).



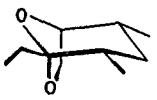
frontalin



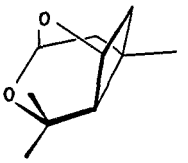
exo-brevicomin



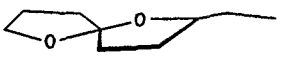
endo-brevicomin



multistriatin



lineatin



chalcogran

9

Figure 10: Cyclic ketal and acetal pheromones.

White *et al.* (1980) have proposed that frontalin, *exo*-, and *endo*-brevicomin are synthesized through specialization of a "hormone type" of metabolic pathway. Silverstein (cited in White *et al.*, 1980) speculated that the biosynthetic pathways may, alternatively, be similar to laboratory routes to these pheromones which utilize short chain, dihydroxy ketones (e.g. **10**, Figure 11) as precursors. The logical biosynthetic precursor of **10** is 6-methyl-6-hepten-2-one (**11**, Figure 11) which could in turn be derived from 6-methyl-5-hepten-2-one (**12**) or sulcatol (**13**) (Brand *et al.*, 1979). The latter two compounds are present in volatiles of the Douglas-fir beetle (Butterfield, 1984; Madden *et al.*, 1987). Brand *et al.* (1979) suggested that microbial symbionts may supply beetles with biosynthetic precursors to frontalin, since mycangial fungi of female *D. frontalis* produce **12** (Brand and Barras, 1977).

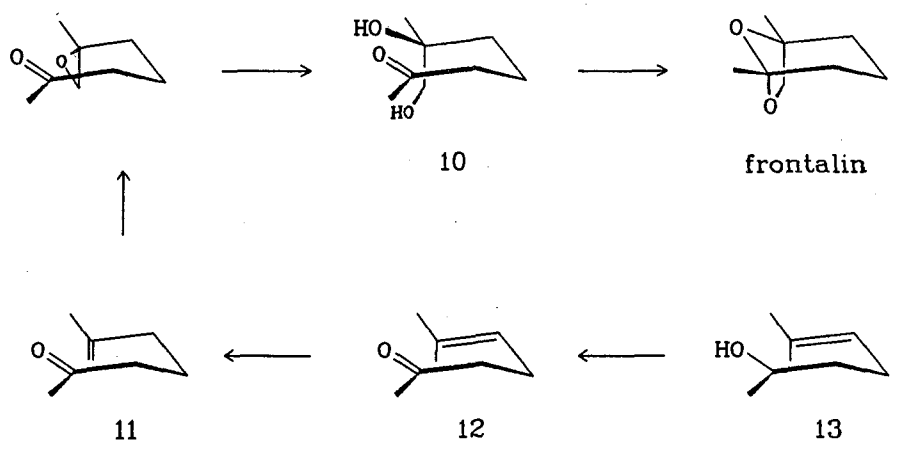


Figure 11: Speculative biosynthetic route to frontalin.

Information concerning the origin of the tricyclic acetal lineatin is also vague. Francke and Vité (1983) pointed out the structural similarity between lineatin and monoterpenoid compounds such as myrcene and grandisol. It is conceivable that lineatin is indeed derived through the oxidation and cyclization of a monoterpenoid precursor (Figure 12), but as yet no experimental evidence for this route is available.

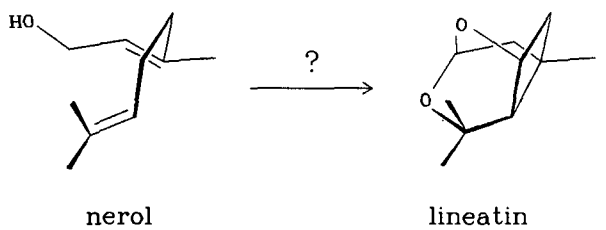


Figure 12: Speculative biosynthetic route to lineatin from a monoterpenoid precursor.

C) Role of microorganisms in "pheromone" biosynthesis

The ability of coleopterans to colonize their host is, in many cases, augmented by their association with microorganisms. For example, attacking scolytids distribute pathogenic fungi which help to render the

host incapable of impairing beetle development (Graham, 1967). Symbiotic microorganisms may also play a role in the production or degradation of chemicals involved in mediating coleopteran behavior.

Examination of the metabolites of coleopteran symbionts has revealed that in some cases, the microorganisms produce the same compounds that are utilized as pheromones by their host. Such symbionts include bacteria isolated from the grass grub beetle, *Costelytra zealandica* (Hoyt *et al.*, 1971), a *Serratia* species isolated from the bark beetle *Phleosinus armathus* (Chararas *et al.*, 1980), and a *Bacillus cereus* strain isolated from *I. paraconfusus* (Brand *et al.*, 1975). This is not surprising since microorganisms, exposed to many of the same compounds as their hosts, could by coincidence metabolize some to the same end products. Thus, the relative contributions by both symbiont and insect to overall pheromone production must be considered.

This is illustrated by earlier work on a *Bacillus cereus* strain isolated from *Ips paraconfusus*. Brand *et al.* (1975) reported that this microorganism was capable of the oxidation of α-pinene to *cis*- and *trans*-verbenol. Subsequent experiments by Byers and Wood (1981) indicated that streptomycin sulfate, to which *B. cereus* is susceptible, did not reduce the production of *cis*-verbenol by *I. paraconfusus*. These results are not necessarily conflicting: *B. cereus* may produce *cis*- and *trans*-verbenol, but this production may make a neglible contribution to total pheromone production by *I. paraconfusus*.

In order to assess the importance of the microbial contribution to overall pheromone production, some studies have attempted to ascertain the metabolic capability of insects made as devoid as possible of their natural *endo*- (intracellular) and *exo*- (extracellular) symbionts. The surface sterilization of eggs, followed by axenic rearing to adulthood on asceptic host material, has been widely used to obtain adults free of readily culturable exosymbionts (Whitney and Spanier, 1982; Conn *et al.*, 1984; Hunt and Borden, 1989). The populations of endosymbionts have been

reduced by administration of membrane-permeable antibiotics to wild (contaminated) adults (Chararas, 1980; Byers and Wood, 1981; Hunt and Borden, 1989).

Experiments with *I. paraconfusus* and *D. ponderosae* have been conducted utilizing these techniques (Conn *et al.*, 1984; Hunt and Borden, 1989). The males of both species convert myrcene to ipsdienol. Normal levels of ipsdienol were produced by axenically-reared and by streptomycin-treated male *D. ponderosae* (Conn *et al.*, 1984; Hunt and Borden, 1989). Thus there is no apparent microbial involvement in ipsdienol production by male *D. ponderosae*. In contrast, ipsdienol production was repressed (but not halted) in streptomycin-treated (Byers and Wood, 1981) and in axenically-reared (Hunt and Borden, 1989) male *I. paraconfusus*. Thus both insectival and microbial involvement are indicated in ipsdienol production by male *I. paraconfusus*.

The meaning of these results is difficult to assess. The decreased ipsdienol production noted in streptomycin-treated *I. paraconfusus* could be due to non-target toxic effects of streptomycin (Gilman *et al.*, 1985). The decreased ipsdienol production observed in axenically-reared insects might be due to the use of a rearing medium of pasteurized phloem. The greatly reduced monoterpene content in this diet could affect the ability of the adult to produce ipsdienol. Insects reared on such a diet would be exposed to only low levels of monoterpenes during larval and teneral adult feeding. The importance of prior exposure to pheromone production by the adult has not been well studied. It is conceivable that PSMO-induction and/or monoterpene-sequestration in earlier life stages could effect higher levels of pheromone production by the adult. Some examples from the literature that support this hypothesis have been presented in this review. Hughes (1975) demonstrated that *D. frontalis* exposed to α-pinene as pupae produced more *trans*-verbenol as adults than beetles that had not been exposed. Conn *et al.* (1984) demonstrated that female *D. ponderosae* could be triggered to produce *trans*-verbenol without exposure to α-pinene.

Pheromone production by male *I. paraconfusus* was stimulated by JH III treatment, without exposure to myrcene (Borden *et al.*, 1969).

Both sexes of *I. paraconfusus* and *D. ponderosae* metabolize α-pinene to *cis*- and *trans*-verbenol and myrtenol. Axenically-reared male *I. paraconfusus* and female *D. ponderosae* contain significantly more α-pinene metabolites than wild beetles, whereas axenically-reared female *I. paraconfusus* contain near normal levels of α-pinene metabolites (Conn *et al.*, 1984; Hunt and Borden, 1989). Similarly, both sexes of wild *D. ponderosae* contained up to six times more α-pinene metabolites after ingestion of a host phloem diet containing streptomycin sulfate (Hunt and Borden, 1989). Thus, in both species studied, reduction of the symbiont populations does not reduce pheromone content, and may actually result in its increase.

These observations parallel those on boll weevils by Gueldner *et al.* (1977). Weevils that were relatively free of bacteria produced significantly more pheromone than those that were heavily contaminated. Increased pheromone production in microbe-suppressed insects could simply be due to healthier insects (Gueldner *et al.*, 1977). Alternatively, the symbionts present in wild (contaminated) insects could decrease pheromone content through competition with the beetle for substrate or through use of the beetle-produced pheromone as a substrate (Conn *et al.*, 1984). Such activities could, under natural conditions, serve to regulate pheromone levels (Conn *et al.*, 1984).

In an extension of this idea, it has been suggested that microorganisms may be involved in the conversion of aggregation pheromones to compounds that inhibit attraction (Léufven *et al.*, 1984). Supporting evidence is provided by studies of the ability of various symbiont isolates to metabolize α-pinene-derived pheromones. Several yeast isolates of the spruce bark beetle, *Ips typographus*, were shown to interconvert the chiral isomers of *cis*- and *trans*-verbenol with the respective verbenones (Leufvén *et al.*, 1984). Verbenone, an anti-

aggregation pheromone found in wild female *D. ponderosae*, was not produced by axenically-reared or streptomycin-fed beetles (Hunt and Borden, 1989). Mycangial fungi of *D. frontalis* perform the verbenol to verbenone conversion as well as oxidation of seudenol, an aggregation pheromone of *D. pseudotsugae*, to 3-methylcyclohex-2-en-1-one (Brand *et al.*, 1976), an antiaggregation pheromone of this species. The conversion of scolytid aggregation pheromones to antiaggregation pheromones by symbionts could play a significant role in terminating the attack of a successfully colonized host (Raffa and Berryman, 1983; Leuven *et al.*, 1984; Borden *et al.*, 1986; Hunt *et al.*, 1989; Hunt and Borden, 1990).

CHAPTER II

MACROLIDE (CUCUJOLIDE) BIOSYNTHESIS IN CUCUJID GRAIN BEETLES

A) INTRODUCTION

This laboratory has been involved in the isolation, identification, and synthesis of the aggregation pheromones of five economically important species of cucujid grain beetles: *Cryptolestes ferrugineus* (Stephens), *C. pusillus* (Schönhen), *C. turcicus* (Grouvelle), *Oryzaephilus mercator* (Fauvel), and *O. surinamensis* (L.) (Borden *et al.*, 1979; Wong *et al.*, 1983; Millar *et al.*, 1985a,b; Pierce *et al.*, 1984 a,b, 1985). Males were found to produce seven structurally similar macrocyclic lactones (Figure 13), given the trivial name "cucujolides" (Oehlschlager *et al.*,1988). Both males and females are attracted to species-specific combinations of these cucujolides (Oehlschlager *et al.*, 1987).

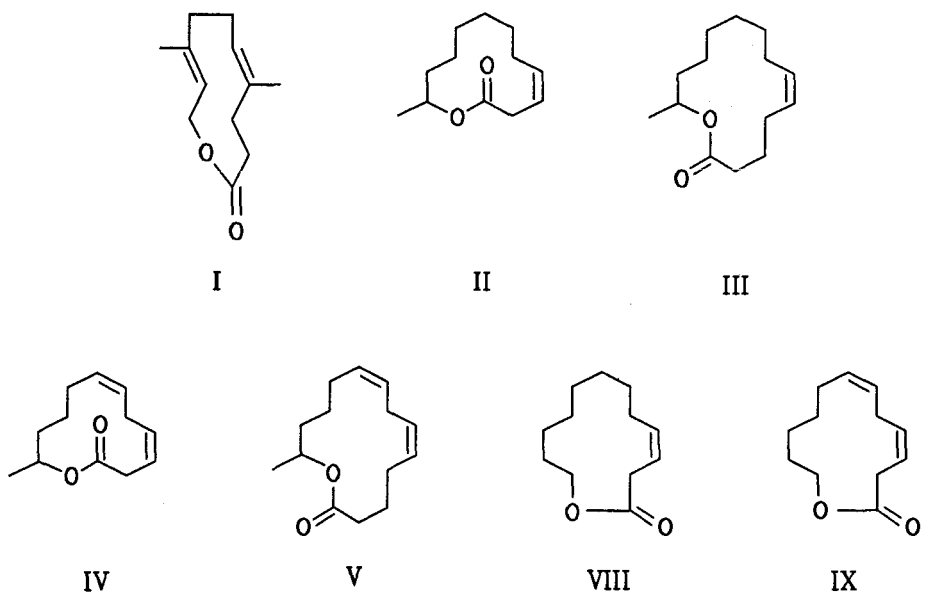
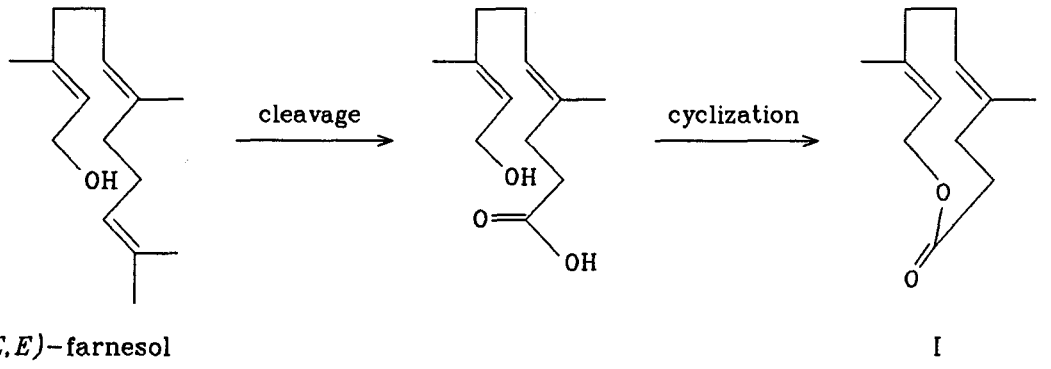


Figure 13: Cucujolides used as aggregation pheromones by cucujid grain beetles (numbered according to Oehlschlager *et al.*, 1988): **I**, 4(*E*),8(*E*)-4,8-dimethyldecadien-10-olide; **II**, 3(*Z*)-dodecen-11-olide; **III**, 5(*Z*)-tetradecen-13-olide; **IV**, 3(*Z*),6(*Z*)-dodecadien-11-olide; **V**, 5(*Z*),8(*Z*)-tetradecadien-13-olide; **VIII**, 3(*Z*)-dodecen-12-olide; and **IX**, 3(*Z*),6(*Z*)-dodecadien-12-olide.

Cucujolide **I** was hypothesized to be of terpenoid origin, due to the characteristic branching pattern and the *E* configuration of the double bonds. Farnesol could conceivably be transformed to **I** through oxidative cleavage of the terminal double bond, followed by cyclization, as shown in Figure 14 (Pierce *et al.*, 1984b; Vanderwel and Oehlschlager, 1987). Oxidative cleavage of the terminal double bond of farnesol has been reported to occur during farnesol catabolism by isolated embryonic *Drosophila* cells (Gonzalez-Pacanowska *et al.*, 1988).



(E,E)-farnesol I

Figure 14: Proposed biosynthesis of cucujolide **I** from (*E,E*)-farnesol.

The other cucujolides (**II-V, VIII** and **IX**) were hypothesized to be of fatty acid origin due to the position (6-7 and/or 9-10 from the terminal carbon) and *Z* geometry of the double bond(s) (Pierce *et al.*, 1984b; Vanderwel and Oehlschlager, 1987). Plausible biosynthetic routes from the fatty acids to cucujolides **II-V, VIII** and **IX** would involve: (1) two (or three) cycles of ß-oxidation; (2) hydroxylation at the penultimate or terminal carbon; and (3) cyclization of the hydroxy-acid intermediate to the macrolide as shown in Figure 15. Steps 1 and 2 could occur in any order. Evidence for the ω-1 oxidation of fatty acids has been reported for termites (Prestwich *et al.*, 1985). The shortening of fatty acid chains by ß-oxidation occurs in the biosynthesis of many lepidopteran pheromones (Roelofs and Bjostad, 1984; Bjostad *et al.*, 1987).

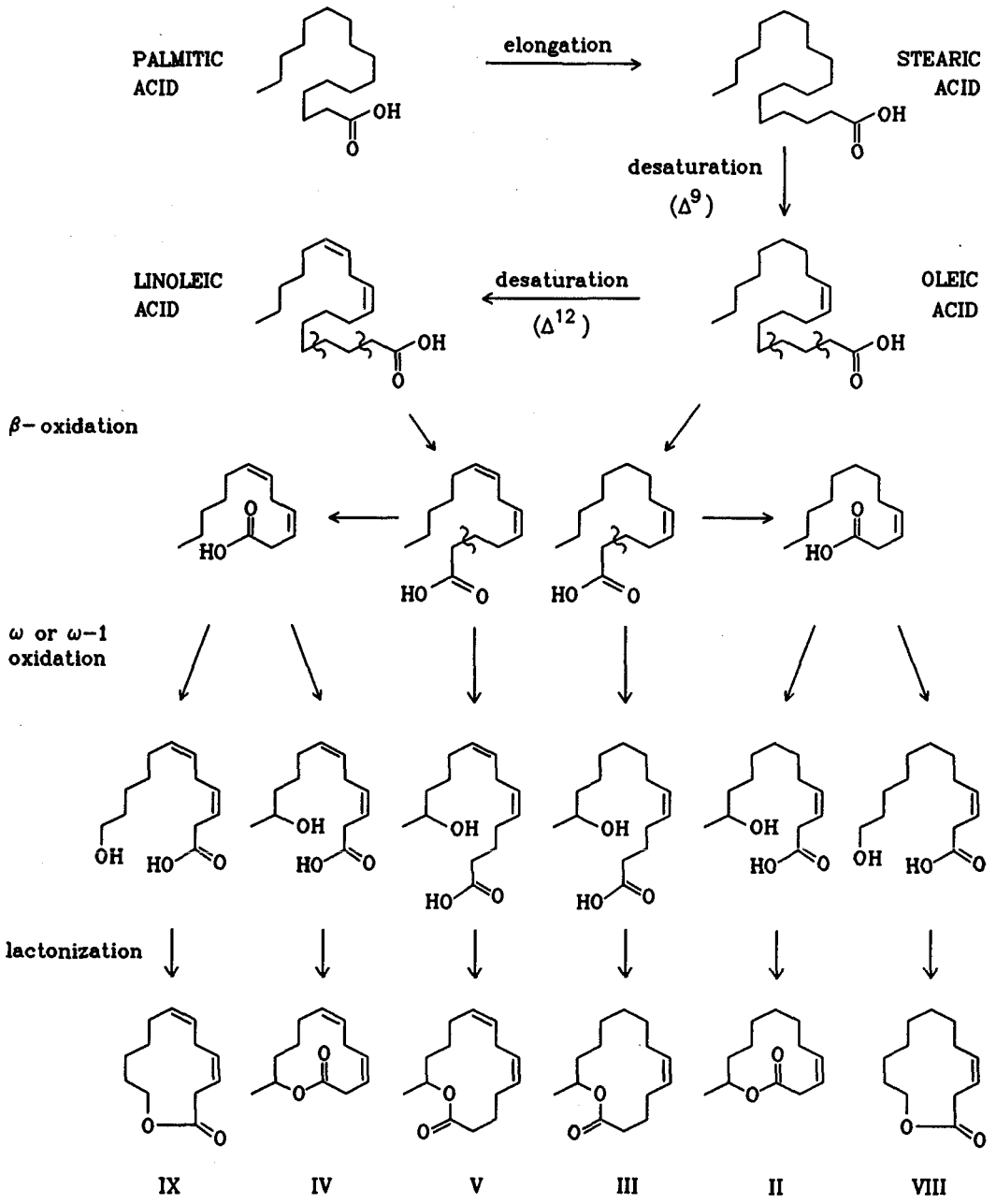


Figure 15: Proposed biosynthesis of cucujolides **II–V, VIII** and **IX** from fatty acids.

The objectives of this project were to:

1. test the hypothesis that cucujolide I is of terpenoid origin;
2. test the hypothesis that the other cucujolides are of fatty acid origin;
3. determine if the cucujolides can be synthesized *de novo*;
4. determine if farnesol can serve as a precursor of cucujolide I *in vivo*;
5. study the stereochemistry of the lactonization reactions leading to cucujolides I and II.

Most studies were performed with *C. ferrugineus* since this species produces five of the cucujolides (I to V) at relatively high levels. However, since *C. ferrugineus* produces *(S)*-II while *O. mercator* produces *(R)*-II (Oehlshlager *et al.*, 1987), the stereochemistry of cucujolide II biosynthesis was examined in both species.

B) EXPERIMENTAL

1. Insects:

C. ferrugineus and *O. mercator* were each reared on 1 kg of rolled oats and brewer's yeast (95/5, w/w) in 3.8 L glass jars at 32 °C and 65% relative humidity (Pierce *et al.*, 1981). Beetles were separated from the oats with a coarse sieve and collected by aspiration as they crawled away from the siftings. The beetles (2.5 g) were then allowed to feed for 7-10 days on 200 g of fresh oats in a cylindrical glass chamber (6 x 10 cm) through which humidified, purified air was drawn. The insects were separated from the oats as described above, and were either transferred directly to culture chambers containing substrate-laced oats (prepared as described below) or were starved for 48 h before transfer (in order to deplete endogenous fatty acid pools and to increase the level of incorporation of exogenously added substrate). During the starvation period, insects were maintained in a glass chamber through which humidified, purified air was drawn.

2. Preparation of experimental cultures:

A measured quantity (specified in tables of results in *Results and Discussion*) of labelled compound was dissolved in 200 mL of pentane, diethyl ether, or ethanol (depending on solubility) in a 500 mL round-bottomed flask. Oats (usually 30 g) were added and the solvent was removed *in vacuo*. Remaining traces of solvent were removed under high vacuum. In several experiments the oats were first "defatted" by Sohxlet extraction with diethyl ether for 30 min.

Oats laced with labelled precursors were transferred from the round-bottomed flask to an aeration chamber *via* a short glass tube fitted with ground glass joints at either end, to prevent the escape of radioactive dust. Mixed-sex beetles (2.0 g, ca. 6000 individuals) were introduced into the aeration chamber.

3. Capture and isolation of culture volatiles:

Culture volatiles were captured on a Porapak Q trap for 10 days at 23-27 °C, by a technique similar to that previously reported (Pierce *et al.*, 1984b). Humidified and purified air was drawn first through a culture of beetles feeding on the labelled oats in a cylindrical glass chamber (6 x 10 cm) and then through a glass trap (13 x 0.8 cm) packed with Porapak Q (50-80 mesh, Applied Science Laboratories). Porapak Q was subjected to Soxhlet extraction with diethyl ether, air dried, and then packed into the trap. Residual ether was removed by forcing helium through the trap, which was warmed to about 65 °C. Captured volatiles were removed from the Porapak Q by back-flushing the trap with 20 mL of 0.1% (v/v) isopropyl alcohol (IPA) in pentane. Although some compounds remained in the Porapak Q after this procedure, no cucujolides were detectable by GC in subsequent 10 mL washes with anhyd diethyl ether.

4. Analyses of cucujolides:

Analyses by gas chromatography (GC) were performed on a Hewlett-Packard 5830A gas chromatograph equipped with a 18835B capillary inlet system and a flame-ionization detector (FID). Samples were introduced by splitless injection onto a glass open-tubular column (30 m x 0.55 mm I.D.) coated with SP-1000. The oven temperature program was: 100°C for 2 min, then 4°C/min to a final temperature of 180°C for 20 min. Cucujolides were identified by comparing their GC retention times with standards. Quantification of cucujolides in soln was accomplished by using methyl myristate as an internal standard.

5. Isolation, purification and analyses of cucujolides:

Initial separation of the cucujolides was carried out by high performance liquid chromatograph (HPLC) on a Varian LC5000 equipped with a series 634 UV/Visible Spectrophotometer. The 0.1% IPA in pentane soln of Porapak Q-trapped volatiles was cooled in ice and concentrated to 75 μL by

a gentle nitrogen stream. This sample (plus a 25 μL hexane rinse) was injected onto a Micropak SI-10 column (30 cm x 4 mm I.D.) which was equilibrated with 0.1% IPA in hexane. The column was eluted with a gradient of IPA in hexane (0.1% for 3 min, 0.1% to 2.0% from 3 to 23 min, and 2% to 10% from 23 to 43 min) at a flow rate of 2 mL/min. Effluent was monitored at 213 nm and fractions were collected as peaks emerged (or in 2 mL volumes, whichever was less). Fractions were analyzed by analytical GC.

HPLC fractions containing cucujolides were further purified by preparative GC on a Varian model 1200 gas chromatograph equipped with a 10:1 effluent splitter, FID and thermal gradient collector. The fractions were concentrated to 30 μL as described above, and injected onto a stainless steel column (3.0 m x 3.2 mm I.D.) packed with 10% SP-1000 on Chromosorb W (100/120). Samples were eluted isothermally at 180 °C and collected in glass tubes (20 cm x 1.6 mm O.D.). The collected samples were washed into half-dram glass vials, usually with 1.0 mL pentane. The purity of collected samples was determined by analytical GC analysis, as previously described.

a) Analyses of incorporation of radiolabelled precursors:

A portion of each sample (typically 250 μL) was counted in Liquifluor (a PPO-POPOP/toluene-based scintillation cocktail, New England Nuclear) on a Beckman LS 8000 scintillation counter. If sufficient quantities of sample remained, this was concentrated and subjected to an additional cycle of preparative GC and subsequent analyses to determine if the specific activity was constant.

For comparison, the specific activity of each labelled cucujolide was calculated. Raw scintillation counting results (in counts/min) were converted to mCi (2.22 x 10^9 decompositions/min) by subtracting the background level of counts/min and correcting for quench (T was counted with 45% efficiency; ^{14}C with 92%). The cucujolides were quantified by

analytical GC, using methyl myristate as an internal standard. Errors in the specific activity were calculated considering volume measurements and twice the standard deviation of the scintillation counting results.

b) Analyses of incorporation of stable isotope-labelled precursors:

GC/MS were recorded on a Hewlett-Packard 5985B GC/MS system by electron impact (EI, 70 eV) or by chemical ionization (CI, using isobutane as the ionizing gas). The GC/MS of the compounds were obtained first in order to confirm the identity of the compounds and to obtain the exact masses of the ions of interest. Then the run was repeated, scanning only for the ions of interest. In cases where the GC was equipped with a DB-1 column (30 m x 0.25 mm I.D.; 0.25 μm film thickness), satisfactory separation of the cucujolides was not achieved. Therefore the Porapak Q extracts were subjected to preliminary purification by HPLC and/or preparative GC as described previously. In cases where the GC was equipped with the SP-1000 column, the Porapak Q extracts were analyzed without preliminary purification.

Unlabelled samples were always analyzed by GC/SIM-MS at the same time as the labelled samples. The absolute values of the relative abundances shifted slightly from day to day (due to such factors as how recently the MS was tuned, the integrity of the GC column, *etc.*), although the difference between two values (of unlabelled and labelled samples) was relatively constant. Authentic samples of unlabelled compounds isolated from insects the same way as the experimental samples were used to minimize potential errors due to the comigration of impurities that might have a MS fragment of the same m/e ratio as the one of interest.

c) Analyses of chirality of cucujolide II

The enantiomeric composition of cucujolide **II** was determined by derivatization with acetyl-(*S*)-lactyl chloride. The ratios of the resulting diastereomeric derivatives were then determined by analytical GC

(Slessor *et al.*, 1985).

Synthesis of acetyl (*S*)-lactyl chloride reagent

The reagent was prepared as described by Slessor *et al.* (1985). *(S)*-(+)-Lactic acid (22 mmol, Sigma) was dissolved in freshly distilled acetylchloride (47 mmol). After the soln was stirred for 2 h at RT under inert atmosphere, the acetylchloride remaining in excess was removed *in vacuo*. Thionylchloride (62 mmol) was added, and the soln was stirred for 8 h at RT. Excess thionylchloride was removed *in vacuo*. The remaining crude acetyl-*(S)*-lactyl chloride was distilled under vacuum (b.p. 52-54 °C, 8.2 mm Hg), yielding 1.81 g (49% yield) product (>99% pure by GC analysis).

Derivatization of cucujolide II

In glass ampoules, cucujolide **II** (up to 10 μg of either crude Porapak Q extracts, or isolates purified by preparative GC) was dissolved in 50-100 μl methanol containing 5% boron trifluoride-etherate. The ampoules were cooled on dry ice, sealed, then kept at 67 °C overnight. Samples were worked up by adding 200 μL pentane and washing three times with 200 μl water. The pentane extracts were dried by filtration through a mini-column, containing ca. 200 mg anhyd Na_2SO_4 in a pasteur pipette. The column was rinsed with 200 μl pentane.

The pentane was evaporated by blowing with a gentle Ar stream. Pyridine [50 μl of an 8% (v/v) soln in anhyd methylene chloride, containing one crystal of DMAP], then acetyl-*(S)*-lactyl chloride [50 μl of a 6% (v/v) soln in anhyd methylene chloride], were then added with mixing. The mixture was allowed to stand for several hours, before being worked up as described above.

The proportion of each diastereomer was determined by analytical GC on a Hewlett-Packard 5890 GC equipped with a capillary inlet system and an FID. Samples were introduced by splitless injection onto a DB-1 column

(15 m x 0.25 mm I.D.; 0.25 μm film thickness). The oven temperature program was: 70°C for 0 min, then 5°C/min to a final temperature of 250°C. The *R* isomer was assumed to elute first, as reported by Slessor *et al.* (1985). Samples of *(S)*-**II** and *(R)*-**II** (from rusty and merchant grain beetles, respectively) were coinjected to ensure that baseline separation of the two isomers was achieved.

d) Isolation, purification, and analyses of unknowns:

GC analyses of the Porapak Q extracts revealed that, in some cases, exposure of the insects to the precursors of interest induced the production of compounds not normally observed. Wherever possible, these metabolites were isolated and identified.

Three unknown compounds, designated **A**, **B**, and **C**, were observed in the Porapak Q extracts of both the *C. ferrugineus* and *O. mercator* cultures exposed to [3,4-D_2]-3-dodecenoic acid. The three unknowns produced by the two beetle species exhibited the same GC retention time and MS (EI), and were assumed to be identical. Unknowns **A** and **B** were isolated from the *C. ferrugineus* volatiles by preparative GC (as described previously). Unknown **C** was isolated from the *O. mercator* volatiles. The samples were eluted directly from the glass collection tubes into NMR tubes with $CDCl_3$. The MS (both EI and IBCI) and ^1H NMR (400 MHz) spectra of the three unknowns are presented in the *Results and Discussion*.

Three compounds of the same retention times as unknowns **A**, **B**, and **C** were also observed in the Porapak Q extract of the *C. ferrugineus* culture exposed to [3,4-D_2]-3-dodecanoic acid. The compounds co-eluting with unknowns **B** and **C** were not produced in large enough quantities to isolate, but they exhibited the same MS (EI). The compound co-eluting with unknown **A** exhibited a MS (EI) closely related, but not identical, to that of unknown **C**. This compound was designated as unknown **D**, and isolated by preparative GC as described above.

Relatively large quantities of a compound designated unknown **E** was

observed in the Porapak Q extracts of the aerations with both [1-D]-*(E,E)*-farnesol and [1-D,1-^{18}O]-*(E,E)*-farnesol. After initial purification by HPLC and preparative GC, as described previously, the two isolates were found to exhibit the same GC retention time and MS fragmentation pattern. Therefore the two isolates were combined for final purification by preparative GC (>98% pure by analytical GC). The sample was eluted directly from the glass collection tube into an NMR tube with $CDCl_3$, and identified as geranyl acetate (6,10-dimethyl-deca-5,9-dien-2-one). ^{1}H NMR ($CDCl_3$): δ 1.61 (3 H, s, vinyl CH_3); 1.63 (3 H, s, vinyl CH_3); 1.68 (3 H, d, $J_{6\text{-}CH3,5}$=1 Hz, 6-CH_3); 1.95-2.00 (2 H, t, $J_{7,8}$=8 Hz, 7-H); 2.00-2.10 (2 H, q, J=8 Hz, 8-H); 2.15 (3 H, s, 1-H); 2.22-2.31 (2 H, q, J=7 Hz, 4-H); 2.43-2.50 (2 H, t, $J_{3,4}$=7 Hz; 3-H); 5.05-5.12 (2 H, m). MS (EI) m/e (relative abundance): 194 (M^+, 10); 176 (12); 161 (14); 151 (100); 136 (72); 126 (38); 121 (20); 107 (50); 93 (20); 69 (60). MS (CI) m/e (relative abundance): 195 (M+1, 42); 177 (100). IR: 3220 (m); 2905 (m); 2900 (s); 2840 (m); 2380 (m); 1710 (s); 1440 (s); 1100 (m); 810 (s) cm^{-1}.

6. Lipid analyses of oats:

a) Method 1: Bligh and Dyer extraction of "defatted" oats:

Rolled oats (49.0 g) were subjected to Soxhlet extraction with 500 mL diethyl ether for one hour. The ether layer was concentrated *in vacuo*, to yield a yellow oil (3.613 g, 7.37%). This oil was dissolved in diethyl ether. A portion of the extract was treated with diazomethane to form the methyl esters of the fatty acids. This derivatized fraction was analyzed for fatty acid methyl esters and farnesol by analytical GC on a Hewlett-Packard 5890 GC equipped with a capillary inlet system and an FID. Samples were introduced by splitless injection onto a DB-1 column (15 m x 0.25 mm I.D.; 0.25 μm film thickness). The oven temperature program was: 75°C for 2 min, then 10°C/min to a final temperature 250°C for 2 min. The fatty acid methyl esters and farnesol were identified by comparison of their GC retention times and MS fragmentation patterns with

standards. Mass spectra were obtained on a Hewlett-Packard 5985 GC/MS system at 70eV.

The remaining "defatted" oats were extracted by an adaptation of Bligh and Dyer's (1959) procedure. A higher solid/liquid ratio was used, to keep volumes at workable levels. A crystal of dihydroquinone (to prevent oxidation), $CHCl_3$ (25 mL), methanol (50 mL), and distilled water (20 mL) were added to the rolled oats (49 g, which were 10% water). Thus the volumes of solvents added brought the $CHCl_3$/MeOH/H_2O ratio to 25/50/20. The oats were ground by mortor and pestle for about 10 min. Then 25 mL $CHCl_3$ and 25 mL 0.034% aqueous $MgCl_2$ were added (to bring the $CHCl_3$/MeOH/H_2O ratio to 50/50/45). Aqueous $MgCl_2$ was used instead of pure H_2O, to decrease the extraction of lipids into the upper aqueous phase (Folch *et al.*, 1957). The mixture was mixed well and filtered. The two layers were separated by bench-top centrifugation. The upper phase (mostly MeOH and H_2O) was gently removed by Pasteur pipette. The lower phase (mostly $CHCl_3$, containing the lipids) was washed with "upper phase wash" (3 x 1 mL of the top layer from a mixture of 4 mL $CHCl_3$, 4 mL MeOH, and 3.6 mL 0.034% $MgCl_2$). The lower layer was concentrated *in vacuo* to 0.489 g (1%) of yellow oil. This oil was dissolved in diethyl ether, and a small portion derivatized with diazomethane. This extract was analyzed for farnesol and fatty acid methyl ester content by analytical GC and MS, as described previously.

b) Method 2: Saponification of extracted lipids

Rolled oats (45.0 g) were ground with mortor and pestle, and extracted by Soxhlet extraction with anhyd diethyl ether for 15 h. The solvent was removed *in vacuo* to an orange-brown oil (3.186 g, 7.1%). This was transferred to a 50 mL round bottomed flask, equipped with a gas inlet tube, reflux condensor, and a magnetic stir bar. A soln of 4.0 g KOH, 4.0 mL H_2O, and 16.0 mL ethanol was added and the soln was refluxed under Ar. After 3 h, the soln was cooled, diluted with 20 mL H_2O, and extracted with

CH_2Cl_2 (4 x 40 mL). The organic extracts were combined, washed with water until neutral, and washed once with brine (20 mL). The organic extract was then dried over anhyd $MgSO_4$ and filtered. The solvent was removed *in vacuo* to a yellow oil (0.437 g, 0.97%). A small portion of this nonsaponifiable oil was dissolved in diethyl ether and analyzed by GC and MS for farnesol as described previously. The sample was not derivatized with diazomethane, since fatty acids were expected to be present in the aqueous phase after saponification.

This procedure was repeated, using oats (40.0 g) that had been finely ground in a coffee grinder and extracted by refluxing with stirring in 500 mL of diethyl ether for 5 h. The diethyl ether was cooled, centrifuged and filtered to remove the solids. The solvent was removed *in vacuo* to a dark orange oil. This oil was saponified as described above to give nonsaponifiable oil (0.164 g, 0.41%). This oil was dissolved in ether and analyzed as described above.

A small portion of farnesol (mixture of isomers, 83% pure by analytical GC, with a *Z,Z : Z,E/E,Z : E,E* ratio of 1 : 2.9 : 2.3) subjected to saponification was found to remain unchanged by analytical GC. The recovered farnesol was 84% pure by GC (final ratio of isomers 1 : 3.1 : 2.3).

7. Purchased/donated chemicals:

[1-^{14}C]-Lauric acid (5.3 mCi/mmol) was purchased from ICN Pharmaceuticals. [U-^{14}C]-Linoleic acid (900.0 mCi/mmol), (R,S)-[5-T]-mevalonolactone (24.0 Ci/mmol), [9,10-T]-oleic acid (9.5 Ci/mmol), [9,10-T]-palmitic acid (30.0 Ci/mmol) and [1-^{14}C]-sodium acetate (61 mCi/mmol) were obtained from New England Nuclear. The sodium salt of [5-T]-mevalonolactone was prepared by hydrolysis with 0.01 M NaOH in sterile water. [9,10-T]-Oleic acid and [U-^{14}C]-linoleic acid were purified by preparative thin layer chromatography on Silica Gel (Merck) using hexane/diethyl ether/acetic acid (70/30/1, v/v/v) as eluent.

[8,8-D_2]-Stearic acid and [16,16,16-D_3]-palmitic acid were generously donated by Professor Robert J. Cushley*.

$NaBD_4$ (98 atom % D) and $NaBD_3CN$ (98 atom % D) were purchased from Sigma; $^{18}OH_2$ (97 atom % ^{18}O) from both MSD Isotopes and Sigma. Palladium (10%) on activated carbon was purchased from Strem Chemicals, Inc. 1-Undecyne was purchased from Alfa. The 11-hydroxy-3-dodecenoic acid used as a starting material in Scheme 4 was generously donated by Dr. Blair Johnson**. All other chemicals used for the syntheses described in the following section were obtained from Aldrich or Sigma.

8. Synthesis of stable isotope-labelled chemicals:

a) Analyses:

^1H Nuclear Magnetic Resonance (NMR) spectra were recorded at 400.13 MHz on a Bruker WM-400 spectrometer. Splitting patterns are described as: s, singlet; d, doublet; t, triplet; q, quartet; p, pentet; m, multiplet; and combinations thereof. Coupling constants are reported in Hertz (Hz). Mass spectra (MS) were recorded on a Hewlett-Packard 5985B GC/MS system by electron impact (EI) ionization (70 eV) or by chemical ionization (CI) using isobutane (IB) as the ionizing gas. Infrared spectra (IR) were recorded neat (NaCl disc) on a Perkin Elmer Model 599B spectrometer.

b) Syntheses of chemicals:

All reactions were performed in flame-dried glassware with magnetic stirring. Where necessary inert atmospheres of either Ar or N_2 were employed. After quenching, the "usual workup" involved extraction of the aqueous phase three times with diethyl ether; combination of the organic extracts and washing once with brine; drying the organic layer with anhyd

---

* Department of Chemistry, Simon Fraser University, Burnaby, BC, Canada. V5A 1S6.

** Department of Chemistry, Simon Fraser University, Burnaby, BC, Canada. V5A 1S6.

$MgSO_4$; filtration; and removal of solvent by rotary evaporation under reduced pressure (without heating). All flash column chromatography was performed on Silica Gel 60 (230-400 mesh, Merck) as described by Still *et al.* (1978). Thin layer chromatography (TLC) was performed on aluminum sheets precoated with 60 F_{254} Silica Gel (layer thickness 0.2 mm, Merck). The composition of chromatography solvents are reported as the ratios of volumes.

Diaminopropane (DAP) was distilled from BaO and stored over KOH pellets. Dimethyl sulfoxide (DMSO) was distilled from CaH_2 and stored over molecular sieves (4 Å). Hexamethylphosphoramide (HMPA) was stored over molecular sieves (4 Å). CH_2Cl_2 was distilled from CaH_2 and stored over molecular sieves (4 Å). Triethylamine was stored over NaOH pellets. Oxalyl chloride was distilled over anhyd Na_2CO_3 and stored in sealed glass ampoules under Ar. Tetrahydrofuran (THF) was freshly distilled from Na metal under Ar.

Analytical samples of the methyl esters of carboxylic acids were prepared with diazomethane.

Boiling points are uncorrected.

Preparation of [3,4-D_2]-3-dodecenoic acid and [3,4-D_2]-lauric acid (Scheme 1):

2-Dodecynoic acid (15):

n-Butyllithium in hexane (23.7 mmol) and 1-undecyne (19.7 mmol) were added to anhyd THF (200 mL) at -20 °C. The soln was stirred for 30 min at -10 °C. The reaction mixture was cooled to -15 °C and CO_2 gas was bubbled through for 45 min. The reaction was quenched by addition to ice-water (100 mL). The aqueous phase was extracted with diethyl ether (3 x 40 mL). The combined ether layers were washed once with water. The pooled aqueous extracts were acidified wtih glacial acetic acid, and extracted with diethyl ether (3 x 40 mL). The combined organic extracts were washed once with brine, and dried over anhyd $MgSO_4$. The solvent was

removed *in vacuo*. Most of the acetic acid was removed by azeotropic distillation with CCl_4, then remaining traces were removed under high vacuum (8 h). The product (3.9 g, quantitative yield) was 100% pure by GC and was used directly in the next step. ^1H NMR ($CDCl_3$): δ 0.87 (3 H, t, $J_{11,12}$=6.5 hz, 12-H); 1.18-1.5 (12 H, m, 6-H to 11-H); 1.54-1.63 (2 H, p, $J_{4,5}=J_{5,6}$=7 Hz, 5-H); 2.35 (2 H, t, $J_{4,5}$=7 Hz, 4-H). MS (IBCI) of methyl ester, m/e (relative abundance): 211 (M+1, 100).

3-Dodecynoic acid (**16**):

Sodium amide (158 mmol) was prepared *in situ*. In a flask cooled with dry ice/acetone bath and equipped with a dry ice condensor, Na metal (52 mmol) was dissolved in 100 mL of liquid NH_3 to form a deep blue soln. A crystal of $Fe(NO_3)_2$ was added and the soln stirred until the blue color faded and an almost colorless soln remained. Two more portions of sodium metal (53 mmol) were added, reviving the blue color. Each time the soln was stirred stirred until only a greyish tinge remained. To this the alkyne **15** (19.7 mmol) dissolved in 45 mL of THF was added dropwise. The soln was stirred for 1 h before the NH_3 was allowed to evaporate. The remaining soln was poured into ice-cold water containing NH_4Cl (1.3 mol). The aqueous phase was acidified with conc HCl, and extracted with diethyl ether (3 x 50 mL). The organic extracts were combined, dried over anhyd $MgSO_4$, and concentrated *in vacuo* to give a brown oil (4.32 g). The crude product was flash chromatographed (hexane/ethyl acetate/acetic acid, 200/100/1) to give a colorless oil (2.73 g, 70% yield). GC analysis revealed that the product was comprised of both **16** (87%) and another product (11%) whose identity as the allene **17** was confirmed by NMR and MS analyses. ^1H NMR ($CDCl_3$): δ 0.89 (3 H, t, 12-H); 1.15-1.41 (10 H, m, 7-H to 11-H); 1.44-1.53 (2 H, m, 6-H); 2.16-2.22 (1.7 H, tt, 5-H); 3.33 (1.7 H, t, 2-H). Multiplets due to the presence of **17** were also visible (δ 5.5-5.59 and 5.64-5.70). MS (IBCI) of the methyl ester, m/e (relative intensity): 211 (M+1, 100); 179 (22); 161 (5); 151 (8); 137 (25).

[3,4-D_2]-3-Dodecenoic acid (**18**):

D_2 gas was bubbled through a vigorously stirred suspension of Lindlar's catalyst (0.4 g) in hexane (50 mL) for approx 10 min before the alkyne **16** (9.9 mmol) was added. After 3 h, the catalyst was removed by filtration and washed thoroughly with diethyl ether. The filtrate and ether washes were combined and the solvent removed *in vacuo*, leaving a yellow oil (1.85 g) 87% pure by GC analysis. Flash chromatography (hexane/ethyl acetate/acetic acid, 200/100/1) followed by bulb-to-bulb distillation (0.05 mm Hg) left a colorless oil (1.4 g, 70% yield), 97% pure by GC analysis. ^{1}H NMR ($CDCl_3$): δ 0.87 (3 H, t, $J_{11,12}$=7 Hz, 12-H); 1.17-1.40 (12 H, m, 6-H to 11-H); 2.02 (2 H, t, $J_{5,6}$=7 Hz, 5-H); 3.14 (2 H, s, 2-H). MS (IBCI) of the methyl ester, m/e (relative intensity): 215 (M+1, 100), 214 (12).

[3,4-D_2]-Lauric acid ([3,4-D_2]-dodecanoic acid) (**19**):

H_2 gas was bubbled through a vigorously stirred suspension of 0.1 g palladium (10%) on activated carbon in anhyd ethanol (10 mL) for 10 min. before D_2-**18** (2 mmol) dissolved in anhyd ethanol (4 mL) was added. After 2 h the catalyst was removed by filtration and washed with diethyl ether. The filtrate and ether washes were combined, dried over anhyd $MgSO_4$ and the solvent removed *in vacuo* to leave a colorless oil which solidified on standing (0.39 g, 97% yield) and was 99% pure by GC analysis. ^{1}H NMR ($CDCl_3$): δ 0.88 (3 H, t, $J_{11,12}$=6.5 Hz, 12-H); 1.23-1.35 (15 H, m, 4-H to 11-H); 1.58-1.66 (1 H, m, 3-H); 2.34 (2 H, d, $J_{2,3}$=7.3 Hz, 2-H). MS (IBCI) of the methyl ester, m/e (relative abundance): 218 (52); 217 (M+1, 100), 216 (65); 215 (23).

Preparation of [11-D]-11-hydroxy-3-dodecenoic acid (Scheme 2):

4-Undecyn-2-ol (**20**):

n-Butyllithium in hexane (22.1 mmol) was added dropwise to a soln of 1-octyne (18.0 mmol) in 20 mL anhyd THF, stirred at -40 °C. After the

addition was complete, the temperature was raised to -4 °C, where it was maintained for 45 min, before cooling to -40 °C. Anhyd HMPA (6 mL) was added in one portion, followed by dropwise addition of propylene oxide (16.3 mmol) dissolved in 6 mL of HMPA. The soln was gradually warmed to RT, and stirred for 2 days. The reaction was quenched by addition of ice-cold brine. The aqueous phase was extracted with diethyl ether (3 x 30 mL). The combined organic extracts were washed several times with 2% HCl (aqueous) to remove the HMPA. The organic phase was then washed once with brine, dried over anhyd $MgSO_4$, filtered, and the solvent removed *in vacuo*. The crude oil (2.57 g) was distilled under vacuum to give a colorless oil (1.77 g, 65% yield), bp 76-79 °C (0.8 mm Hg), 95% pure by GC analysis. ^1H NMR ($CDCl_3$): δ 0.88 (3 H, t, 11-H); 1.23 (3 H, d, 1-H); 1.25-1.53 (8 H, m, 7-H to 10-H); 2.11-2.18 (2 H, m, 6-H); 2.23-2.30 (1 H, ddt, J_{gem}=16 Hz, $J_{2,3}$=7 Hz, $J_{3,6}$=2 Hz, 3-H); 2.32-2.41 (1 H, ddt, J_{gem}=16 Hz, $J_{2,3'}$=5 Hz, $J_{3',6}$=2 Hz, 3-H'); 3.94-3.84 (1 H, m, 2-H). IR: 3330 (m, broad); 2940 (s); 2910 (s); 2840 (s); 1450 (m); 1420 (w); 1365 (m); 1110 (m); 1075 (m); 930 (m); cm^{-1}. MS (EI) m/e (relative abundance): 168 (M^+, 0.3); 153 (3); 124 (6); 109 (10); 95 (95); 81 (82); 67 (100); 54 (90); 45 (86). MS (IBCI): 169 (M+1, 100); 151 (36); 109 (25); 95 (32); 81 (14).

10-Undecyn-2-ol (**21**):

KH (20.6 mmol, as a 35% wt dispersion in paraffin oil) was washed with hexane (4 x 30 mL) to remove the paraffin, and dried first by a gentle stream of Ar, and then *in vacuo* for 30 min. DAP (21 mL) was added to the residue in one portion. The liquid was stirred for 2 h at RT, during which time the KH dissolved and the color changed from pale yellow to dark orange-brown. The soln was cooled to -4 °C and **20** (9.8 mmol) was added dropwise. DAP (2 mL) was used to complete the transfer. The soln was stirred for 24 h at RT, then quenched by pouring into ice water. The usual workup yielded a yellow oil (1.82 g) which was purified by distillation under vacuum (0.81 g, 49% yield), bp 75 °C (0.2 mm Hg), 95%

pure by GC analysis. ^{1}H NMR ($CDCl_3$): δ 1.19 (3 H, d, 1-H); 1.25-1.61 (12 H, m, 3-H to 8-H); 1.93 (1 H, t, 11-H); 2.15-2.23 (2 H, dt, 9-H); 3.74-3.84 (1 H, br s, 2-H). IR: 3350 (m, broad); 3280 (s); 2940 (s); 2900 (s); 2830 (s); 2100 (w); 1450 (m); 1360 (m); 625 (s) cm^{-1}. MS (IBCI) m/e (relative intensity): 169 (M+1, 1.2); 151 (10); 109 (38); 95 (100); 81 (50)..

10-Undecyn-2-one (**22**):

PCC (9.3 mmol) was added to 200 mL anhyd CH_2Cl_2 and stirred for 30 min at RT, at which time **21** (4.8 mmol) was added in one portion. The ensuing reaction was slightly exothermic and the soln immediately turned black. After stirring for 1 h at RT, anhyd diethyl ether (200 mL) was added, and the resulting mixture filtered through a 3 cm x 12 cm Florisil column. The solvent was removed *in vacuo*. The resulting greenish oil was purified by bulb-to-bulb distillation under vacuum to give a yellow oil (0.802 g, 100% yield), 95% pure by GC analysis. ^{1}H NMR ($CDCl_3$): δ 1.31-1.61 (10 H, m, 4-H to 8-H); 1.94 (1 H, t, 11-H); 2.15 (3 H, s, 1-H); 2.16-2.20 (2 H, dt, 9-H); 2.43 (2 H, t, 3-H). IR: 3300 (s); 2940 (s); 2860 (s); 2120 (w); 1720 (s); 1710 (s); 1465 (m); 1430 (m); 1410 (m); 1360 (s); 1260 (w); 1175 (w); 630 (s) cm^{-1}. MS (IBCI) m/e (relative intensity): 167 ((M+1, 66); 149 (100); 109 (36); 97 (8); 85 (22).

[2-D]-10-Undecyn-2-ol (**23**):

To the ketone **22** (4.8 mmol) dissolved in 2 mL of ethanol at 0 °C was added $NaBD_4$ (2.2 mmol). The soln was stirred 1 h at RT. The usual workup yielded a pale yellow oil (0.77 g, 94% yield), 95% pure by GC analysis. ^{1}H NMR ($CDCl_3$): δ 1.17 (3 H, s, 1-H); 1.26-1.54 (12 H, m, 3-H to 8-H); 1.93 (1 H, t, 11-H); 2.13-2.20 (2 H, dt, 9-H). IR: 3300 (s, broad); 2960 (s); 2920 (s); 2850 (s); 2120 (w); 1630 (m); 1450 (m); 1370 (m); 630 (m) cm^{-1}. MS (IBCI) m/e (relative abundance): 170 (M+1, 4); 152 (38); 110 (36); 109 (34); 96 (84); 95 (100).

[11-D]-11-Hydroxy-2-dodecynoic acid (**24**):

To 75 mL of anhyd THF at -10 °C was added *n*-butyllithium (11.3 mmol) in hexane. To this soln was added dropwise **23** (4.5 mmol), dissolved in 5 mL of THF. The soln was warmed to RT and stirred for 2.5 h before cooling to 0 °C. Then CO_2 was bubbled through the soln for 30 min, at which point a heavy white precipitate developed. The reaction mixture was carefully extracted with H_2O (100 mL) and diethyl ether (30 mL). The ether layer (containing the unreacted alkyne) was discarded. The aqueous layer was acidified with HCl, and worked up as usual. The dark yellow oil obtained was purified by flash chromatography (hexane/ethyl acetate/acetic acid, 100/100/1) to give a colorless oil (0.624 g, 65% yield), 85% pure by GC analysis. ^{1}H NMR ($CDCl_3$): δ 1.19 (3 H, s, 12-H); 1.25-1.62 (12 H, m, 5-H to 10-H); 2.36 (2 H, t, 4-H); 8.0-8.9 (br s, -OH). IR: 3400 (s, broad); 2960 (s); 2920 (s); 2840 (s); 2600 (m); 2230 (m); 1940 (w); 1700 (s); 1450 (m); 1375 (m); 1250 (m); 1100 (m) cm^{-1}. MS (IBCI) of the methyl ester, m/e (relative intensity): 228 (M+1, 32); 210 (100); 178 (15); 150 (82); 139 (8).

[11-D]-11-Hydroxy-3-dodecynoic acid (**25**):

Sodium amide (23 mmol) was prepared *in situ* as described previously (Scheme 1, preparation of **16**). To this **24** (2.9 mmol) dissolved in 5 mL of anhyd THF was added dropwise. The soln was stirred for 1 h before the NH_3 was allowed to evaporate. Using diethyl ether the remaining reaction mixture was transferred to a flask containing NH_4Cl (200 mmol) dissolved in ice water. The soln was acidified with HCl. The usual workup yielded a yellow oil that was purified by flash chromatography (hexane/ethyl acetate/acetic acid, 100/100/1) to a colorless oil (0.325 g, 52% yield), 92% pure by GC analysis. ^{1}H NMR ($CDCl_3$): δ 1.19 (3 H, s, 12-H); 1.22-1.54 (10 H, m, 6-H to 10-H); 2.16-2.24 (2 H, m, 5-H); 3.3 (2 H, t, 2-H); 4.80-5.13 (br s, -OH). Small (non-integratable) multiplets due to the presence of allene (δ 5.54-5.59 and 5.62-5.68) were also visible. IR:

3400 (s, broad); 2920 (s); 2860 (s); 2600 (s); 2240 (m); 1955 (m); 1700 (s); 1450 (s); 1250 (s); 1050 (s); 900 (m); 760 (m); 660 (m). MS (IBCI) of methyl ester, m/e (relative abundance): 228 (M+1, 28); 210 (100); 196 (5); 182 (15); 178 (36); 150 (40); 136 (80); 85 (12). The compound was 95% enriched in D by MS.

[11-D]-11-Hydroxy-3-dodecenoic acid (D-**26**):

P2Ni was prepared *in situ* by adding $NaBH_4$ (0.076 mmol) dissolved in 1 mL of ethanol to $Ni(OAc)_2 \cdot 4(H_2O)$ (0.076 mmol) dissolved in 10 mL of 95% ethanol in a flask flushed with H_2. Ethylene diamine (0.24 mmol) was added to the black catalyst suspension. Then **25** (1.51 mmol) was added and the mixture stirred with a H_2 gas purge for 16 h at RT. The usual workup followed by flash chromatography (hexane/ethyl acetate/acetic acid, 100/100/1), yielded a colorless oil (0.20 g, 61% yield) 91% pure by GC analysis (the remaining 9% was the fully reduced analogue, [11-D]-hydroxy-3-dodecanoic acid, D-**27**). ^1H NMR: δ 1.19 (3 H, s, 12-H); 1.23-1.49 (10.7 H, m, 6-H to 10-H); 1.97-2.08 (1.7 H, m, 5-H); 3.08-3.15 (1.5 H, d, 2-H); 4.5-4.8 (br s, -OH); 5.49-5.65 (1.7 H, m, 3-H and 4-H). Integration of the vinyl region suggested the presence of excess alkyl residues, and there was an extra signal at δ 3.03-3.07 (0.3 H, m, 2-H of sat'd analogue) due to the presence of **27**. MS (IBCI) of methyl ester, m/e (relative intensity): 230 (M+1, 28); 212 (100); 180 (38); 152 (6); 38 (11).

Preparation of D_2-26; ^{18}O,D_2-26; and D_4-27 (Scheme 3):

[3,4-D_2]-11-Hydroxy-3-dodecenoic acid (D_2-**26**):

11-Hydroxy-3-dodecynoic acid (unlabelled **25** prepared as described in Scheme 2, except in 34% overall yield) was reduced with P2Ni and D_2 gas. D_2 gas was bubbled through a stirred suspension of alkyne (6.3 mmol), P2Ni [prepared by the reduction of $Ni(OAc)_2 \cdot 4(H_2O)$ (0.32 mmol) with $NaBD_4$ (0.32 mmol)] and ethylene diamine (1.0 mmol) in ethanol (2.5 mL) overnight at RT. The reaction was worked up as described previously

(Scheme 2, for the preparation of D-26) to give a colorless oil (0.98 g, 72% yield), 90% pure by GC analysis. The product also contained (by GC) 7% of the fully reduced isomer, D_2-27. ^{1}H NMR ($CDCl_3$): δ 1.16 (3 H, d, 12-H); 1.20-1.50 (10 H, m, 6-H to 10-H); 2.03 (2 H, t, 5-H); 3.11 (2 H, s, 2-H); 3.72-3.84 (1 H, m, 11-H); 6.56-6.92 (br s, -OH). MS (IBCI) of the methyl ester, m/e (relative intensity): 231 (M+1, 24); 213 (100); 181 (36); 165 (4); 153 (8); 139 (14); 125 (8).

[3,4-D_2]-11-Keto-3-dodecenoic acid (**28**):

2.5 M Chromic acid (about 2.3 mmol) was added dropwise to a soln of D_2-**26** (3.2 mmol) in acetone (80 mL) at 0 °C, until the orange color persisted. The soln was stirred an additional 15 min before filtration through Celite. The acetone was removed *in vacuo*. The remaining oil was dissolved in diethyl ether, washed with brine and dried over anhyd $MgSO_4$. Removal of the solvent *in vacuo* gave a yellow oil (0.80 g, 116%), 79% pure by GC analysis. ^{1}H NMR ($CDCl_3$): δ 1.21-1.39 (6 H, m, 6-H to 8-H); 1.48-1.59 (2 H, m, 9-H); 2.02 (2 H, m, 5-H); 2.12 (3 H, s, 12-H); 2.36-2.44 (2 H, t, 10-H); 3.12 (2 H, s, 2-H). MS (IBCI) of the methyl ester, m/e (relative intensity): 229 (M+1, 100); 197 (37).

[3,4-D_2]-11-Keto-3-dodecenoic acid 2,2-dimethyl-1,3-propane diol ketal (**29**):

A toluene (20 mL) soln containing **28** (3.20 mmol), 2,2-dimethyl-1,3-propanediol (3.24 mmol) and a catalytic quantity of *p*-toluenesulfonic acid (0.03 mmol) was refluxed for 4 h, during which time water was removed as it formed by Dean-Stark extraction. The toluene was removed *in vacuo* and the crude product was purified by flash chromatography (hexane/ethyl acetate/acetic acid, 80/40/1) to give a colorless oil (0.58 g, 60% yield), 83% pure by GC analysis. ^{1}H NMR ($CDCl_3$): δ 0.89 (3 H, s, ketal methyl); 1.00 (3 H, s, ketal methyl); 1.22-1.44 (8 H, m, 6-H to 9-H); 1.64-1.70 (2 H, m, 10-H); 1.98-2.06 (2 H, m, 5-H); 2.10 (3 H, s, 12-H); 3.13 (2 H, s,

2-H); 3.43 (2 H, d, ketal methylene); 3.56 (2 H, d, ketal methylene). MS (IBCI) of the methyl ester, m/e (relative abundance): 316 (M+2, 100); 229 (62); 197 (15); 129 (92).

[11-^{18}O-3,4-D_2]-11-Hydroxy-3-dodecenoic acid (^{18}O,D_2-**26**):

Gaseous HCl was bubbled through 0.5 g $^{18}OH_2$ for about 20 sec. The flask was cooled to 0 °C and ketal **29** (2.5 mmol) was added. Transfer was completed with 0.2 mL anhyd hexane. Vigorous stirring of the biphasic reaction was continued at RT for 1 h. The reaction vessel was again cooled to 0 °C, $NaCNBH_3$ (5 mmol) was added in one portion, and the soln was stirred for 1 h at RT. After the usual workup the crude product was flash chromatographed on silica (hexane/ethyl acetate/acetic acid, 100/60/1) to give a colorless oil (0.193 g, 35% yield), 93% pure by GC analysis. ^{1}H NMR ($CDCl_3$): identical to that of D_2-**26**. MS (IBCI) of the methyl ester, m/e (relative intensity): 233 (M+1, 22); 213 (100); 181 (32); 139 (10). The product was 82% enriched for both ^{18}O and two deuterium atoms, and was 18% enriched for either ^{18}O or two deuterium atoms.

[3,3,4,4-D_4]-11-Hydroxy-dodecanoic acid (D_4-**27**):

D_2-**26** (0.23 mmol), prepared as previously described, was added to 20 mL of anhyd ethanol and 5% palladium (10%) on activated carbon in a Caries tube, flushed with D_2 gas. The tube was sealed with D_2 gas at 20 PSI. After stirring overnight at RT, the catalyst was removed by filtration. The catalyst was washed several times with diethyl ether, the combined organic extracts were concentrated *in vacuo* and the crude product was purified by flash chromatography (hexane/ethyl acetate/acetic acid, 100/60/1) to give a colorless oil (0.035 g, 69% yield), 96% pure by GC analysis. ^{1}H NMR ($CDCl_3$): δ 1.12 (3 H, d, 12-H); 1.22-1.57 (11.5 H, m, 5-H to 10-H); 2.33 (1.7 H, s, 2-H); 3.75-3.82 (1 H, m, 11-H). Non-integer integration ratios due to over-deuteration of the hydrocarbon chain were

confirmed by MS. MS (IBCI) of the methyl ester, m/e (relative abundance): 241 (3); 240 (4); 239 (5); 238 (6); 237 (8); 236 (10); 235 (M+1, 17); 234 (15); 233 (10); 232 (3); 231 (1); 227 (2); 226 (4); 225 (7); 224 (10); 223 (14); 222 (19); 221 (25); 220 (28); 219 (38); 218 (58); 217 (100); 216 (73); 215 (41); 214 (10); 213 (1.4); 153 (1); 152 (8); 151 (1).

Preparation of D-26; D,^{18}O-26 and D-27 (Scheme 4):

11-Keto-3-dodecenoic acid (**30**):

Hydroxy acid **26** (11.2 mmol) was dissolved in acetone (200 mL) and cooled to 0 °C with mechanical overhead stirring. Chromic acid soln (ca. 3 mL of 2.5 M in H_2O) was added dropwise until the green color persisted. The soln was stirred an additional 20 min at 0 °C, then allowed to warm to RT. Isopropanol was added dropwise until a yellow color persisted. The soln was filtered through glass fiber filter paper, then concentrated *in vacuo*. Aqueous 5% HCl (30 mL) was added to the remaining green oil, and the aqueous phase was extracted with diethyl ether (3 x 50 mL). The combined organic phases were washed with 5% HCl (2 x 20 mL) and then with brine (20 mL) before drying over anhyd Na_2SO_4. Removal of the solvent *in vacuo* left a pale yellow oil (1.44 g), 91% pure by GC analysis. Flash chromatography (hexane/ethyl acetate/acetic acid, 50/20/0.5) afforded two usable fractions (combined yield 1.05 g, 76% yield): the first was a colorless oil which solidified on standing (0.68 g), 98% pure by GC analysis; the second was a colorless oil (0.37 g), 83% pure by GC analysis. The major impurity in the second fraction was 11-ketododecanoic acid (formed from 11-hydroxydodecanoic acid, present in the original sample of **26**). Thus the second fraction was used for the preparation of D-**27**. ^{1}H NMR (first fraction, in $CDCl_3$): δ 1.23-1.41 (6 H, m, 6-H to 8-H); 1.51-1.60 (2 H, p, $J_{8,9}=J_{9,10}=7$Hz, 9-H); 1.99-2.07 (2 H, q, $J_{4,5}=J_{5,6}=7$Hz; 5-H); 2.13 (3 H, s, 12-H); 2.41 (2 H, t, $J_{9,10}=7$Hz, 10-H); 3.13 (2 H, d, $J_{2,3}=6$Hz, 2-H); 5.50-5.64 (2 H, m, 3-H and 4-H). IR: 3020 (w); 2930 (m); 2860 (m); 2250 (m); 1710 (s); 1415 (w); 1360 (w); 1220 (w);

1170 (w); 910 (s); 730 (s); 650 (m) cm^{-1}. MS (IBCI) of the methyl ester, m/e (relative abundance): 227 (M+1, 100); 195 (22).

[11-D]-11-Hydroxy-3-dodecenoic acid (D-26):

To a soln comprised of **30** (1.4 mmol), D_2O (0.1 mL), anhyd THF (2 mL) and a trace of methyl orange was added 1.4 mmol $NaBD_3CN$. A soln of DCl/DOAc in THF (prepared by the addition of 0.3 mL of D_2O and 0.3 mL of acetyl chloride to 2 mL of anhyd THF cooled to 0 °C) was added dropwise to maintain the red color. After approximately 5 min, the red color persisted. Stirring was continued for 1 h at RT, after which the reaction was quenched by addition to 50 mL of 1% HCl. The aqueous phase was extracted with diethyl ether (3 x 20 mL). The combined organic extracts were washed with brine (10 mL), then dried over anhyd $MgSO_4$, concentrated *in vacuo* and left under high vacuum for 15 min. The crude product was flash chromatographed (hexane/ethyl acetate/acetic acid, 50/25/1). The appropriate fractions were pooled and concentrated *in vacuo*. The residue was dissolved in diethyl ether (60 mL) and washed with 1% HCl (3 x 15 mL) and then with brine (10 mL) to remove most of the acetic acid. The organic extract was dried over anhyd $MgSO_4$ and concentrated *in vacuo* to give a colorless oil (0.28 g, 92% yield), 99% pure by GC analysis. ^1H NMR ($CDCl_3$): δ 1.17 (3 H, br s, 12-H); 1.20-1.50 (10 H, m, 6-H to 10-H); 1.98-2.06 (2 h, q, $J_{4,5}=J_{5,6}=6.5$ Hz, 5-H); 3.10 (2 H, d, $J_{2,3}=7$ Hz, 2-H); 5.49-5.62 (2 H, m, 3-H and 4-H); 6.11-6.51 (2 H, br s, -OH). IR: 3300 (m); 2990 (m); 2950 (s); 2880 (m); 2150 (w); 1715 (s); 1390 (m); 1305 (m); 1200 (m); 1140 (m); 1100 (m); 950 (m) cm^{-1}. MS (IBCI) of the methyl ester, m/e (relative abundance): 230 (M+1, 24); 213 (16); 212 (100); 211 (12); 180 (22); 138 (5). D-**25** was 94% enriched in D as judged by GC/SIM-MS analysis.

[11-D]-11-Hydroxydodecanoic acid (D-27):

Keto acid **30** (1.8 mmol prepared as described above, using the "impure" fraction of **30** which contained 12% of the saturated analogue, D-27) was dissolved in 10 mL of anhyd ethanol and added to a vigorously stirred suspension of 60 mg palladium (10%) on activated carbon in 10 mL of anhyd ethanol, through which H_2 gas was bubbled. After approximately 5 h, the catalyst was removed by filtration and washed with diethyl ether. The filtrate and ether washes were combined and concentrated *in vacuo*. The crude product was flash chromatographed (hexane/ethyl acetate/acetic acid, 500/150/7). Appropriate fractions were combined and treated as described above in the preparation of D-**26**. The product was a white solid (0.22 g, 58% yield), 99% pure by GC analysis. ^{1}H NMR ($CDCl_3$): δ 1.17 (3 H, s, 12-H); 1.20-1.48 (14 H, m, 4-H to 10-H); 1.58-1.67 (2 H, m, 3-H); 2.31-2.37 (2 H, dt, $J_{2,3}$=7.4, 2-H). MS (IBCI) of the methyl ester, m/e (relative abundance): 232 (M+1, 6); 215 (16); 214 (100); 213 (18); 182 (5). D-27 was 95% enriched in D as judged by GC/SIM-MS.

[11-D,11-^{18}O]-11-Hydroxy-3-dodecenoic acid (D,^{18}O-**26**):

Gaseous HCl was bubbled through $^{18}OH_2$ (1.00 g) containing a few crystals of methyl orange until the methyl orange turned red. The soln was cooled to 0 °C and 1.8 mmol of **30** dissolved in 2 mL of anhyd ethanol was added. The soln was stirred for 1 h at RT. At this time, most of the ethanol was removed *in vacuo*, and the remaining mixture was extracted with anhyd ethyl ether (2 x 2 mL). The ether was removed from the combined organic extracts with a gentle stream of Ar. The remaining oil was dissolved in 2 mL of anhyd ethanol and cooled to 0 °C, then 3.6 mmol $NaBD_4$ in anhyd ethanol (0.5 mL) was added dropwise. The reaction was stirred for 1 h at 0 °C and quenched by addition to 1% HCl (20 mL). After the usual workup, the crude product was flash chromatographed (hexane/ethyl acetate/acetic acid, 50/50/1) to give a colorless oil (0.23 g, 59% yield) 98% pure by GC analysis. ^{1}H NMR ($CDCl_3$): identical to that of D-**25**. MS

(IBCI) of the methyl ester, m/e (relative abundance): 232 (M+1, 10); 213 (16); 212 (100); 211 (25); 180 (24); 179 (7); 138 (5). By GC/SIM-MS analysis, compound D,^{18}O-**26** was 87% enriched in D and 87% enriched in ^{18}O. Sixty-eight per cent of the molecules were enriched in both isotopes.

Preparation of [1-D]-(*E,E*)-farnesol (Scheme 5):

(E,E)-Farnesal [(E,E)-3,7,11-trimethyl-2,6,10-dodecatrienal] (**31**):

Oxalyl chloride (0.27 mL, 3.15 mmol) was added to 15 mL anhyd CH_2Cl_2, and the soln was cooled to -60 °C in a dry ice/acetone bath. DMSO (0.46 mL, 6.48 mmol) in 2 mL CH_2Cl_2 was added slowly, while maintaining the temperature between -50 and -60 °C. The soln was stirred for 15 min, then 1.35 mmol of (*E,E*)-farnesol (97% pure by GC) in 5 mL CH_2Cl_2 was gradually added. The soln was stirred for 1 h at -50 to -60 °C before 2 mL triethylamine was slowly added. After 5 min at -50 °C the soln was warmed to RT. A heavy white precipitate developed. Then 30 mL of H_2O were added and the reaction was worked up in the usual way to yield a yellow oil (0.52 g), 84% pure by GC analysis. This oil was purified by flash chromatography (hexane/ethyl acetate, 3/1) to give a colorless oil (0.278 g, 93% yield), 90% pure by GC analysis. ^{1}H NMR: agreed with literature values (Johnston *et al.*, 1976). IR: 2980 (s); 2930 (s); 2860 (s); 1680 (s); 1450 (m); 1380 (m); 1200 (w); 1120 (w) cm^{-1}. MS, m/e (relative intensity): 220 (M^+, 1); 205 (2); 191 (2); 189 (2); 177 (4); 136 (20); 121 (20); 107 (10); 95 (20); 84 (90); 69 (100).

[1-D]-(E,E)-Farnesol (D-**32**):

Farnesal, **31** (1.3 mmol) was dissolved in 2 mL of ethanol. $NaBD_4$ was added and the reaction was stirred at RT for 40 min. The reaction was quenched by pouring into a stirred ice-cold aqueous soln of 1% HCl. The usual workup yielded a colorless oil (0.225 g, 80% yield), 82% pure by GC analysis, which was purified by flash chromatography (CH_2Cl_2) to give a colorless oil (0.176 g, 62%), 96% pure by GC analysis. ^{1}H NMR ($CDCl_3$): δ

1.13 (1 H, s, 1-OH); 1.60 (6 H, s, vinyl CH_3); 1.68 (6 H, s, vinyl CH_3); 1.93-2.17 (8 H, m, 4-H, 5-H, 8-H, 9-H); 4.15 (1 H, br d, $J_{1,2}$=6Hz, 1-H); 5.05-5.13 (2 H, m, 6-H, 10-H); 5.42 (1 H, d, $J_{1,2}$=6Hz, 2-H). This agrees with literature values for (*R,E,E*)-(-)-[1-D]-farnesol (Noyori *et al.*, 1984), except that the authors did not report the methylene multiplet. IR: agrees with literature values. MS, m/e (relative intensity): 223 (M^+, 0.5); 140 (20); 81 (30); 69 (100). By GC/SIM-MS, the (*E,E*)-farnesol was 95% enriched in D.

Preparation of [1-D,1-^{18}O]-(*E,E*)-farnesol (Scheme 6):

[1-D]-(E,E)-Farnesyl bromide (33):

Labelled farnesol, D-32 (3.6 mmol) prepared as described above (except that a quantitative yield of product, 88% pure by GC, 97 atom% D by GC/SIM-MS analysis, was obtained) was dissolved in anhyd diethyl ether (ca. 15 mL) and cooled to 0 °C. PBr_3 (1.6 mmol) was added dropwise and the soln was stirred at 0 °C for 1 h. The reaction was stopped by addition to ice-cold saturated $NaHCO_3$. The usual workup yielded a yellow oil (0.99 g, 96% yield). The product decomposed when analyzed by GC. ^{1}H NMR ($CDCl_3$): δ 1.68 (6 H, s, vinyl CH_3); 1.68 (3 H, s, vinyl CH_3); 1.74 (3 H, s, 3-CH_3); 1.93-2.17 (8 H, m, 4-H, 5-H, 8-H, 9-H); 4.02 (1 H, br d, $J_{1,2}$=7 Hz, 1-H); 5.06-5.15 (2 H, m, 6-H, 10-H); 5.53 (1 H, br d, $J_{1,2}$=7 Hz, 2-H). IR: 2980 (s); 2940 (s); 2880 (s); 1740 (w); 1655 (m); 1450 (s); 1395 (s); 1390 (s); 1170 (m) cm^{-1}. MS (EI) m/e (relative abundance): 287 (M^+, 0.2); 285 (M^+, 0.1); 244 (0.3); 242 (0.3); 206 (1); 162 (0.8); 150 (2); 81 (39); 69 (100).

[1-D,1-^{18}O]-(E,E)-Farnesol (D,^{18}O-32):

Sodium metal (0.05 g) was dissolved in 1.00 mL of $^{18}OH_2$ under argon at 0 °C, then 33 (2.1 mmol) in 3 mL of anhyd THF was added. The flask was sealed with a serum stopper, covered in aluminium foil, and stirred vigorously at RT for 2 weeks. The reaction was monitored periodically by

GC and TLC. Additional THF and Na metal were added (with cooling) as needed. The usual workup yielded a pale yellow oil (0.516 g). Flash chromatography (hexane/ethyl acetate, 3/1) followed by ^{1}H NMR analysis revealed that the crude oil was comprised of the desired product (24%) unreacted farnesyl bromide (27%) and another major product (38%). The unreacted bromide was resubmitted to the reaction conditions. Flash chromatography of the pooled products yielded a colorless oil (0.128 g, 27% yield), 95% pure by GC analysis. ^{1}H NMR, IR: identical to those of [1-D]-*(E,E)*-farnesol. MS (EI) m/e (relative intensity): 225 (M^+, 0.8); 136 (40); 123 (34); 121 (34); 109 (32); 93 (58); 81 (92); 69 (100). By GC/SIM-MS, the product was >95% enriched in D, and 84% enriched in ^{18}O.

The other major product was isolated by flash chromatography (0.18 g, 38% yield, 82% pure by GC) and identified as [1-D,3-^{18}O]-3,7,11-trimethyldodeca-1,6,10-trien-3-ol (**34**). ^{1}H NMR ($CDCl_3$): δ 1.28 (3 H, s, 3-CH_3); 1.60 (6 H, s, vinyl CH_3); 1.68 (3 H, s, vinyl CH_3); 1.92-2.12 (8 H, m, H-4, H-5, H-8, H-9); 5.05-5.17 (3 H, m, 1-H, 6-H and 10-H); 5.88-5.97 (1 H, m, 2-H). IR: 3340 (m, broad); 2910 (s); 2860 (s); 2800 (s); 1415 (m); 1350 (m); 960 (w); 790 (w) cm^{-1}. MS (EI) m/e (relative abundance): 225 (M^+, 0.3); 205 (1.4); 190 (5); 162 (28); 136 (45); 123 (25); 121 (25); 108 (38); 94 (75); 81 (40); 69 (100).

C) RESULTS AND DISCUSSION

Since many of the required techniques were new to the study of pheromone biosynthesis in coleoptera, the methodology chosen or developed for this study will be discussed first. Then the results of the biosynthetic studies will be presented and discussed.

1. Methodology

Four methods have typically been used to introduce labelled precursors into insects to study pheromone biosynthesis: injection (e.g. Mitlin and Hedin, 1974; Thompson and Mitlin, 1979); topical application onto the pheromone-producing gland (e.g. Bjostad *et al.*, 1987); addition of a precursor to cell-free extracts of excised glands (e.g. Wolf and Roelofs, 1983); and force-feeding of precursor-laced food (e.g. Thompson and Mitlin, 1979). The last technique is most practical for small insects and was the approach used in this study.

a) Radiolabels versus stable isotope labels: pros and cons

In situations where the incorporation efficiency is expected to be low, radiolabels are usually the isotopes of choice to study incorporation of a precursor into a product. In this study, low levels of incorporation of added substrates into the cucujolides were expected since the label was introduced *via* the food source (effecting a large isotope dilution). Therefore, initial studies were performed using commercially available radiolabelled precursors.

However the use of radiolabelled precursors to study pheromone biosynthesis has some drawbacks. For example, to ensure that radioactivity attributed to the cucujolides was not actually due to the presence of a radiolabelled contaminant, it was necessary to purify the sample exhaustively. This is technically difficult when the compound is volatile and present in only small quantities: significant (if not total) losses are unavoidable. This limitation can be overcome by using stable

isotope-labelled precursors. Incorporation can be measured by gas chromatography/mass-spectroscopy (GC/MS) removing the requirement for extensive purification. Other advantages of using stable isotope-labelled precursors include:

1. the location of a stable isotope label in the ultimate product can be confirmed by the MS fragmentation pattern and NMR;
2. the syntheses of stable isotope labelled substrates are usually safer, easier and cheaper than the syntheses of the corresponding radiolabelled compounds;
3. studies following the fate of stable isotope-labelled oxygen are feasible. Unlike radioactive oxygen (^{20}O), the heavy isotope of oxygen (^{18}O) is readily available in useful forms, such as $^{18}O_2$ and $^{18}OH_2$.

Stable-isotope labelled precursors have been used successfully to study pheromone biosynthesis in several orders of insects, including beetles (Fish *et al.*, 1979; Hendry *et al.*, 1980); moths (Bjostad *et al.*, 1987); flies (Blomquist *et al.*, 1987) and cockroaches (Chase *et al.*, 1990). In these studies, high levels of incorporation were achieved either by incubation of the labelled precusor *in vitro* with the pheromone-producing gland (Bjostad *et al.*, 1987) or enzymes (Blomquist *et al.*, 1987); or by exposure of unfed live insects to the labelled precursor (Fish *et al.*, 1979; Hendry *et al.*, 1980). These techniques were not practical to implement with *C. ferrugineus* and *O. mercator*, since the sites of pheromone biosynthesis are not known and cucujolide production is sharply reduced if the insects are not feeding (Pierce *et al.*, 1984a). It was expected that a prohibitively high dilution of the isotope label would occur if the insects were allowed to feed normally in the presence of an added labelled substrate. However, preliminary studies (described below) indicated that this isotopic dilution could be reduced to workable levels if the oats were defatted and the beetles were starved for 48 hours prior to exposure to the labelled precursors. Selected Ion Monitoring-MS (SIM-

MS) was used to increase the sensitivity of the MS measurements to the ions of interest.

b) Increased incorporation efficiency

Due to the necessity for *C. ferrugineus* and especially *O. mercator* to feed for normal cucujolide production, added substrates become diluted in the food source. Based on estimates from whole wheat (Mecham, 1971), the 30 g of oats typically used in an experiment could contain 0.3 - 1.5 g of lipid, which is a large quantity when compared to the quantities of radio- or stable isotope-labelled substrates added (typically <1 mg or about 100 mg, respectively). Clearly this would cause significant isotope dilution.

An obvious solution to this problem would be to supply the beetles with an artificial food source devoid of digestible materials, spiked with the labelled substrate of interest. Cucujolide production might be initiated, since a trigger to initiate pheromone production in other beetles has been proposed to be the "gut stretching" associated with feeding (Borden, 1985). The labelled substrate would thus be streamlined into cucujolide biosynthesis with little dilution. However, the beetles did not seem to feed on filter paper or α-cellulose (Dr. Harold Pierce, Jr., personal communication*; personal observations). Choices of medium were limited by the requirement to pass air through the feeding beetles so that the volatiles could be continuously collected on Porapak Q.

Rolled oats that had been "defatted" prior to being coated with the stable isotope-labelled precursors provided a medium that supported cucujolide production but minimized isotope dilution. Eighty-eight per cent of the oil was removed from the rolled oats by soxhlet-extraction with diethyl ether. The lipid that remained in the oats was rich in palmitic, oleic and linoleic acids (Table 3).

---

* Department of Chemistry, Simon Fraser University, Burnaby, BC, Canada. V5A 1S6.

Table 3: Free fatty acid content of oil retained in the oats, as compared to that removed.

Fatty acid	Fatty acid content (% of total)	
	Oil removed[1]	Oil retained[2]
linoleic acid	29.1	30.1
myristic acid	0.2	1.5
oleic acid	20.6	14.0
palmitic acid	16.1	16.2
stearic acid	2.6	1.4
others	31.4	36.8

[1]"Oil removed" by soxhlet-extraction with diethyl ether (Method 1 of *Experimental*); [2]"Oil retained" in the defatted oats was removed for analysis by Bligh and Dyer (1959) extraction (Method 1 of *Experimental*).

Another step taken to decrease the dilution of the isotope-labelled precursor with unlabelled compounds was to starve the beetles for 48 hours prior to their exposure to labelled precursors. This was expected to deplete endogenous fatty acid pools.

Table 4 summarizes the results of an experiment designed to determine if the techniques of defatting the oats and starving the beetles did, in fact, increase the incorporation efficiency of added substrate. The incorporation of [9,10-T]-palmitic acid into cucujolide II by unstarved and by starved beetles feeding on defatted oats were compared to that obtained by unstarved beetles feeding on untreated oats. In order to mimic more closely the situation in which larger quantities of stable isotope-labelled precursors would be used, unlabelled palmitic acid was added to the [9,10-T]-palmitic acid.

Table 4: Comparison of treatments designed to increase substrate incorporation efficiency.

Beetle treatment:	unstarved	unstarved		starved
Oat treatment:	untreated	defatted[1]		defatted
		30 min	12 h	
% Radioactivity incorporated into volatiles (±0.01%)	0.49	0.70	0.70	0.78
Specific activity of [9,10-T]-palmitic acid precursor (mCi/mol):	3.00×10^7	8.3×10^4		8.3×10^4
Specific activity of **II** product (mCi/mol):	12.6 ± 0.7	30.7 ± 0.7		57 ± 2
% **II** made from added precursor:	4.2×10^{-5}	3.7		6.9

[1]Oats were defatted by Soxhlet-extraction with diethyl ether for both 30 min and 12 h. Since extraction of the oats for 12 h did not increase the % incorporation of radiolabel into volatiles, the cucujolides from these two treatments were combined for subsequent analyses.

Prestarved *C. ferrugineus* feeding on oats that had been extracted with diethyl ether prior to being coated with [9,10-T]-palmitic acid were able to incorporate a greater proportion of the added substrate into cucujolide **II** (Table 4). Thus it was expected that the incorporation of deuterium into the cucujolide **II** produced by starved insects feeding on defatted oats coated with stable-isotope labelled fatty acids would be detectable by GC/SIM-MS. To ascertain if this would in fact occur, the incorporation of [16,16,16-D_3]-palmitic acid and [8,8-D_2]-stearic acid into the cucujolides was studied.

c) Trial of stable isotope labelling method

The incorporation of [16,16,16-D_3]-palmitic acid into cucujolides **II** and **III** was clearly discernable by GC/SIM-MS. The relative abundances of the M^+, $(M+1)^+$, $(M+2)^+$, and $(M+3)^+$ peaks of cucujolides produced by beetles feeding on unlabelled oats ("unlabelled" values, Table 5) were compared to those of beetles feeding on oats coated with D_3-palmitic acid ("D_3-palmitate" values, Table 5). Only the $(M+3)^+$ peak (corresponding to the incorporation of three deuterium atoms) showed enrichment due to the incorporation of D_3-palmitic acid (Table 5). Cucujolides **II** and **III** were clearly enriched for 3 deuterium atoms while, as expected, cucujolide **I** (of suspected terpenoid origin) was not labelled.

Table 5: Incorporation of [16,16,16-D_3]-palmitic acid into cucujolides[1]: relative abundances of GC/SIM-MS peaks of interest.

Sample	M^+	$(M+1)^+$	$(M+2)^+$	$(M+3)^+$
Cucujolide **I**				
unlabelled:	100.0	12.7	1.2	n.d.[2]
D_3-palmitate:	100.0	13.2	1.1	n.d.
Cucujolide **II**				
unlabelled:	100.0	20.9	1.8	n.d.
D_3-palmitate:	100.0	21.7	1.8	**2.8**
Cucujolide **III**				
unlabelled:	100.0	34.0	2.6	n.d.
D_3-palmitate:	100.0	32.4	2.5	**1.9**

[1] 2.01 g of starved beetles were fed on 15 g of defatted oats, coated with 30 mg [16,16,16-D_3]-palmitic acid; [2] "n.d." = "not detectable".

The proportion of cucujolide **II** molecules enriched with three deuterium atoms is termed the "enrichment factor" (EF), and can be approximated as described by Campbell (1974). In the present case, the EF is 2.7%, while the EF of cucujolide **III** for three deuterium atoms is 1.9%.

A similar experiment was performed with [8,8-D_2]-stearic acid. The

results are summarized in Table 6. As expected cucujolide I was not enriched for deuterium. Cucujolide III was enriched for two deuteriums [as shown by the larger relative abundance of the $(M+2)^+$ peak, EF = 4.5%]. Cucujolide II did not show an enrichment for two deuteriums. Initially this was surprising, as stearic acid was expected to be an effective precursor of II (Figure 15). However, as shown in Figure 16, after ß-oxidation, the two deuterium atoms of [8,8-D_2]-stearic acid are α to a carbonyl and can thus exchange with unlabelled protons of water.

Table 6: Incorporation of [8,8-D_2]-stearic acid into cucujolides[1]: relative abundances of GC/SIM-MS peaks of interest.

Sample	M^+	$(M+1)^+$	$(M+2)^+$	$(M+3)^+$
Cucujolide I				
unlabelled:	100.0	14.2	1.7	n.d.
D_2-stearate:	100.0	12.9	0.8	n.d.
Cucujolide II				
unlabelled:	100.0	13.9	1.7	n.d.
D_2-stearate:	100.0	15.2	1.5	n.d.
Cucujolide III				
unlabelled:	100.0	20.5	1.9	n.d.
D_2-stearate:	100.0	21.4	**6.6**	8n.d.

[1] 1.70 g of starved beetles were fed on 15 g of defatted oats, coated with 30 mg [8,8-D_2]-stearic acid.

Thus it is technically feasible to use stable isotope-labelled precursors to study cucujolide biosynthesis. Since the techniques of defatting the oats and prestarving the beetles improved incorporation efficiencies, many of the radiolabelling studies were performed under these conditions. The results are presented in the following discussion.

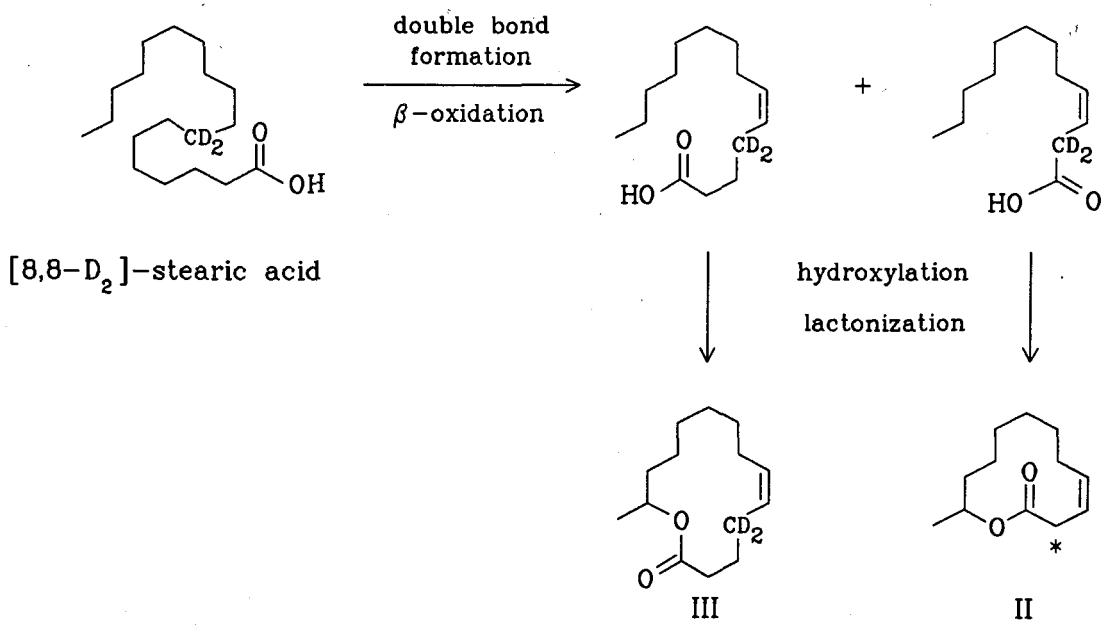


Figure 16: Biosynthesis of cucujolides **II** and **III** from [8,8-D_2]-stearic acid.

2. Investigation of cucujolide biosynthesis

a) Determination of biosynthetic origin of cucujolides

Cucujolides **I** to **V** typically accounted for about 97% (by analytical GC) of the Porapak Q-trapped volatiles emanating from *C. ferrugineus* fed on oats. Typically, 840 μg **I**; 670 μg **II**; 160 μg **III**; 20 μg **IV**; and 6.2 μg **V** were produced by 2 g of beetles (about 6000 individuals) over 10 days of aeration. When the oats were impregnated with radiolabelled substrates, 0.169% to 2.80% of the total amount of radiolabel supplied was incorporated into the volatiles (Table 7). These levels are comparable to those observed in pheromone-labelling studies in other insects (Mitlin and Hedin, 1974; Schmidt and Munroe, 1976; Thompson and Mitlin, 1979; Dillwith *et al.*, 1981; Wolf and Roelofs, 1983). Due to the non-specific method by which labelled substrates were introduced to the beetles in this study, the percent incorporation was not an accurate measure of a substrate's ability to serve as a cucujolide precursor. Incorporation is also

affected by such factors as the rate of absorption, and by the rate of transport of the substrate or its derivatives to the site of pheromone biosynthesis. For comparison, the specific activity of each labelled cucujolide was calculated (Table 7). In some cases, the specific activities of some cucujolides were not recorded. Data were not reported if the scintillation counts were not statistically different from background or if the sample was depleted before the cucujolide could be purified from unknown (potentially radiolabelled) contaminants. Failure to detect incorporation of radiolabel could be due to the technical difficulties involved in the isolation and purification of small quantities of volatile compounds.

Table 7: Incorporation of radiolabelled fatty acid substrates into cucujolides by *C. ferrugineus*[1].

	Radiolabelled substrate		
	[U-^{14}C]-linoleic acid	[9,10-T]-oleic acid	[9,10-T]-palmitic acid
beetles starved?	yes	yes	yes
oats defatted?	no	no	yes
Quantity used (μCi)	2.52 ± 0.05	21.7 ± 0.4	200.1 ± 0.1
Incorporation into volatiles (μCi)	0.009 ± 0.002	0.125 ± 0.007	5.60 ± 0.14
Incorporation into volatiles (% of total)	0.37 ± 0.07	0.58 ± 0.04	2.80 ± 0.07
Specific Act. (mCi/mol)			
I:	0.22 ± 0.04	0.38 ± 0.09	2.35 ± 0.09
II:	n.d.	3.1 ± 0.2	30.7 ± 0.7
III:	n.d.	5.3 ± 0.6	25.0 ± 1.0
IV:	1.7 ± 0.6	n.d.	10.1 ± 0.3
V:	n.d.	n.d.	11.7 ± 0.7

[1]Errors were calculated as described in the *Experimental*.

Fatty acid origin of cucujolides II - V

When *C. ferrugineus* was fed on oats coated with [9,10-T]-palmitic acid, the cucujolides **II**, **III**, **IV** and **V** produced were radiolabelled (Table 7), indicating that palmitic acid can serve as a precursor of these cucujolides. Similarly, [9,10-T]-oleic acid was incorporated into **II** and **III**; while [U-^{14}C]-linoleic acid was incorporated into **IV**. These results support the hypothesized fatty acid origin of these cucujolides.

Contrary to expectations (Figure 15), incorporation of linoleic acid into **V** was not observed. Although this could mean that linoleic acid did not serve as a precursor of **V**, it is more likely that the incorporation was not detected due to technical difficulties. Significant losses of cucujolides were suffered at each step of the purification, due to such factors as removal of sample for scintillation counting and evaporation. The incorporation of linoleic acid into **V** would have been especially difficult to detect, since **V** was produced at the lowest level of all the cucujolides studied, and since relatively little radiolabelled linoleic acid was added.

Terpenoid origin/*de novo* biosynthesis of cucujolide I

The specific activities of **I** were about ten-fold less than those of the "fatty acid derived" cucujolides when *C. ferrugineus* was fed labelled fatty acid substrates (Table 7). The low activities of **I** observed support the hypothesis that this cucujolide is not derived directly from fatty acids. The radiolabel observed in **I** was likely due to the incorporation of radiolabelled acetate, formed by ß-oxidation of the labelled fatty acids. This hypothesis is supported by the observation that radiolabelled acetate was incorporated into **I** (Table 8). The ten-fold ratio observed between the two routes (*de novo* biosynthesis versus the derivatization of the fatty acids) is comparable to ratios reported in the waxmoth (Schmidt and Monroe, 1976) and the housefly (Dillwith *et al.*, 1981), but is much greater than reported in boll weevils (Thompson and Mitlin, 1979).

Table 8: Incorporation of radiolabelled substrates into cucujolides by *C. ferrugineus*.

	Radiolabelled substrate		
	[1-^{14}C]-acetate	[5-T]-mevalonate	[1-^{14}C]-lauric acid
beetles starved?	yes	no	no
oats defatted?	yes	no	no
Quantity used (μCi)	50.00 ± 0.05	20.0 ± 0.3	5.0 ± 0.2
Incorporation into volatiles (μCi)	0.084 ± 0.004	0.140 ± 0.004	0.020 ± 0.001
Incorporation into volatiles (% of total)	0.169 ± 0.008	0.70 ± 0.03	0.41 ± 0.04
Specific Act. (mCi/mol)			
I:	1.19 ± 0.06	0.20 ± 0.08	0.53 ± 0.05
II:	1.14 ± 0.07	n.d.	0.33 ± 0.03
III:	1.1 ± 0.1	n.d.	0.45 ± 0.08
IV:	2.5 ± 0.4	n.d.	n.d.
V:	2.0 ± 0.4	n.d.	n.d.

Label was detected in **I**, but not in **II-V**, when the beetles were fed on oats laced with radiolabelled mevalonate (Table 8). These results support the hypothesized terpenoid origin of **I**.

***De novo* biosynthesis of cucujolides II - V**

Labelled acetate (but not mevalonate) was incorporated into **II**, **III**, **IV** and **V** (Table 8), indicating that *C. ferrugineus* can produce these cucujolides through *de novo* biosynthesis. The incorporation of labelled acetate into **IV** and **V** is particularly interesting since these cucujolides were hypothesized to be derived from linoleic acid (Figure 15). Although

linoleic acid is an essential dietary nutrient for most animals, some insect species have been shown to synthesize this fatty acid *de novo* (Blomquist et al., 1982; de Renobales et al., 1987). This has not previously been demonstrated in any species of Coleoptera but the present result indicates that *C. ferrugineus* might be able to synthesize linoleic acid *de novo*. An alternative explanation is that symbiotic microorganisms might supply *C. ferrugineus* with linoleic acid precursors. Although data are not available for *C. ferrugineus* specifically, the mycetomes of a closely related cucujid, *Oryzaephilus surinamensus*, are reported to contain "bacterium-like microorganisms" which are transmitted transovarially (Pant and Dang, 1972). *C. ferrugineus* might similarly possess endosymbionts.

I do not believe that the apparent *de novo* synthesis of linoleic acid is an artifact. As shown in Table 7, palmitic acid was also an effective precursor of **IV** and **V**. The conversion of palmitic acid to linoleic acid would require the action of the Δ^{11} desaturase. The palmitic acid and acetate-labelling experiments were repeated with similar results (to avoid duplication, the data are not presented in this thesis). The incorporation of oleic acid into **IV** and **V** was not detectable, likely due to technical difficulties previously mentioned. These difficulties were magnified by the small quantity of oleic acid used, which was ten times less radiolabel than in the palmitic acid experiment, and effectively five times less than in the acetate experiment (after correction for the greater quench of T).

Incorporation of [1-^{14}C]-lauric acid

Labelled lauric acid showed poor incorporation into all cucujolides. Labelling of **I** was likely due to the incorporation of labelled acetate formed by ß-oxidation of the lauric acid administered. Since the specific activities of **II** and **III** were comparable to that of **I** when labelled lauric acid was administered, these cucujolides were likely also labelled by the

incorporation of acetate. Incorporation of label from lauric acid into **IV** and **V** was not detected. These cucujolides were shown to be synthesized *de novo* from acetate (Table 8), but this route would have been technically difficult to detect in the lauric acid-labelling experiment. Ten times more radiolabel was used in the acetate-labelling experiment than in the lauric acid experiment, which would help to overcome the technical difficulties involved in evaluating incorporation into **IV** and **V** (which are produced at much lower levels than **I - III**).

Undegraded lauric acid might be incorporated into cucujolides **II-V** through one of two routes: (1) a path involving direct desaturation (*via* a unique Δ^3 desaturase), ω-1 oxidation, and lactonization; or (2) a path involving elongation to stearic acid, desaturation (*via* the Δ^9 desaturase), ß-oxidation and finally lactonization (Figure 17). Pheromone biosynthesis *via* chain elongation has been shown to occur in some species of Lepidoptera (Bjostad *et al.*, 1987). It is not possible to determine from the data in Table 8 if undegraded lauric acid was incorporated into cucujolides **II** and **III**. This problem was explored using stable isotope-labelled lauric acid, as discussed in the next section.

b) Biosynthesis of cucujolide II: further studies

The results thus far support the hypotheses that cucujolide **I** is of terpenoid origin and that cucujolides **II - V** are of fatty acid origin. The results also indicate that *C. ferrugineus* can synthesize these cucujolides *de novo*.

With the stable isotope-labelling technique as a viable alternative to the use of radiolabelled substrates, it was possible to study other aspects of cucujolide biosynthesis.

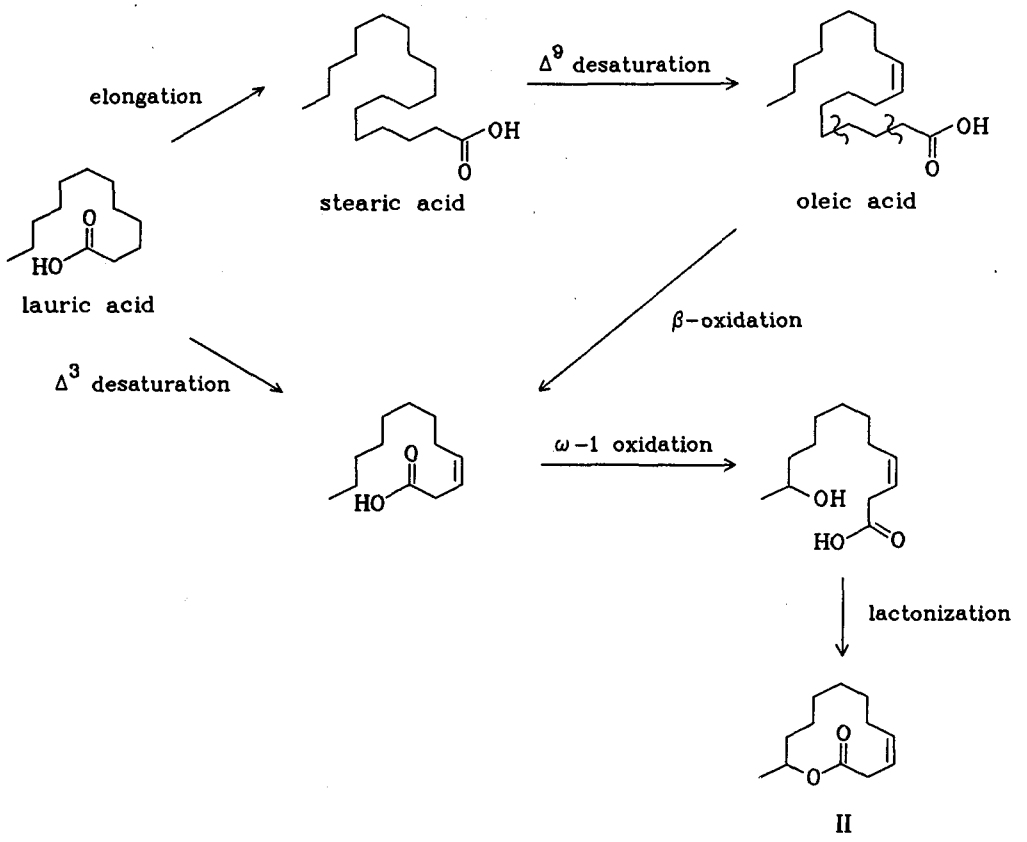


Figure 17: Two hypothetical biosynthetic routes by which undegraded lauric acid might be incorporated into cucujolide **II**.

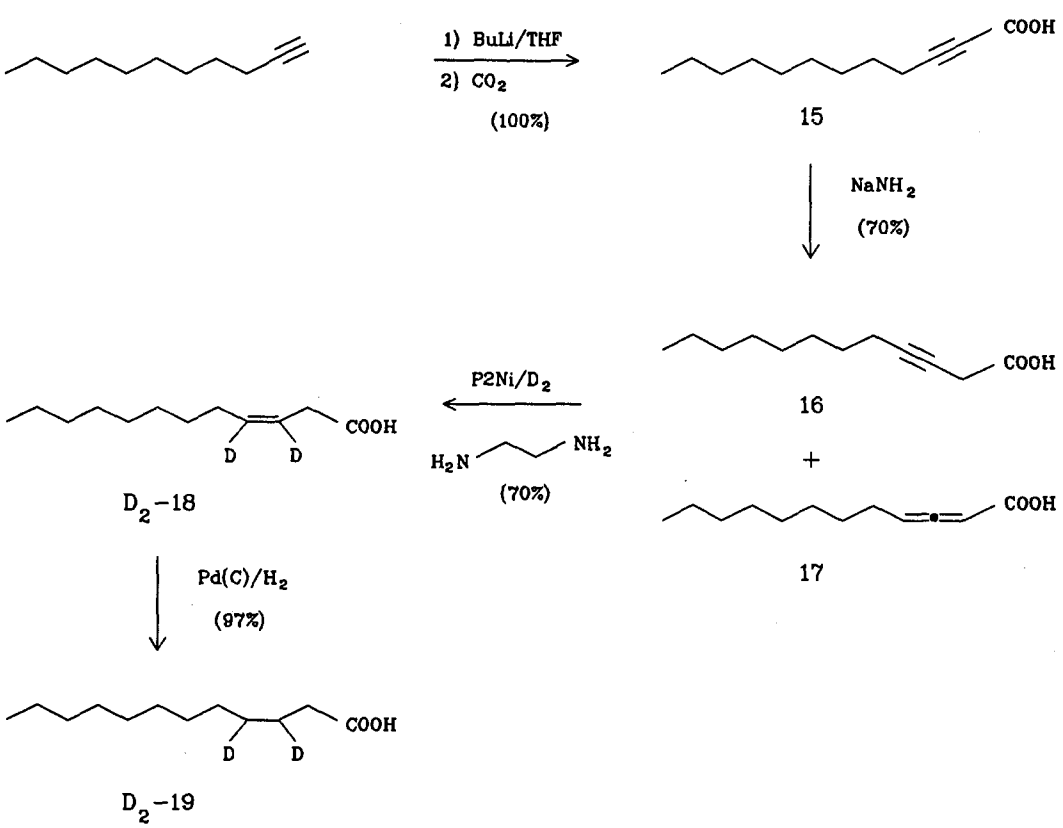
Incorporation of lauric acid into cucujolide II by *C. ferrugineus* and *O. mercator*

One objective was to investigate the possibility that undegraded lauric acid might be incorporated into cucujolide **II** according to one of the biosynthetic routes shown in Figure 17. My approach was to determine if insects exposed to deuterated lauric acid produced cucujolide **II** enriched in deuterium. As discussed earlier, failure to detect deuterium incorporation could be due to failure of the labelled precursor to reach the site of pheromone biosynthesis. Therefore the insects were also exposed to deuterated 3-dodecenoic acid (expected to be an effective precursor of cucujolide **II**, according to Figure 15). This compound is

very similar to lauric acid: if the insects could transport 3-dodecenoic acid to the site of pheromone biosynthesis, they should also be able to transport lauric acid.

Synthesis of labelled lauric and 3-dodecenoic acid

[3,4-D_2]-3-Dodecenoic acid (D_2-**18**) and [3,4-D_2]-lauric acid (D_2-**19**) were synthesized according to Scheme 1. Carbon dioxide was added to the anion of commercially available 1-undecyne in quantitative yield. The resulting 2-dodecynoic acid (**15**) was deconjugated with sodium amide in liquid ammonia (Brandsma, 1971; Oehlschlager *et al.*, 1986) to give 3-dodecynoic acid (**16**) and the corresponding allene (**17**) in an 87/11 ratio



Scheme 1: Synthetic route to [3,4-D_2]-3-dodecenoic acid (D_2-**18**) and [3,4-D_2]-lauric acid (D_2-**19**).

by analytical GC (70% yield). The mixture of **16** and **17** was reduced with deuterium gas over Lindlar's catalyst to give [3,4-D_2]-3-dodecenoic acid (D_2-**18**) in 70% yield. Some scrambling of the deuterium label was observed: the final product was 89% enriched for two deuteriums and 11% enriched for one deuterium (by IBCI GC/SIM-MS analysis). [3,4-D_2]-Lauric acid (D_2-**19**) was produced through the reduction of D_2-**18** with hydrogen gas over palladium on activated carbon (97% yield). This catalyst scrambled the deuterium labels further: the final product was 22% enriched for three deuteriums; 42% enriched for two deuteriums; 27% enriched for one deuterium; and 10% unenriched (by IBCI SIM/GC-MS). In retrospect, this could have been avoided through the use of a catalyst such as *tris*(triphenylphosphine)-rhodium(I) chloride (Monsen, 1971).

Incorporation of 3-dodecenoic acid and lauric acid into cucujolide II

C. ferrugineus were fed on oats coated with either D_2-3-dodecenoic acid (D_2-**18**) or D_2-lauric acid (D_2-**19**). Cucujolide **II** produced upon exposure of the insects to D_2-**18** was greatly enriched (EF=12%) for two deuterium atoms (Figure 18, Table 9). In contrast, D_2-**19** was not efficiently incorporated into cucujolide **II**.

O. mercator was also exposed to D_2-**18**, using the methods described for *C. ferrugineus*. Figure 19 compares the GC/MS of cucujolide **II** produced by *O. mercator* fed on defatted oats coated with D_2-**18** to that produced by a culture fed on untreated oats. As seen in Table 10, cucujolide **II** produced by insects fed D_2-**18** was greatly enriched for two deuteriums (EF=37%) while, as expected (Figure 15), cucujolide **IV** was not. Enhancement of cucujolide **IV** for one deuterium was not observed. It was possible that D-acetate (formed from the degradation of D_2-**18**) would be incorporated into this cucujolide, since the radio-labelling experiments (Table 8) indicated that this cucujolide could be formed through *de novo* synthesis in *C. ferrugineus*. However cucujolide production by *O. mercator* is extremely sensitive to food deprivation (Oehlschlager *et al.*, 1988; Dr.

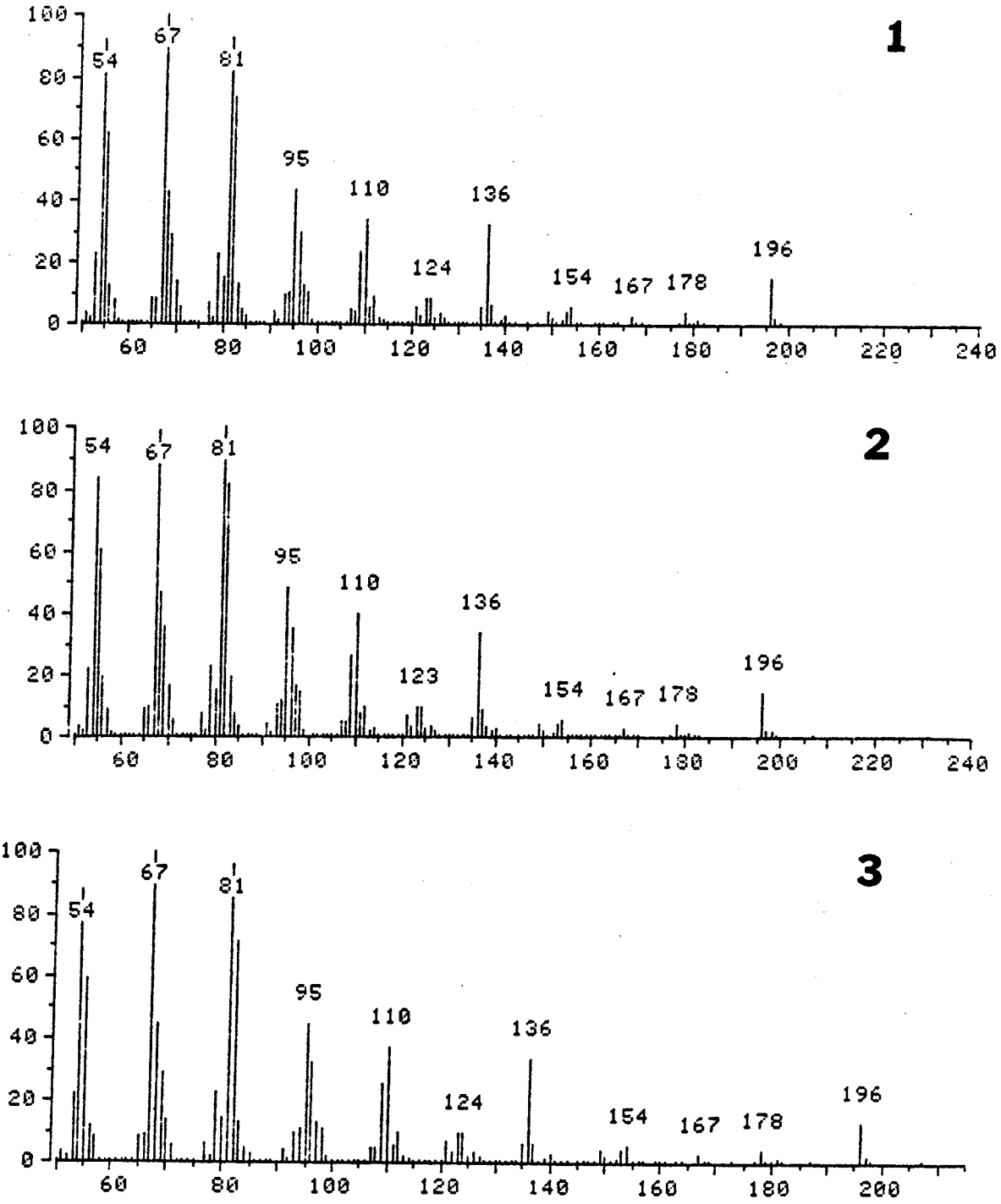


Figure 18: GC-MS (EI) of cucujolide II produced by *C. ferrugineus* feeding on: 1, untreated oats; 2, defatted oats coated with D_2-18; and 3, defatted oats coated with D_2-19.

Table 9: Incorporation of D_2-dodecenoic acid (D_2-18)[1] and D_2-lauric acid (D_2-19)[2] into cucujolides by *C. ferrugineus*: relative abundances of GC/SIM-MS peaks of interest.

	M^+	$(M + 1)^+$	$(M + 2)^+$	$(M + 3)^+$
Cucujolide I				
unlabelled:	100.0	13.6	1.4	0.1
D_2-18:	100.0	15.2	1.5	0.1
D_2-19:	100.0	16.8	2.0	0.2
Cucujolide II				
unlabelled:	100.0	13.1	1.4	0.1
D_2-18:	100.0	14.9	15.4	2.2
D_2-19:	100.0	15.0	1.7	n.d.
Cucujolide III				
unlabelled:	100.0	16.3	1.6	n.d.
D_2-18:	100.0	16.2	1.8	0.1
D_2-19:	100.0	18.3	2.3	0.2

[1] 1.00 g of beetles were fed on 15 g of defatted oats, coated with 150 mg D_2-18; [2] 1.00 g of beetles were fed on 15 g of defatted oats, coated with 380 mg D_2-19.

H. D. Pierce, Jr., personal communication*). The defatted oats supplied as food in these experiments were sufficient to support normal cucujolide biosynthesis in *C. ferrugineus*, but in *O. mercator* cucujolide biosynthesis was reduced about one hundred fold compared to normally feeding insects. Thus, *de novo* cucujolide biosynthesis by *O. mercator* was likely sharply curtailed under the conditions of this experiment.

* Department of Chemistry, Simon Fraser University, Burnaby, BC, Canada. V5A 1S6.

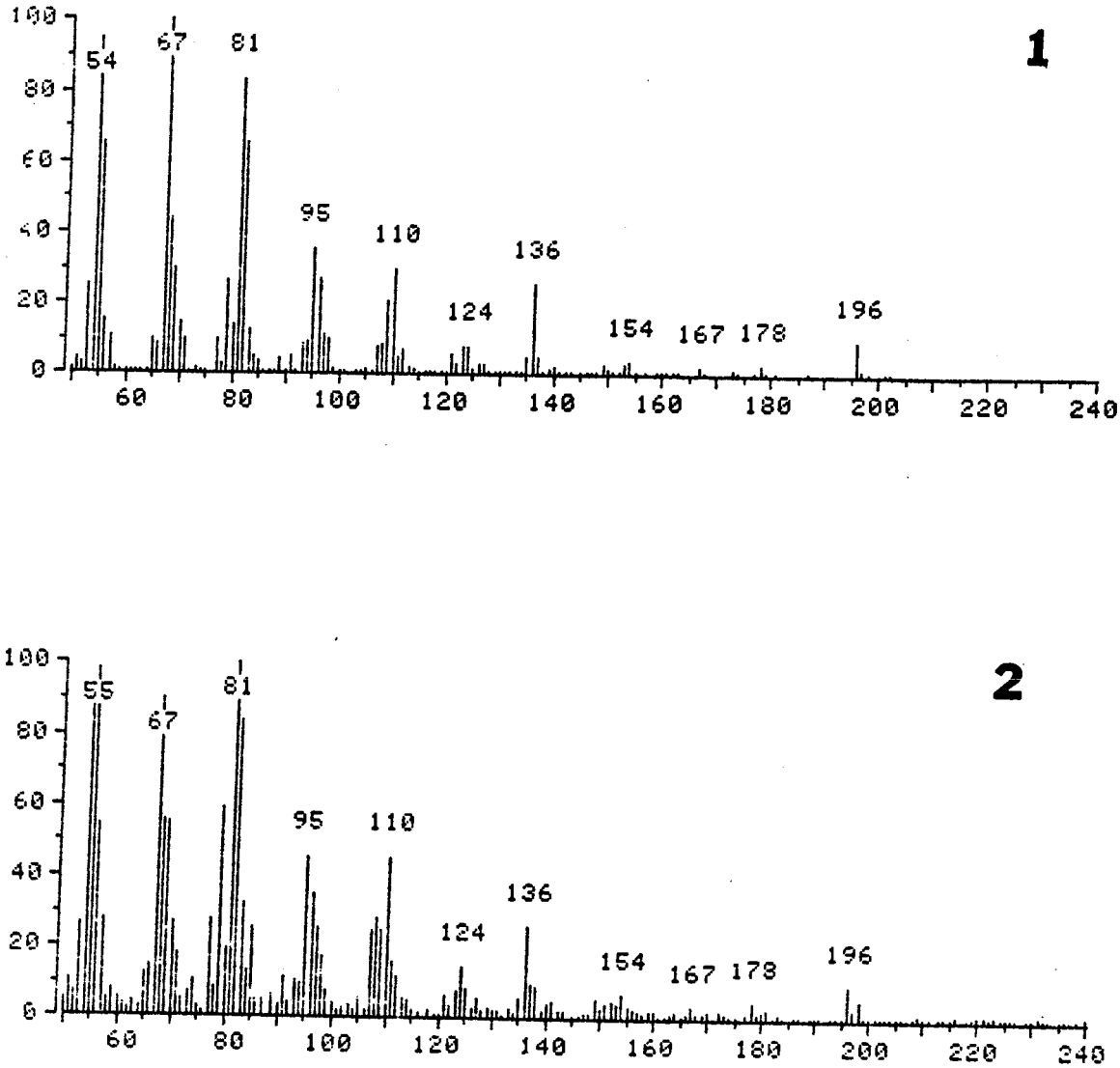


Figure 19: GC-MS (EI) of cucujolide II produced by *O. mercator* feeding on: 1, untreated oats; or 2, defatted oats coated with D_2-18

Table 10: Incorporation of D_2-dodecenoic acid (D_2-**18**)[1] into cucujolides by *O. mercator*: relative abundances of GC/SIM-MS peaks of interest.

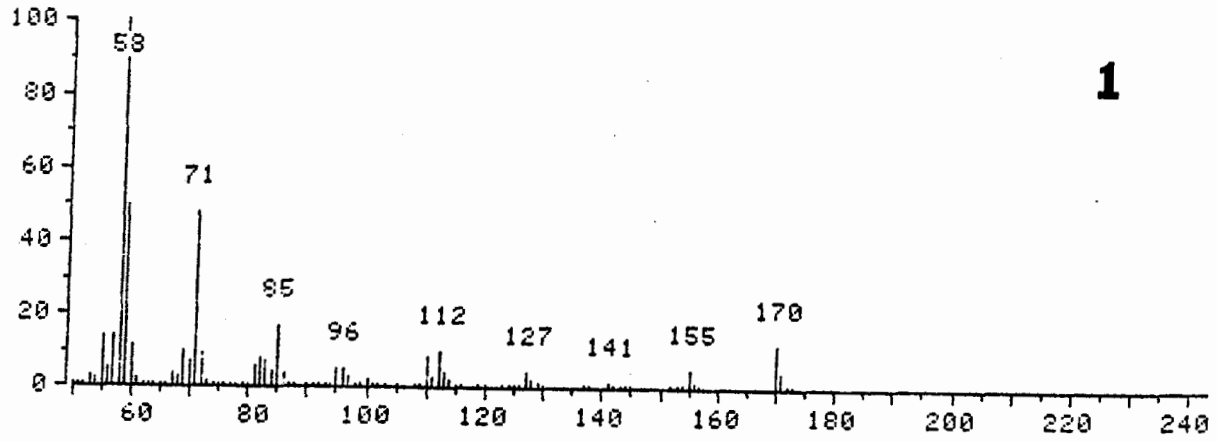
	M^+	$(M + 1)^+$	$(M + 2)^+$	$(M + 3)^+$
Cucujolide II				
unlabelled:	100.0	13.3	1.6	0.2
D_2-**18**:	100.0	18.1	**64.4**	7.4
Cucujolide IV				
unlabelled:	100.0	13.8	1.4	0.2
D_2-**18**:	100.0	13.5	1.3	n.d.

[1] 2.00 g of beetles were fed on 30 g of defatted oats, coated with 150 mg D_2-**18**.

*Biosynthesis of unknowns **A**, **B**, **C** and **D***

Three unknown compounds were observed in the Porapak Q extracts of both the *C. ferrugineus* and *O. mercator* cultures exposed to [3,4-D_2]-3-dodecenoic acid. The three unknowns exhibited the same GC retention times (R.T.s, namely 10.49, 12.45, and 14.29 min, respectively, with the conditions as stated in *Experimental*) and MS (EI) in the two extracts, and were assumed to be identical. These compounds (designated **A**, **B** and **C**) comprised 3%, 22% and 5% (equivalent to 70 μg, 616 μg and 135 μg), respectively, of the *C. ferrugineus* extract, and 7%, 48% and 22% (equivalent to 51 μg, 341 μg and 174 μg), respectively, of the *O. mercator* extract. Unknowns **A** and **B** from the *C. ferrugineus* extract were purified by preparative GC (to 90% and 98% purity, respectively). Unknown **C** from the *O. mercator* extract was purified to 77% purity, but still contained 7% **A** and 3% **B**. The MS (both EI and IBCI) and ^{1}H NMR (400 MHz) spectra of the three unknowns are presented in Figures 20, 21 and 22.

Three compounds of the same GC R.T.s as unknowns **A**, **B**, and **C** were also observed in the Porapak Q extract of the *C. ferrugineus* culture exposed to D_2-**19**. Compounds co-eluting with unknowns **A**, **B** and **C** comprised



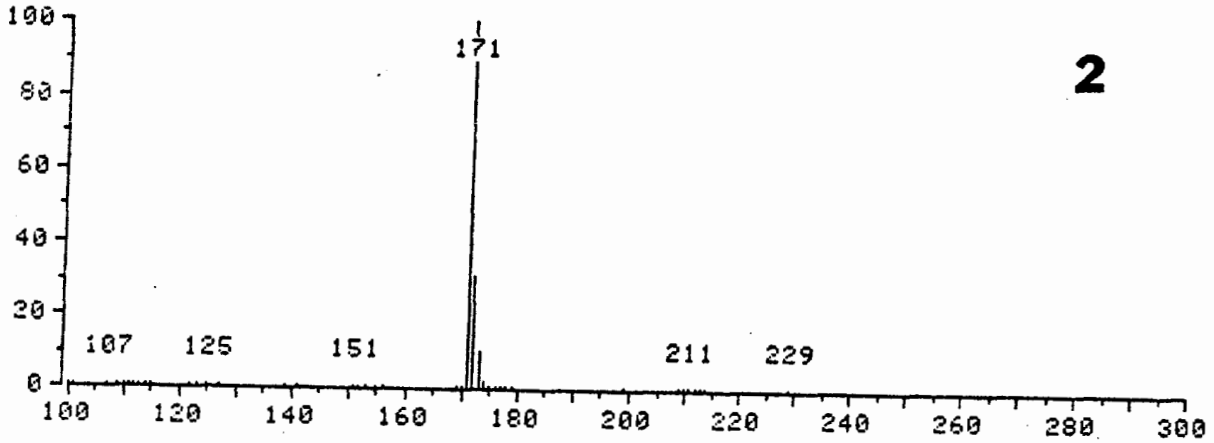
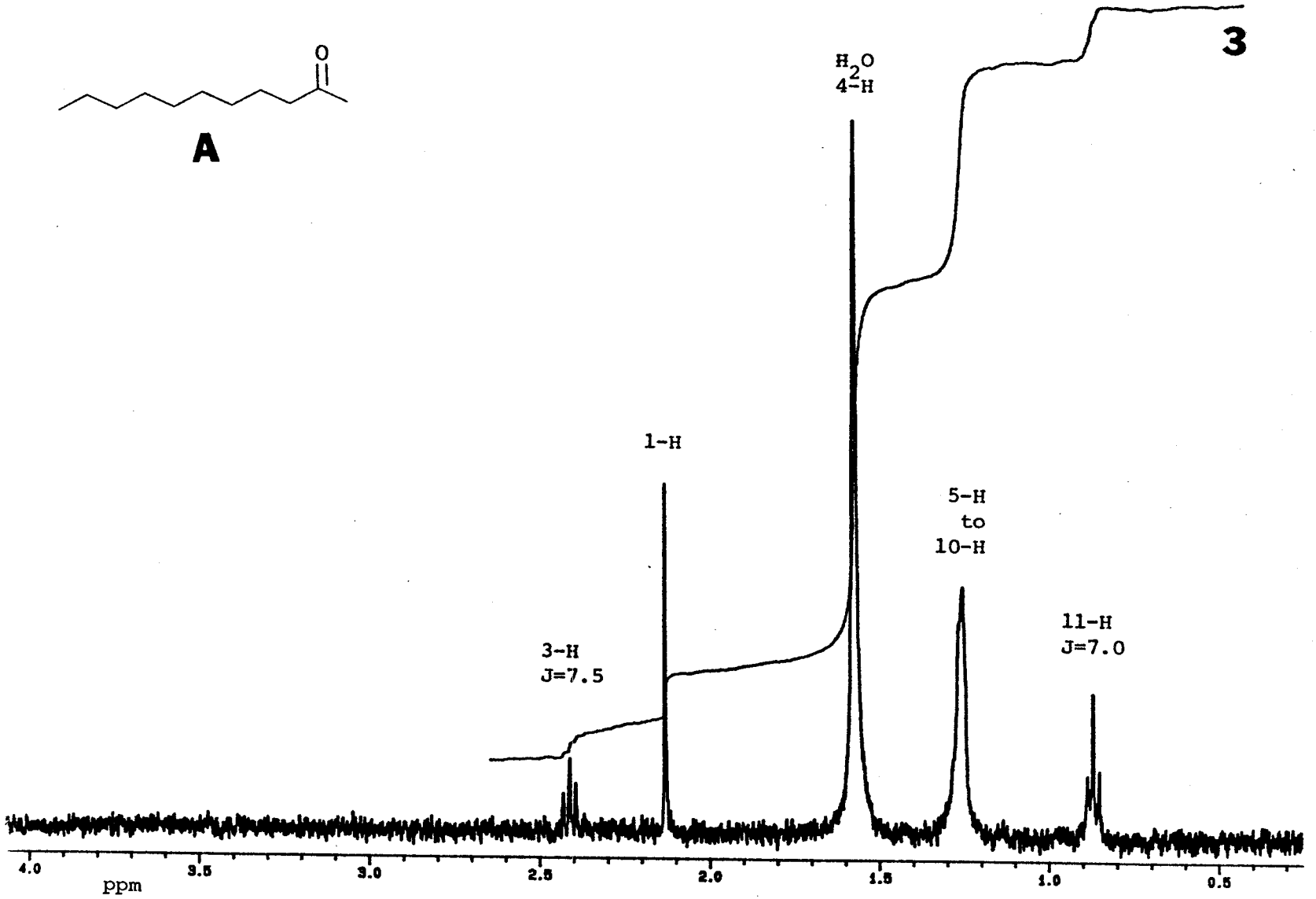
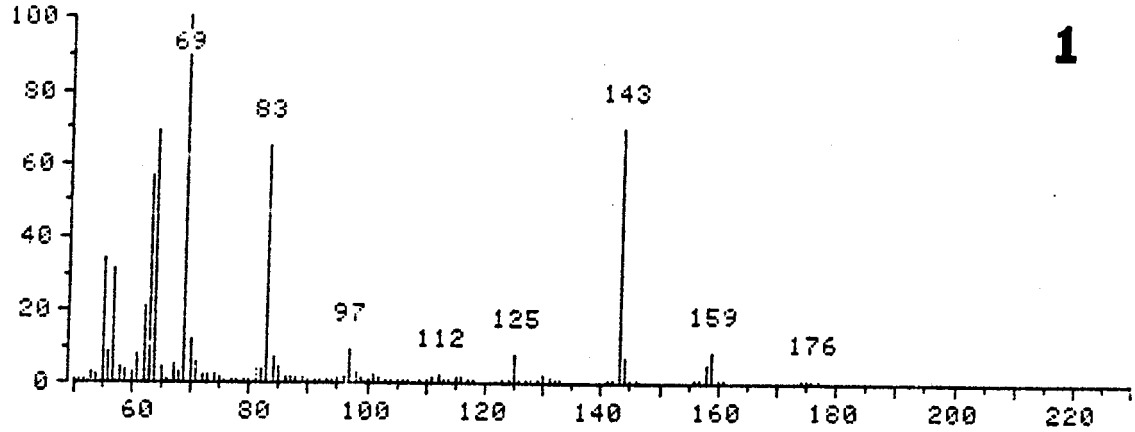


Figure 20: Spectra of Unknown **A** (90% pure by analytical GC): 1, MS (EI); 2, MS (IBCI); 3, ^{1}H NMR (400 MHz in $CDCl_3$) (next page).


A
3
H2O
4-H
1-H
5-H
to
10-H
11-H
J=7.0
3-H
J=7.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
ppm



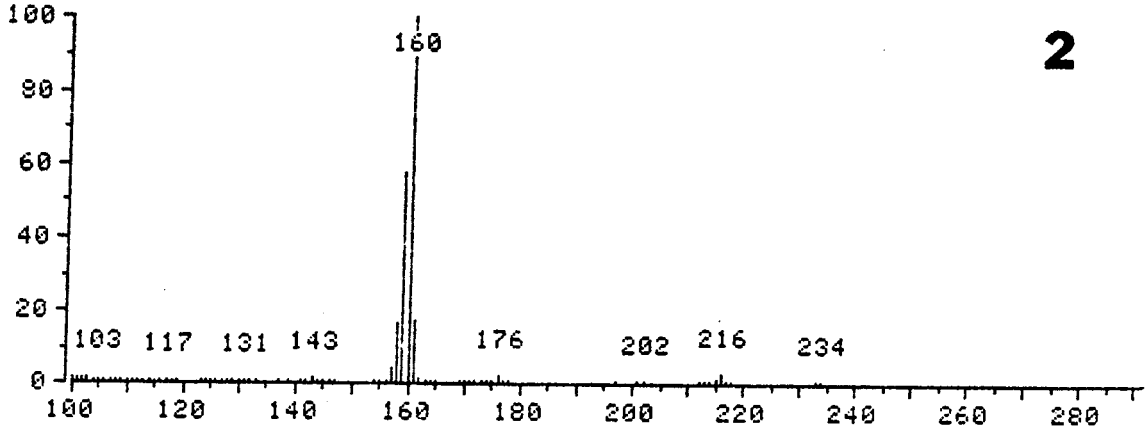
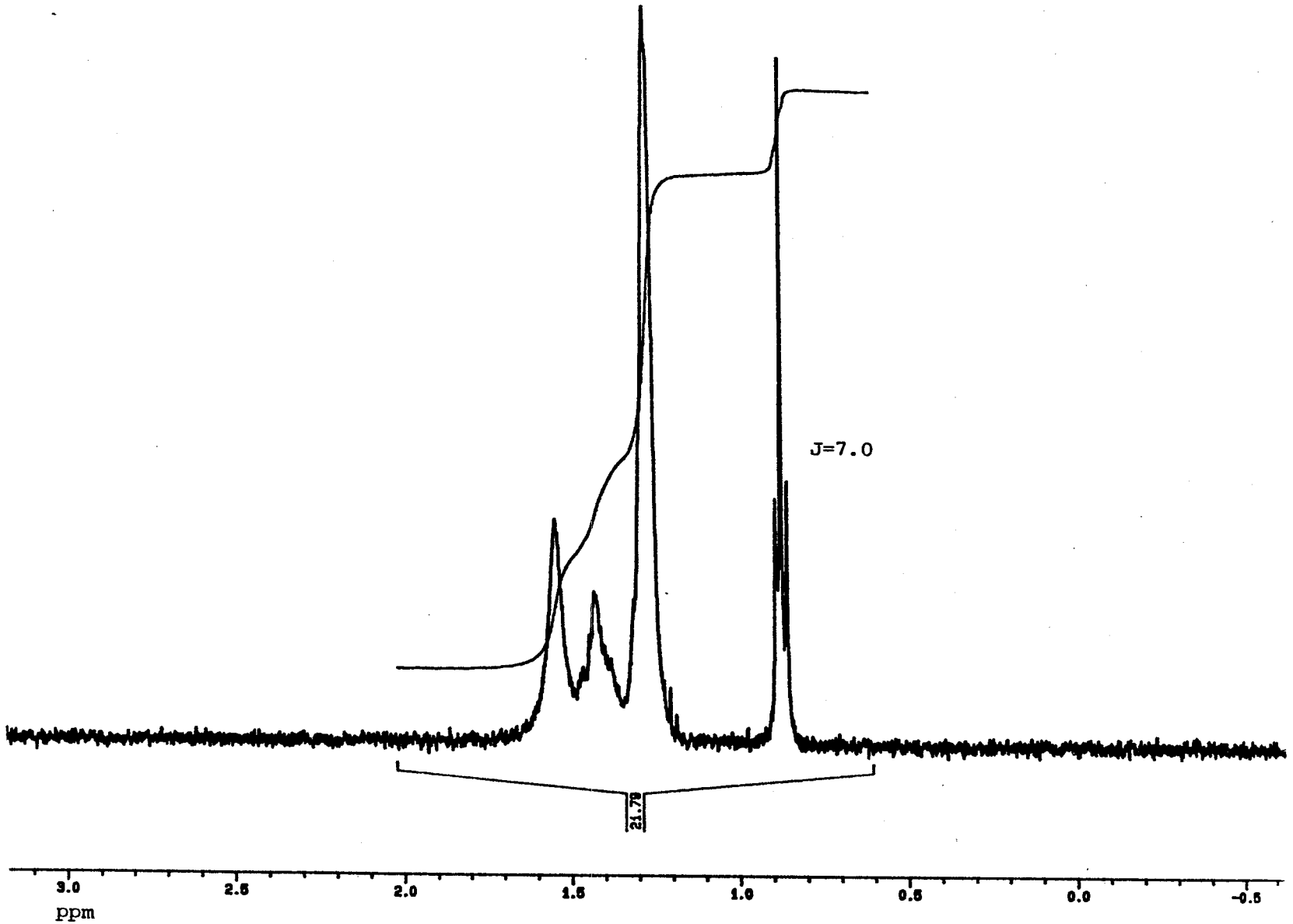


Figure 21: Spectra of Unknown B (98% pure by analytical GC): 1, MS (EI); 2, MS (IBCI); 3, ^{1}H NMR (400 MHz in $CDCl_3$) (next page).

3



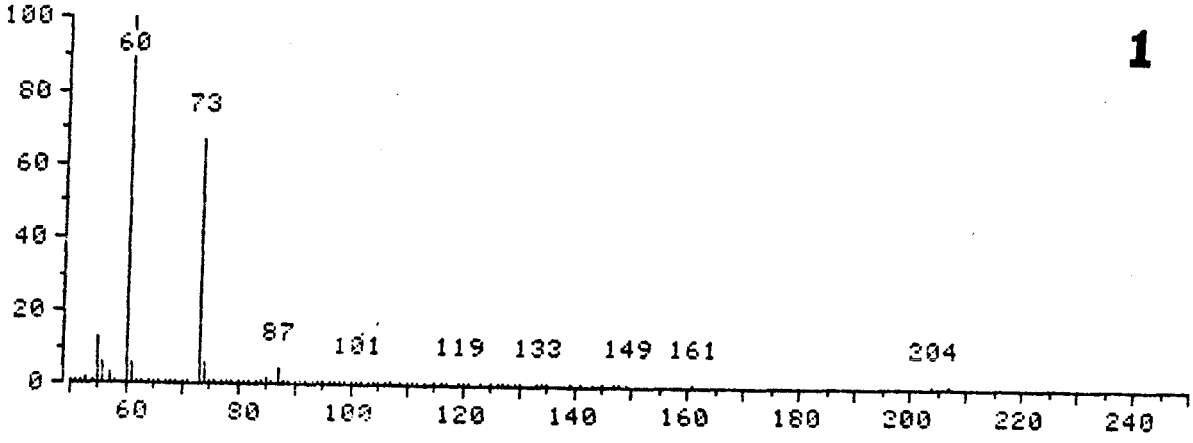
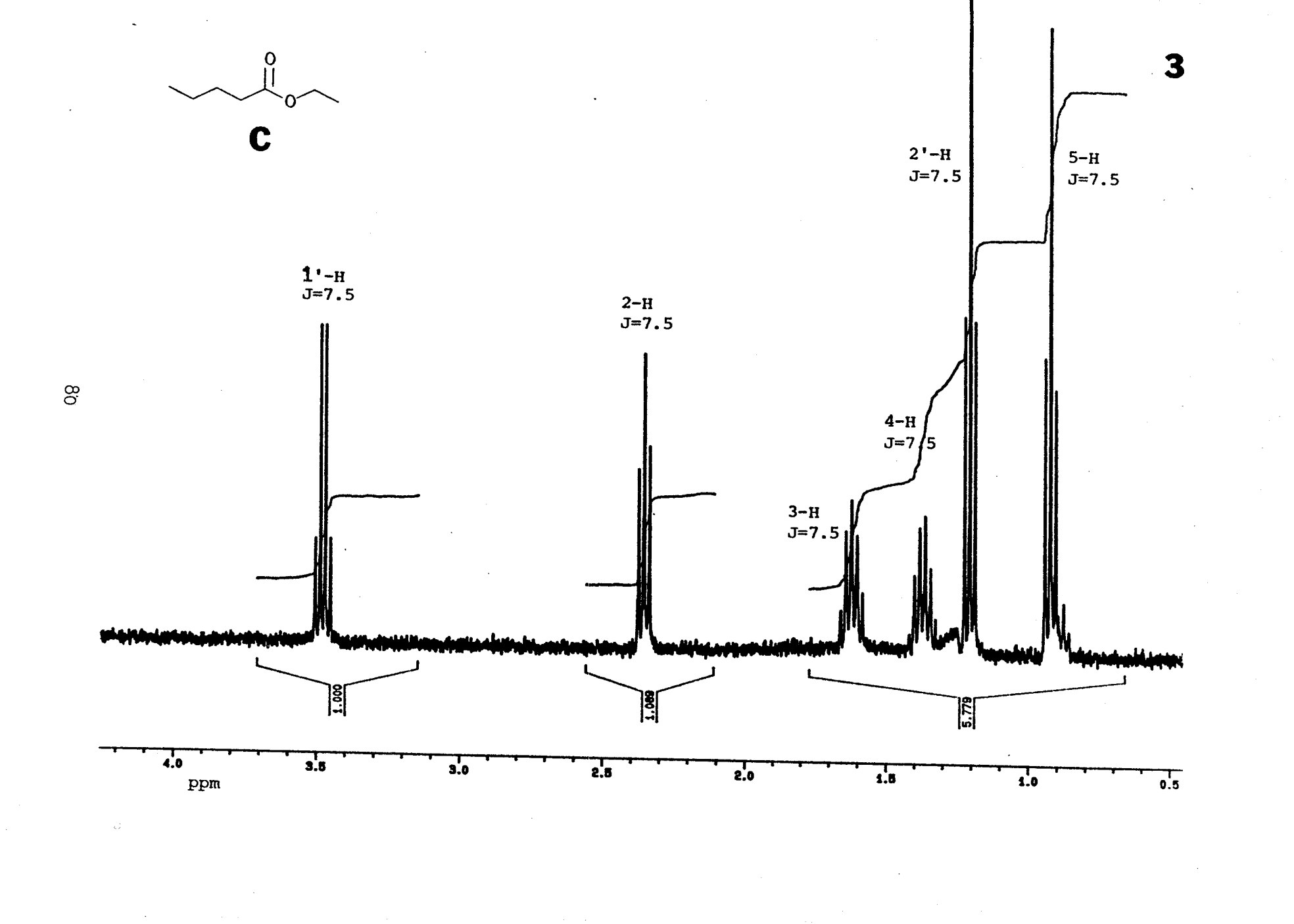
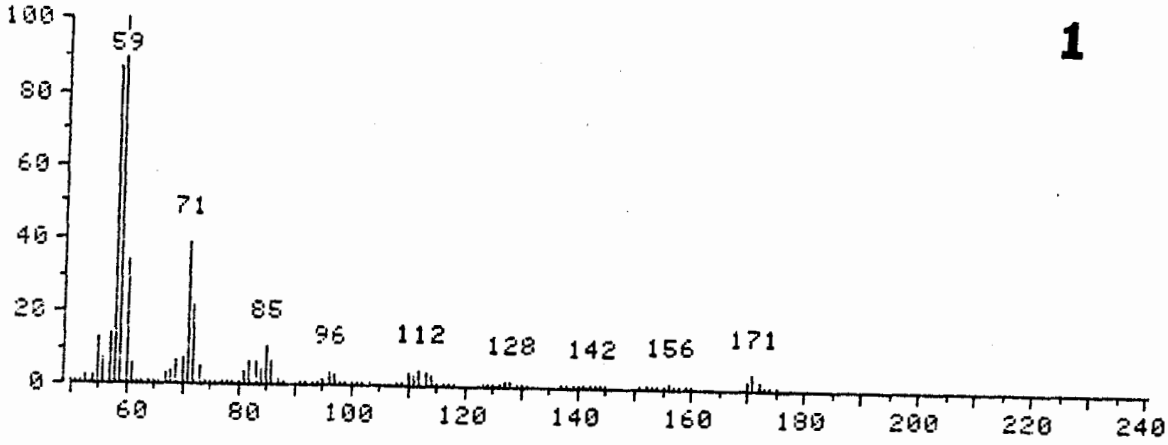


Figure 22: Spectra of Unknown C (77% pure by analytical GC): 1, MS (EI); 2, ^{1}H NMR (400 MHz in $CDCl_3$) (next page).


3
C
1'-H
J=7.5
2-H
J=7.5
3-H
J=7.5
4-H
J=7.5
2'-H
J=7.5
5-H
J=7.5
1.000
1.008
5.779
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
ppm



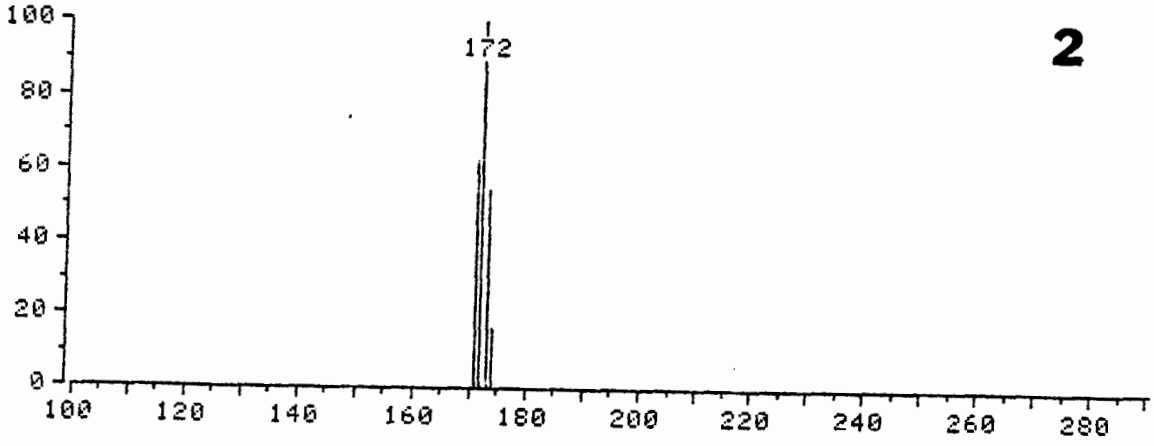
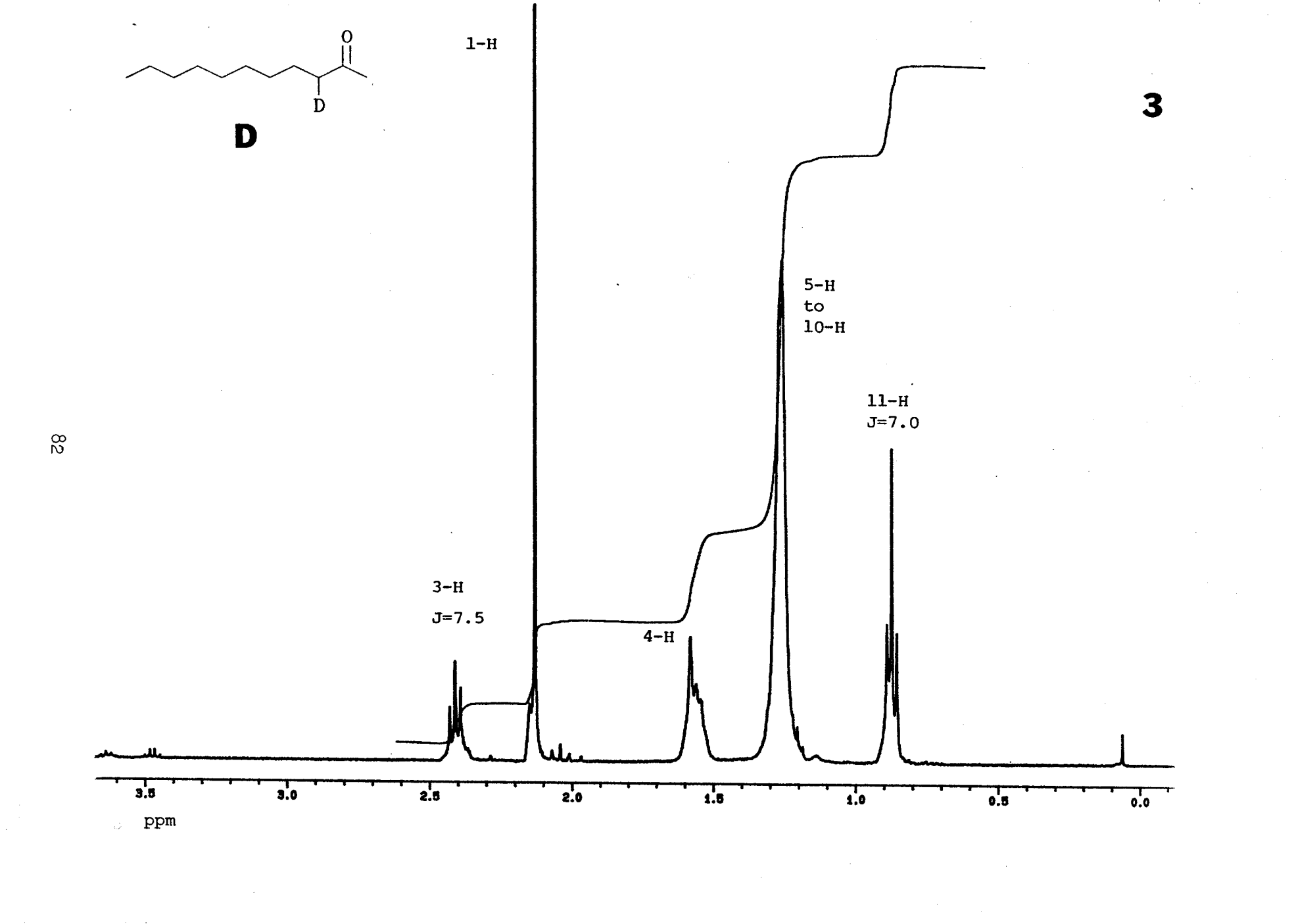


Figure 23: Spectra of Unknown D (92% pure by analytical GC): 1, MS (EI); 2, MS (IBCI); 3, ^{1}H NMR (400 MHz in $CDCl_3$) (next page).


O
D
D
1-H
3
5-H
to
10-H
11-H
J=7.0
3-H
J=7.5
4-H
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
ppm

72%, 5% and 0.4% (equivalent to 1.1 mg, 75 μg and 7 μg), respectively, of the extract. It was not possible to isolate in sufficient purity the compounds coeluting with unknowns **B** and **C** but these compounds exhibited the same MS (EI) as their counterparts. The MS (EI) of the compound coeluting with unknown **A** was closely related, but not identical, to that of unknown **C**. This compound was designated as unknown **D** and was purified by preparative GC. The MS (both EI and IBCI) and ^{1}H NMR (400 MHz) spectra are presented in Figure 23.

Figures 20 and 23 reveal that unknowns **A** and **D** are closely related. In addition to possessing the same GC R.T.s, their molecular ion peaks (m/e 170 and 171, respectively) differ by only one unit, and their ^{1}H NMR spectra are very similar (the large peak at δ 1.56 in Figure 20 is likely due to absorption by water protons). Most likely, unknowns **A** and **D** are the same, with **D** possessing one more deuterium than **A**. If **A** and **D** were formed through the metabolism of D_2-**18** (M^+ = 200) and D_2-**19** (M^+ = 202),the MS indicate that the transformations involved net losses of 30 and 31 mass units, respectively. These numbers indicate a NET loss of one carbon atom, one oxygen atom, and an appropriate number of ^{1}H or D atoms per molecule. The ^{1}H NMR spectra showed singlets (3H) at δ 2.13, indicative of methyl ketones. These two factors led to the tentative identification of the unknowns as undeca-2-one. If this assignment is correct, unknown **A** is not labelled with deuterium (M^+ = 170), while unknown **D** is labelled with one deuterium at C-3 (M^+ = 171). Biosynthesis of **D** from D_2-**19** would presumably involve ß-hydroxylation, reductive decarboxylation or decarbonylation, and oxidation of the resulting hydroxy-hydrocarbon to the corresponding ketone. The last step mentioned would cause loss of the D atom originally at C-3. Biosynthesis of **A** from D_2-**18** would be similar, but would have to involve reduction of the double bond and loss of the D atom originally at C-4.

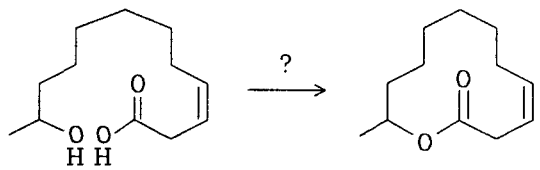
Unknown **C** was tentatively identified as pentyl acetate on the basis of its ^{1}H NMR spectrum. The reason for the relatively large production of

this compound when *C. ferrugineus* was exposed to D_2-**19** is not clear.

A structure was not assigned to unknown **B**.

Investigation of the mechanism of the lactonization reaction to form cucujolide II.

In this portion of the study, the ability of 11-hydroxy-3-dodecenoic acid (**26**) to serve as a precursor of **II** *in vivo* (Figure 24) was investigated. The approach taken was to determine if the cucujolide **II** produced in the presence of deuterated **26** was enriched for deuterium.



26 II

Figure 24: Hypothesized biosynthesis of cucujolide **II** from 11-hydroxy-3-dodecenoic acid, **26**.

If **26** was found to be a precursor of cucujolide **II**, the next objective was to study the direction of the cyclization of **26** to form the macrolide product. The cyclization of **26** could conceivably occur in either of the two directions shown in Figure 25. To distinguish between the two, *C. ferrugineus* and *O. mercator* were exposed to deuterated **26** that was also enriched for ^{18}O at C-11. Any cyclization mechanism involving retention of the C-11 hydroxyl oxygen, such as that through the Coenzyme A (CoA) intermediate shown in Figure 25, would result in the retention of ^{18}O. Cucujolide **II** thus produced would be enriched for both deuterium and ^{18}O. In contrast, any cyclization mechanism involving loss of the C-11 hydroxyl oxygen, such as that through the pyrophosphate intermediate, would result in loss of the ^{18}O. The cucujolide **II** produced through such a mechanism would be enriched for deuterium but not ^{18}O.

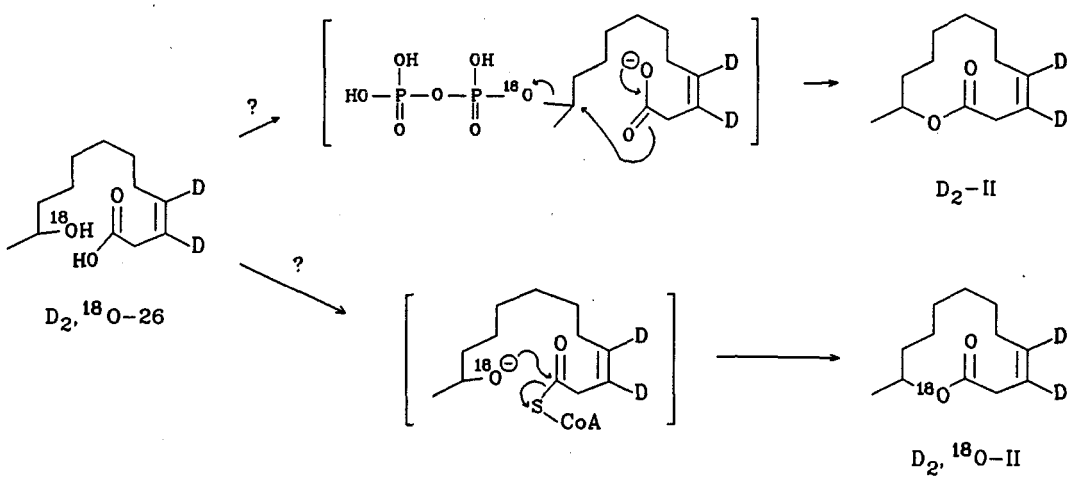


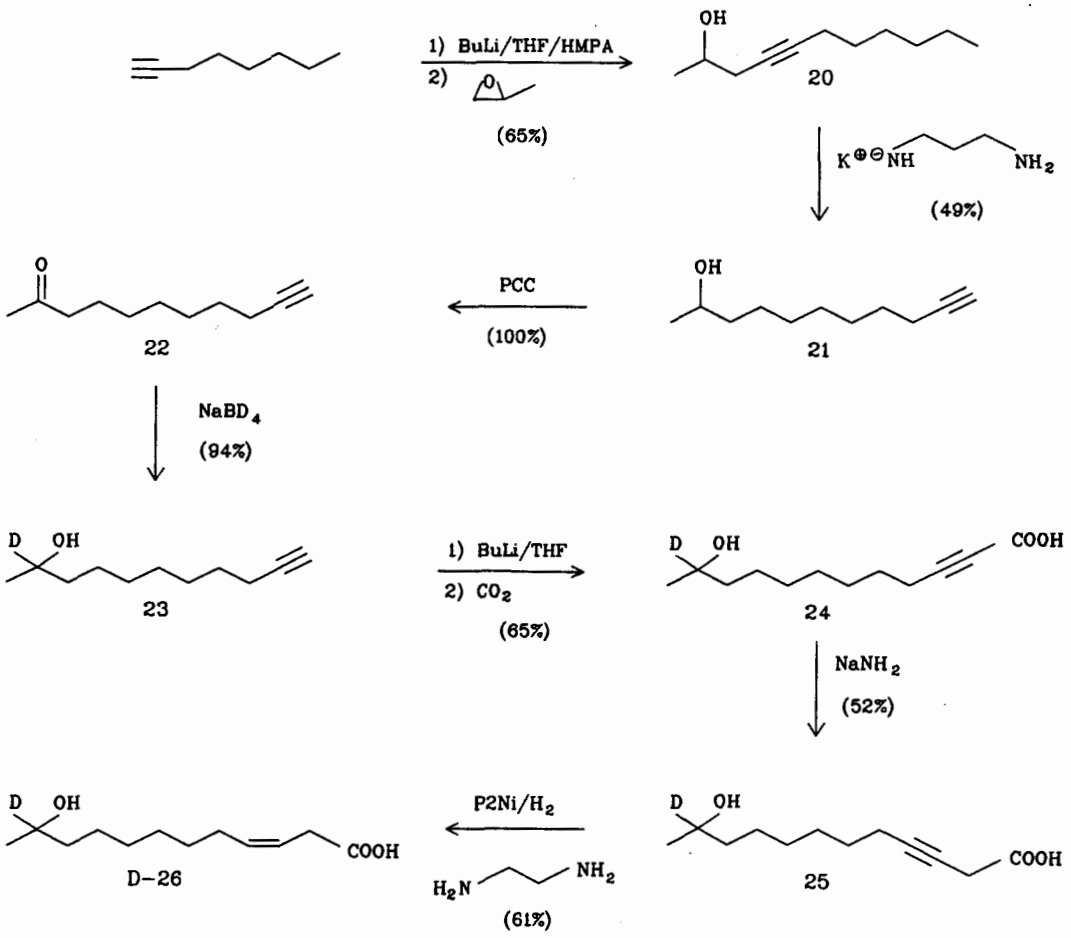
Figure 25: Examples of the two possible directions of cyclizations of the hydroxy acid **26** to cucujolide **II**. Differentiation between the two types of mechanisms is possible with ^{18}O- and D_2-labelled **26**.

Syntheses of labelled 11-hydroxy-3-dodecenoic acid

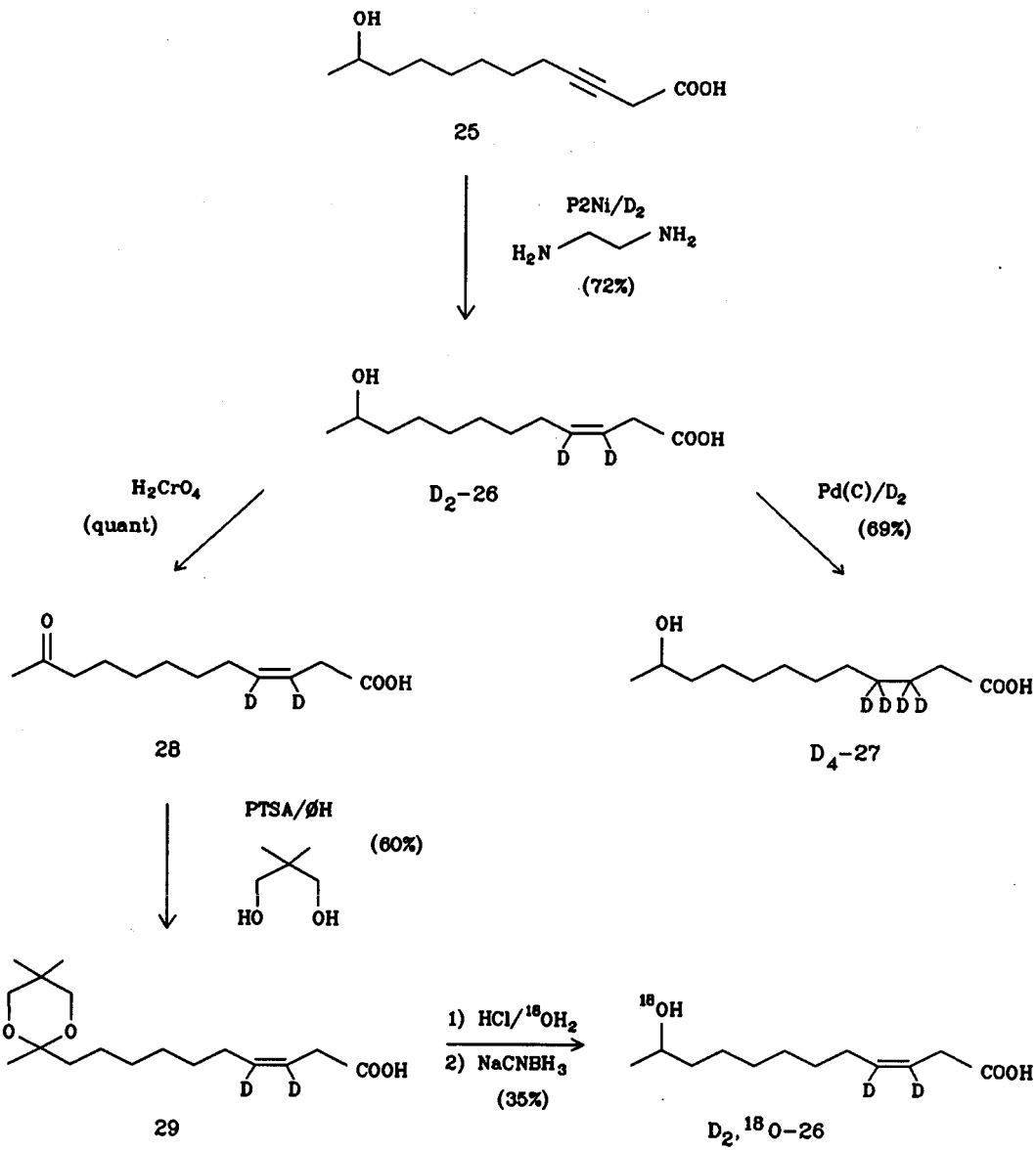
The synthetic routes (Schemes 2, 3 and 4) were basically variations on the route reported by Oehlschlager, *et al.* (1986). As required, detours were taken to insert ^{18}O and/or deuterium.

Two basic strategies were used to insert deuterium: reduction of a carbonyl group with deuteride ion; and reduction of a triple bond with D_2 gas. Schemes 2 and 4 utilized the first approach. In Scheme 2, 10-undecyn-2-ol (**21**) was oxidized with PCC (Corey and Suggs, 1975) to the corresponding ketone (quantitative yield) which was reduced to the alcohol with $NaBD_4$ (Walker, 1976; 94% yield). The final product D-**26** was 95% enriched in D and 91% pure. In Scheme 4, **26** was oxidized to 11-keto-3-dodecenoic acid **30** by Jones reagent (quantitative yield), prior to reduction with $NaBD_3CN$ (Borch, 1971; quantitative yield). The D-**26** was 94% enriched in D and 99% pure. The alternate approach was used in Scheme 3: 11-hydroxy-3-dodecynoic acid (**25**) was reduced to [3,4-D_2]-**26** with D_2 gas, using P2Ni as a catalyst (Brown and Ahuja, 1973; 90% pure,

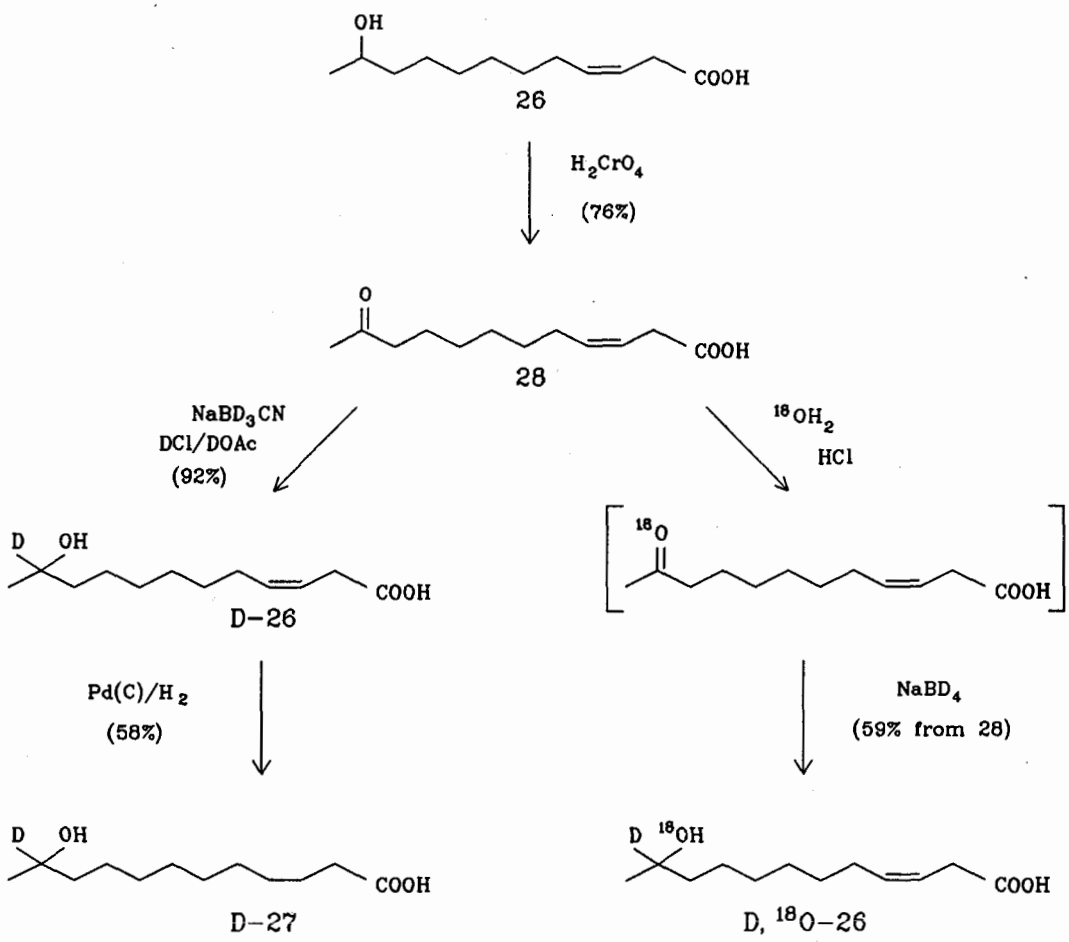
72% yield). As discussed previously, some scrambling of the deuterium label was observed. The D_2-**26** thus formed was 81% enriched in two deuteriums and 19% enriched in one deuterium.



Scheme 2: Synthetic route to D-**26**.



Scheme 3: Synthetic routes to D_2-26, D_2,^{18}O-26, and D_4-27.



Scheme 4: Synthetic routes to D-26, D,^{18}O-26, and D-27.

Two methods were used to insert ^{18}O: acid-catalyzed hydrolysis of a ketal in the presence of ^{18}O-water; and the more direct acid catalyzed exchange of a carbonyl oxygen with the oxygen of $^{18}OH_2$. In Scheme 3, ^{18}O was inserted through hydrolysis of ketal **29** in the presence of $^{18}OH_2$. The ^{18}O-labelled ketone intermediate was reduced *in situ* with $NaBH_3CN$ (35% yield). In Scheme 4, the ketone-oxygen of **30** was exchanged with $^{18}OH_2$ under acidic conditions. In this case, the ^{18}O-ketone was not directly reduced with $NaBH_3CN$ to allow insertion of deuterium at this stage. The

reduction was not performed with $NaBD_3CN$, since the deuteriums would have exchanged with the protons of $^{18}OH_2$ under the acidic conditions of the reaction (Borch *et al.*, 1971). Adding D_2O to counteract this effect would result in the loss of ^{18}O. Thus the product was reduced with $NaBD_4$ to give D,^{18}O-**26** (59% yield).

Incorporation of 26 into cucujolide II: preliminary experiments with C. ferrugineus

C. ferrugineus was fed on oats coated with D-**26** (prepared according to Scheme 2). The Porapak Q-trapped volatiles were purified by preparative GC, then analyzed by GC/MS (using a DB-1 column). As revealed in Table 11, cucujolide **II** was enriched for deuterium (EF=3.1%). Since the precursor only had one deuterium, enrichment was revealed by the enhanced $(M+1)^+$ peak. The $(M+1)^+$ peak is about 11% for molecules of the molecular formula of cucujolide **II** due to natural isotopic abundance. Therefore this experiment was repeated to ensure that the small enrichment seen was not an artifact. The repeat experiment gave a result similar to the first experiment, in which the **II** produced was 1.3 % enriched for deuterium (Table 11).

Unequivocal interpretation of these results is hindered by two factors. First, the D-**26** contained 9% (by GC) of the fully reduced analogue [11-D]-11-hydroxy-docecanoic acid (D-**27**) formed during the P2Ni reduction of **25**. Second, the EFs were low compared to the natural abundance of the $(M+1)^+$ peak.

The first problem was addressed by exposure of *C. ferrugineus* to deuterated 11-hydroxydodecanoic acid (D_4-**27**). As seen in Table 12, the cucujolide **II** produced was not enriched for four deuteriums, indicating that **27** was not an effective precursor. Thus, the deuterium enrichment detected in cucujolide **II** when *C. ferrugineus* was exposed to deuterated **26** was not due to incorporation of the deuterated **27** impurity.

Table 11: Incorporation of [11-D]-11-hydroxy-3-dodecenoic acid (D-**26**) into cucujolides by *C. ferrugineus*: relative abundances of GC/SIM-MS peaks of interest.

	Trial 1			Trial 2		
	M^+	$(M+1)^+$	$(M+2)^+$	M^+	$(M+1)^+$	$(M+2)^+$
Cucujolide I						
unlabelled:	100.0	12.8	1.1	100.0	11.2	1.1
D-**26**:	100.0	13.4	1.2	100.0	11.0	1.1
Cucujolide II						
unlabelled:	100.0	13.5	1.4	100.0	14.8	1.2
D-**26**:	100.0	**16.7**	2.0	100.0	**16.1**	**1.5**
Cucujolide III						
unlabelled:	100.0	17.7	1.8	100.0	15.6	**1.6**
D-**26**:	100.0	17.5	1.9	100.0	14.6	**1.6**

[1] 1.96 g of starved beetles were fed on 25 g of defatted oats, coated with 100 mg D-**26**; [2] 1.11 g of starved beetles were fed on 15 g of defatted oats, coated with 50 mg D-**26**.

The second problem was addressed by labelling **26** with two deuteriums (D_2-**26**, Scheme 3). This gave a clearly significant enhancement of the $(M+2)^+$ peak in cucujolide **II** (EF=2.4%, Table 12). Although the EF is not larger than in the previous experiment (Table 9), the natural abundance of the $(M+2)^+$ peak is much lower than that of the $(M+1)^+$ peak, so that the small enhancement is more convincing.

Cucujolide **II** produced by *C. ferrugineus* in the presence of D_2,^{18}O-**26** exhibited an $(M+4)^+$ peak that was 3.1% enriched, indicating enrichment for both two deuterium atoms and for ^{18}O. This cucujolide was not significantly enriched for two mass units, indicating that very little (if any) **II** was formed from D_2,^{18}O-**26** with the loss of ^{18}O. Thus, **II** is formed by a mechanism that involves retention of the hydroxyl oxygen. This is intuitively satisfying since, *in vivo*, **26** is expected to be formed through ß-oxidation of a longer chain fatty acid and thus is likely present as an acyl-CoA derivative. Cyclization of such an intermediate would proceed with retention of the hydroxyl oxygen and loss of CoA (Figure 25).

Table 12: Incorporation of D_2-26[1], D_4-27[2], and D_2,^{18}O-26[3] into cucujolides by *C. ferrugineus*: relative abundances of GC/SIM-MS peaks of interest.

	M^+	$(M+1)^+$	$(M+2)^+$	$(M+3)^+$	$(M+4)^+$	$(M+5)^+$
Cucujolide I						
unlabelled:	100.0	13.7	2.5	0.2	n.d.	n.d.
D_2-26:	100.0	14.2	0.6	n.d.	n.d.	n.d.
D_4-27:	100.0	15.5	0.8	n.d.	n.d.	n.d.
D_2,^{18}O-26:	100.0	14.1	0.7	n.d.	n.d.	n.d.
Cucujolide II						
unlabelled:	100.0	14.1	1.6	0.1	n.d.	n.d.
D_2-26:	100.0	14.9	4.1	0.5	0.1	n.d.
D_4-27:	100.0	15.1	1.5	0.1	0.1	n.d.
D_2,^{18}O-26:	100.0	14.0	1.8	1.0	3.3	0.7

[1] 1.00 g of starved beetles were fed on 15 g of defatted oats, laced with 100 mg of D_2-26; [2] 0.3 g of starved beetles were fed on 4 g of defatted oats, laced with 30 mg of D_4-27; [3] 0.8 g of starved beetles were fed on 12 g of defatted oats, laced with 80 mg of D_2,^{18}O-26.

Incorporation of D-26 and D,^{18}O-26 into cucujolide II

Preliminary experiments indicating that it was feasible to study the incorporation of 26 into cucujolide II using stable isotope-labelled precursors were repeated using very pure labelled substrates (prepared according to Scheme 4). The D-26 and D-27 used were 99% pure by analytical GC; the D,^{18}O-26 was 98% pure.

Cucujolide II produced by *C. ferrugineus* exposed to D-27 was not significantly enriched in D (Table 13, Figure 26). In contrast, the cucujolide II produced by beetles exposed to D-26 was clearly enriched in D (EF=3.8%). The II produced upon exposure to D,^{18}O-26 was enriched in both D and ^{18}O (EF=2.9% for both D and ^{18}O). Some II was enriched in D but not ^{18}O (EF=0.3%) or in ^{18}O but not D (EF=0.4%). This is due to the fact that the D,^{18}O-26 presented to the insects was not 100% doubly labelled.

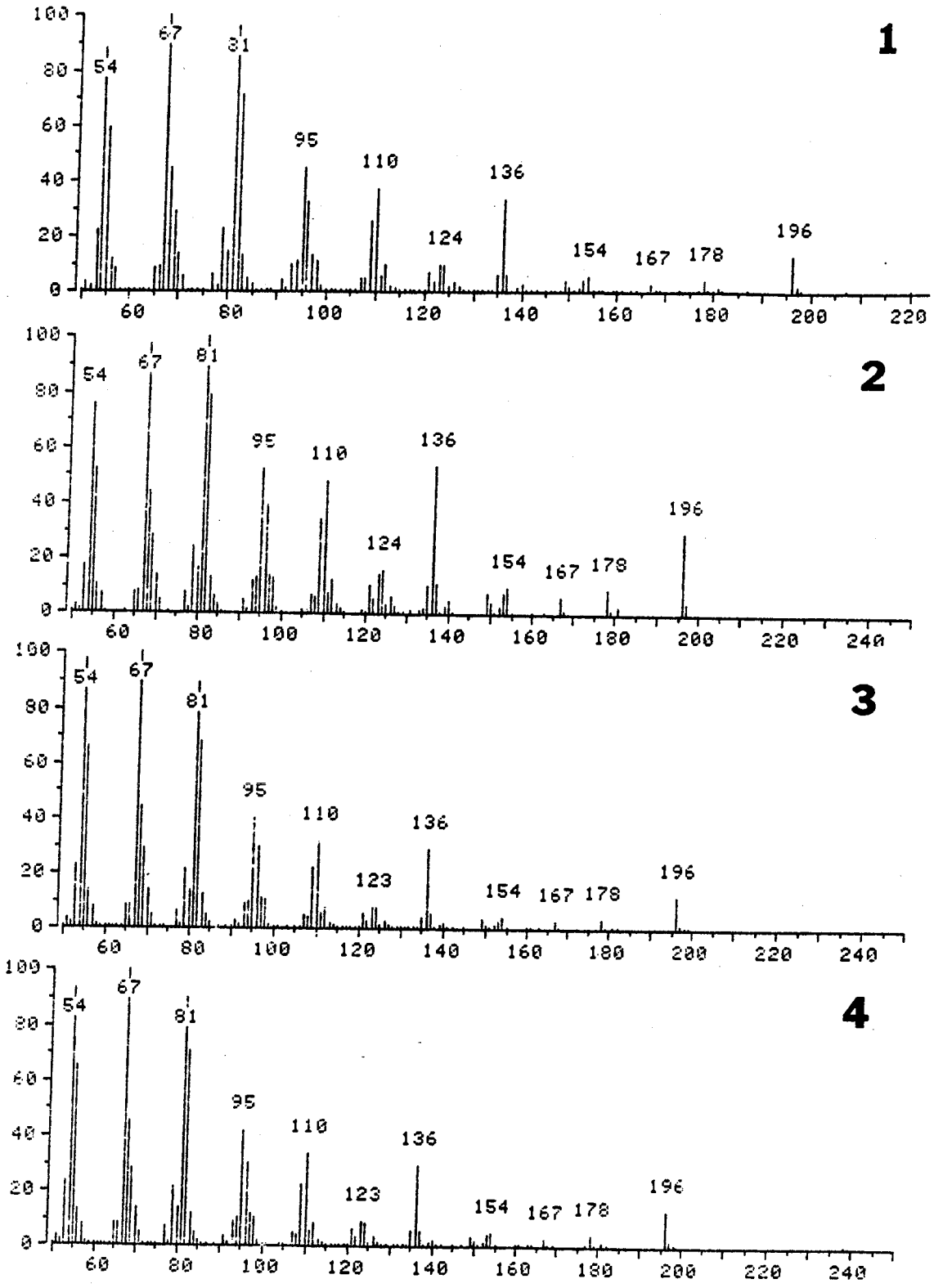


Figure 26: GC-MS (EI) of cucujolide II produced by *C. ferrugineus* feeding on: 1, untreated oats; 2, defatted oats coated with D-26; 3, defatted oats coated with D,^{18}O-19; or 4, defatted oats coated with D-27.

Table 13: Incorporation of D-26[1], D,^{18}O-26[2] and D-27[3] into cucujolides by *C. ferrugineus*: relative abundances of GC/SIM-MS peaks of interest.

	M^+	$(M+1)^+$	$(M+2)^+$	$(M+3)^+$	$(M+4)^+$
Cucujolide I					
unlabelled:	100.0	13.6	1.4	0.1	n.d.
D-26:	100.0	14.0	1.4	0.1	n.d.
D,^{18}O-26:	100.0	13.9	1.4	0.1	n.d.
D-27:	100.0	13.8	1.4	0.1	n.d.
Cucujolide II					
unlabelled:	100.0	13.1	1.4	0.1	n.d.
D-26:	100.0	**17.0**	1.9	0.2	0.0
D,^{18}O-26:	100.0	13.5	1.7	**3.1**	0.5
D-27:	100.0	13.3	1.3	0.1	n.d.
Cucujolide III					
unlabelled:	100.0	16.3	1.6	0.1	n.d.
D-26:	100.0	15.6	1.7	0.1	n.d.
D,^{18}O-26:	100.0	15.7	1.8	0.1	n.d.
D-27:	100.0	15.8	1.7	0.2	n.d.

[1] 1.00 g of starved beetles were fed on 15 g of defatted oats, coated with 100 mg D-26; [2] 1.00 g of starved beetles were fed on 15 g of defatted oats, coated with 100 mg D,^{18}O-26; [3] 1.00 g of starved beetles were fed on 15 g of defatted oats, coated with 100 mg D_2-27.

Of the labelled molecules of **26**, 8% were enriched in D but not ^{18}O, while 15% were enriched in ^{18}O but not D. Since 2.9% of cucujolide **II** was enriched in both D and ^{18}O, one would expect 0.2% enrichment in D only, and 0.4% enrichment in ^{18}O only. It is clear that the cyclization of **26** to cucujolide **II** occurred with retention of the C-11 hydroxyl oxygen.

O. mercator was also fed on oats coated with D-26, D,^{18}O-26, or D-27 (Figure 27 and Table 14). When *O. mercator* was exposed to D-26, cucujolide **II** was greatly enriched in D (EF=95%) whereas after exposure to D,^{18}O-26 it was enriched in both D and ^{18}O (EF=84%). In the latter

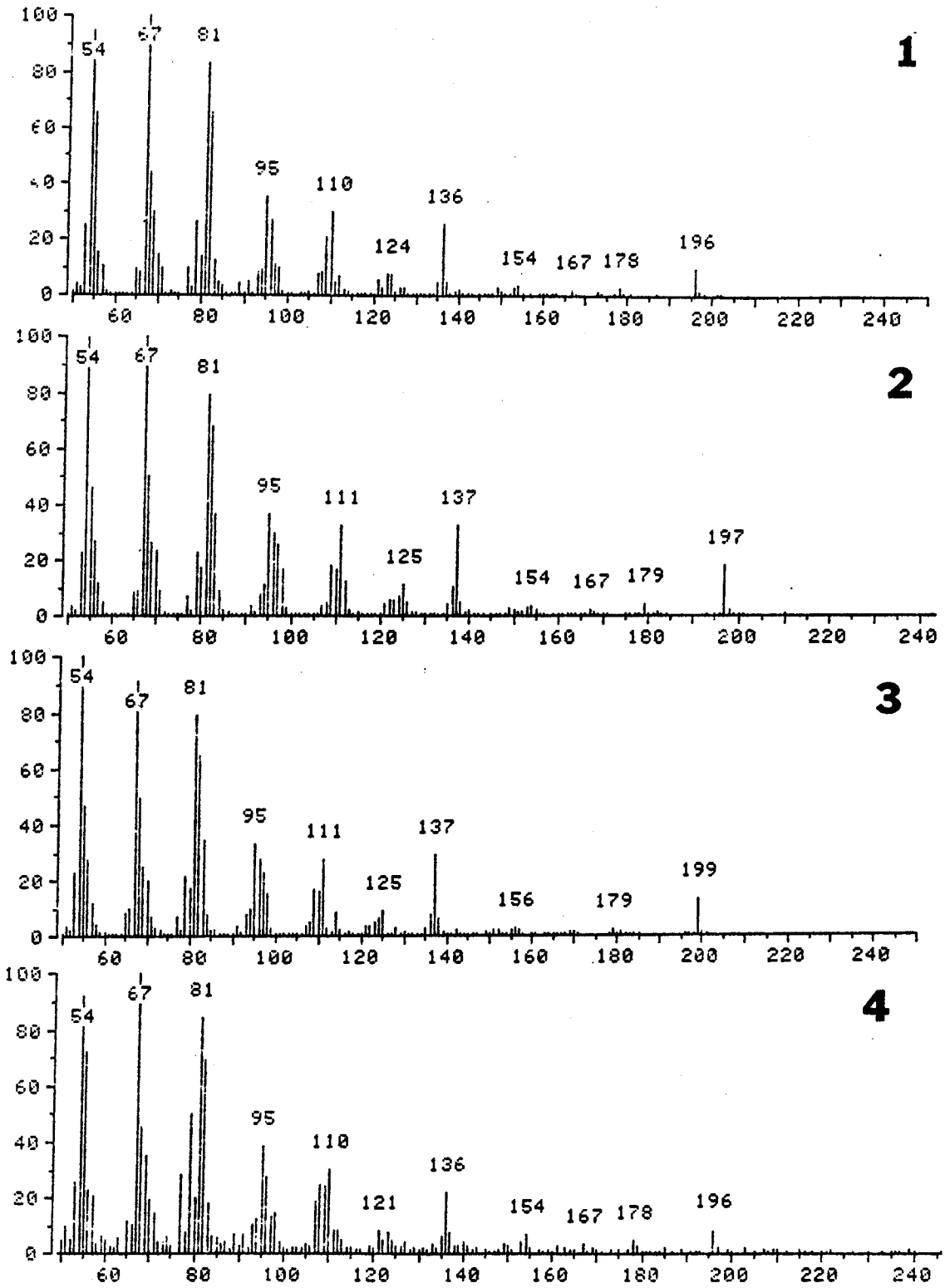


Figure 27: GC-MS (EI) of cucujolide II produced by *O. mercator* feeding on: 1, untreated oats; 2, defatted oats coated with D-26; 3, defatted oats coated with D,^{18}O-19; *or* 4, defatted oats coated with D-27.

Table 14: Incorporation of D-**26**[1], D,^{18}O-**26**[2] and D-**27**[3] into cucujolide **II** by *O. mercator*: relative abundances of GC/SIM-MS peaks of interest.

	M^+	$(M+1)^+$	$(M+2)^+$	$(M+3)^+$	$(M+8\ 4)^+$
Cucujolide **II**					
unlabelled:	100.0	13.3	1.6	0.2	n.d.
D-**26**:	4.8	**100.0**	14.3	1.8	0.3
D,^{18}O-**26**:	2.1	6.7	10.1	**100.0**	14.6
D-**27**:	100.0	15.5	1.7	n.d.	n.d.

[1] 2.00 g of starved beetles were fed on 30 g of defatted oats, coated with 100 mg D-**26**; [2] 2.00 g of starved beetles were fed on 30 g of defatted oats, coated with 100 mg D,^{18}O-**26**; [3] 2.00 g of starved beetles were fed on 30 g of defatted oats, coated with 100 mg D-**27**.

treatment, some **II** was enriched only for D (EF=5.4%) while some was enriched only for ^{18}O (EF=8.4%). As discussed previously, this is because the D,^{18}O-**26** precursor was not 100% doubly labelled. It is clear that in *O. mercator*, as in *C. ferrugineus*, **26** is cyclized to cucujolide **II** and that this cyclization proceeds with retention of the C-11 hydroxyl oxygen.

As a control to determine if the hydroxy acid would cyclize spontaneously, unlabelled **26** was coated onto defatted oats, aerated, and the volatiles collected as usual on Porapak Q. Spontaneous cyclization was not expected to occur, since the cyclization of 12 membered ring lactones is not favored (Galli *et al.*, 1977; Mandolini *et al.*, 1978). Therefore, a greater quantity of precursor than usual was aerated for longer than usual, in order to favor the detection of cucujolide **II** that might "spontaneously" form*. The Porapak Q extract contained a peak with the same GC-R.T. and GC-MS fragmentation pattern as cucujolide **II**, even

* The entire supply left over from Dr. Johnson's gift (158 mg of unlabelled **26**) was coated onto 24.7 g of defatted oats (in order to maintain the usual ratio of 100 mg precursor/15 g of oats). These oats were aerated for 17 days, rather than the usual 10.

though no insects had been allowed to feed on the oats! It is difficult to believe that the cucujolide **II** was formed through spontaneous cyclization of **26**. Perhaps some enzymes of the oats were still active, or perhaps the oats were infected with a microorganism capable of catalyzing the transformation.

The quantity of cucujolide **II** formed was equivalent to 2.7 μg/100 mg **26**/10 days. This is a small quantity when compared to the quantities of cucujolide **II** formed by *C. ferrugineus* (287 ± 24 μg during aerations with **D-26**, **D^{18}O-26** and **D-27**) and by *O. mercator* (310 μg and 76 μg during the aeration with **D-26** and **D,^{18}O-26**, respectively*).

The chirality of the cucujolide **II** produced in the various cultures was assessed. Diastereomeric acetyl (*S*)-lactyl derivatives were prepared by the method of Slessor *et al.* (1985), and analyzed by GC. The cucujolide **II** produced by *C. ferrugineus* feeding on oats (with no labelled precursors added) was 98% *S* while that produced by *O. mercator* was 97% *R* (Table 15), in agreement with previous reports (Slessor *et al.*, 1985; Oehlschlager *et al.*, 1987). The cucujolide **II** produced by oats coated with unlabelled **26** in the absence of insects was racemic (51% *S*, Table 15).

It is clear that the cucujolide **II** produced by *O. mercator* in the presence of labelled **26** was formed largely as a result of beetle activity. The quantities of cucujolide **II** produced from the **D-26** and **D,^{18}O-26** precursors (EFs = 95% and 99%, respectively, Table 14) were much greater than would be expected from "spontaneous" cyclization alone (EF = 0.9% and 3.6%, respectively). In addition, the **II** produced was 97% *R*, not racemic. As an aside, it is interesting that the beetles were able to produce **II** that was 97% *R* when presented with racemic **26**. It appears that the cyclization reaction is highly enantioselective in *O. mercator*.

---

* The reason for the apparent wide variation in cucujolide production by *O. mercator* is not understood. The quantities of oat volatiles were also much lower in the second extract.

Table 15: Enantiomeric composition of cucujolide **II** emitted by a no-insect control, *C. ferrugineus* and *O. mercator* fed on rolled oats coated with various precursors.

Substrate coated onto oats	Ratio of *S/R* in various cultures		
	C. ferrugineus	*O. mercator*	no insects
no substrate (standard):	98/2	3/97	-[1]
unlabelled **26**:	-	-	51/49
D-**26**:	95/5	3/97	-
D,^{18}O-**26**:	96/4	3/97	-

[1]the dash indicates experiments not performed.

The EFs of the cucujolide **II** produced by *C. ferrugineus* after exposures to D-**26** and D,^{18}O-**26** were 3.3% and 3.1%, respectively (Table 13). The quantity expected to be contributed by the oats was 0.9%. Thus, although some of the labelled cucujolide **II** might have formed "spontaneously", most was likely formed by the beetles.

Implications for the enzymatic step subject to regulation

Pheromone production by *Oryzaephilus* and *Cryptolestes* beetles is believed to be under hormonal control. Pierce *et al.* (1986) revealed that the production of aggregation pheromones by male *O. mercator, O. surinamensis* and *C. ferrugineus* was enhanced by methoprene, a juvenile hormone analogue. Feeding may trigger pheromone production in some way other than by simply relieving a constriction in the supply of pheromone-precursors, since *C. ferruginues* is capable of *de novo* cucujolide biosynthesis (Table 8). The biosynthetic step that is subject to regulation in the presence and absence of food is indicated in this study. As mentioned previously, cucujolide production by *O. mercator* was reduced approximately one hundred-fold when the insects were fed on defatted oats. *O. mercator* normally emits cucujolides **II** and **IV** in a ratio of about

1.5 : 1 (Table 16). This ratio was basically undisturbed even when cucujolide production was sharply decreased by feeding on defatted oats, coated with either D_2-**18** or D-**27**. When *O. mercator* was fed on defatted oats coated with labelled **26**, cucujolide **IV** production was still depressed. This treatment, however, increased cucujolide **II** production to normal levels: the production of **II** was enhanced roughly 100 fold relative to **IV**. Qualitatively, it is clear that precursor **26** bypassed whichever biosynthetic step(s) is (are) "turned off" in the absence of an adequate food source. In contrast, the presentation of precursor **18** did **not** allow this (these) step(s) to be bypassed. Since **18** is presumably the biosynthetic precursor of **26**, the ω-1 hydroxylation of **18** to form **26** in *O. mercator* is one (or perhaps is **the**) regulated biosynthetic step (see Figure 28).

Table 16: Comparison of ratios of cucujolide **II** : cucujolide **IV** emitted by *O. mercator* after exposure either to untreated oats or to defatted oats coated with various precusors.

Treatment of oats	Ratio of **II** : **IV**
none	1.5 : 1
defatted; coated with D_2-**18**	1.5 : 1
defatted; coated with D-**27**	2 : 1
defatted; coated with D-**26**	102 : 1
defatted; coated with D,^{18}O-**26**	137 : 1

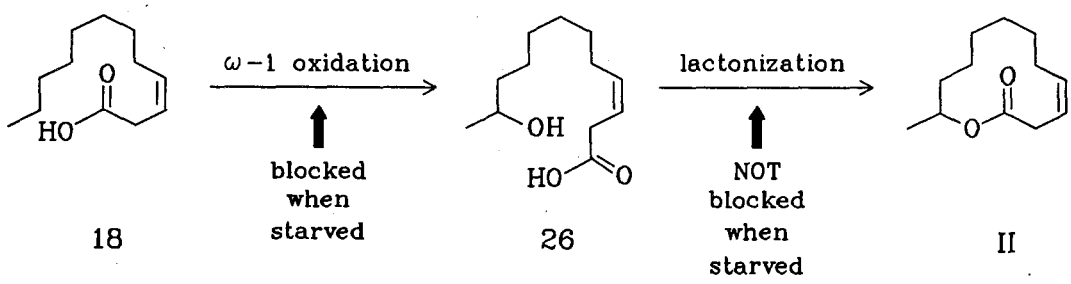


Figure 28: Regulation of cucujolide **II** production in *O. mercator* in the presence and absence of food.

c) Biosynthesis of cucujolide I: further studies

A biosynthetic route to **I** from the sesquiterpene farnesol was proposed by Pierce *et al.* (1984b). To test this hypothesis I determined if labelled cucujolide **I** was formed when *C. ferrugineus* was exposed to labelled farnesol (Figure 29).

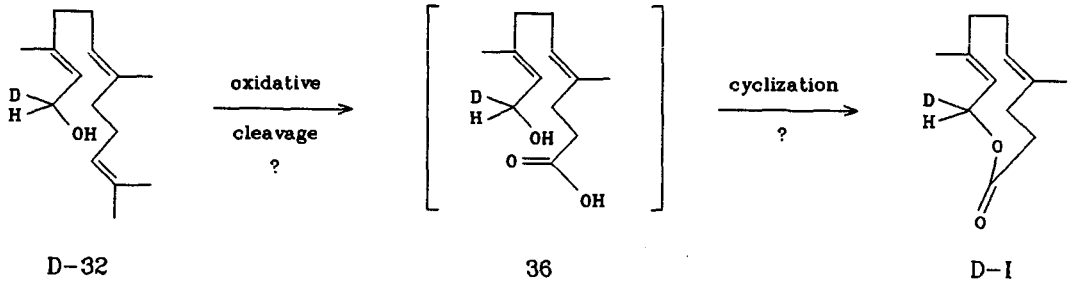


Figure 29: Incorporation of deuterium into cucujolide **I** from the putative precursor [1-D]-*(E,E)*-farnesol (**D-32**).

Cyclization of **36**, the hydroxy-acid precursor of cucujolide **I**, could occur in either of the two directions shown in Figure 30. To distinguish between these, a tactic similar to that described in the study of cucujolide **II** biosynthesis was utilized. *C. ferrugineus* was exposed to [1-D]-*(E,E)*-farnesol that was also enriched for ^{18}O at C-1. Any

cyclization mechanism involving loss of the C-10 hydroxyl oxygen of **36** would result in loss of ^{18}O. The cucujolide **I** thus produced would be enriched only for D. In contrast, retention of the C-10 hydroxyl oxygen of **36** would result in the production of cucujolide **I** enriched in both D and ^{18}O.

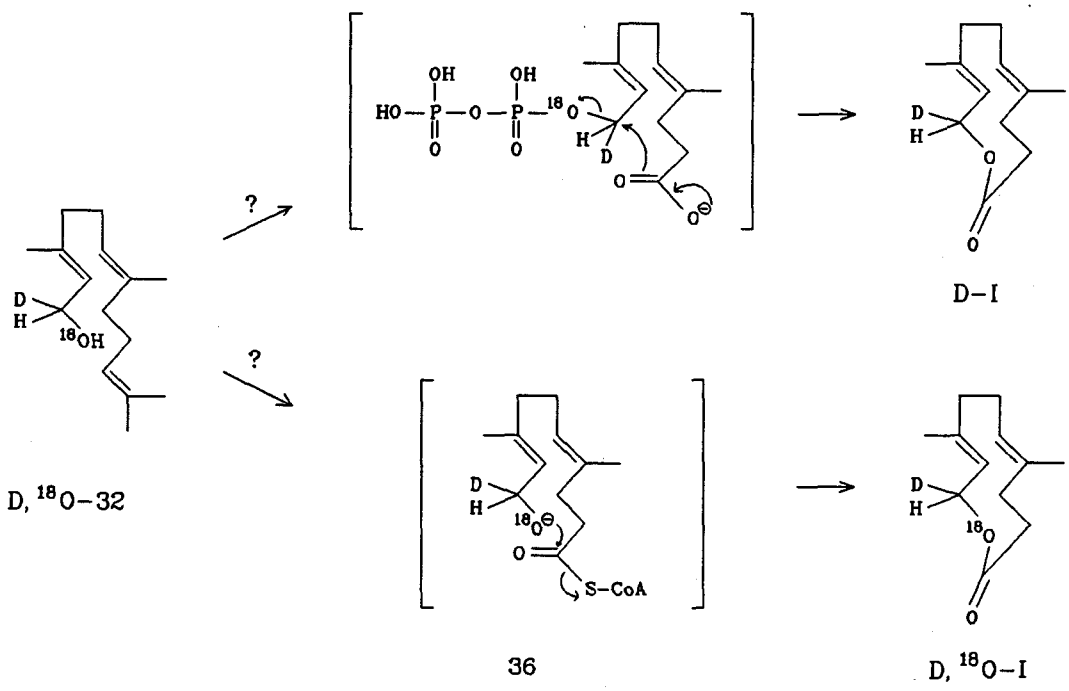
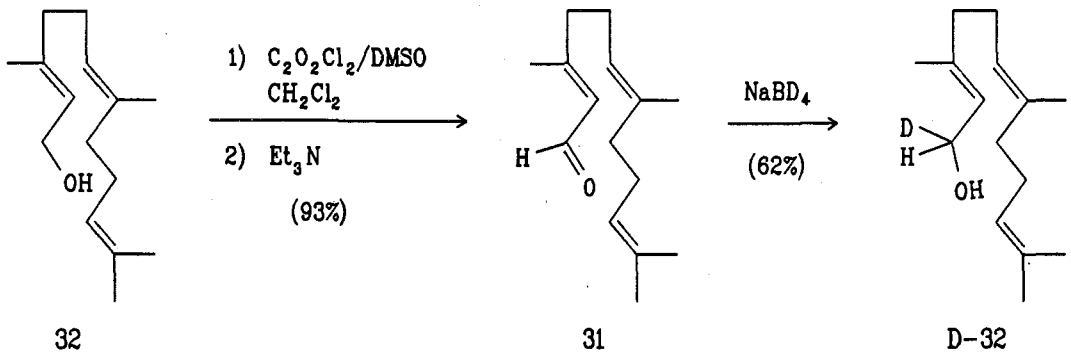


Figure 30: Examples of the two possible directions of cyclizations of the hydroxy acid **36** to cucujolide **I**. Differentiation between the two is possible with D,^{18}O-*(E,E)*-farnesol (D,^{18}O-**32**).

Synthesis of labelled *(E,E)*-farnesol

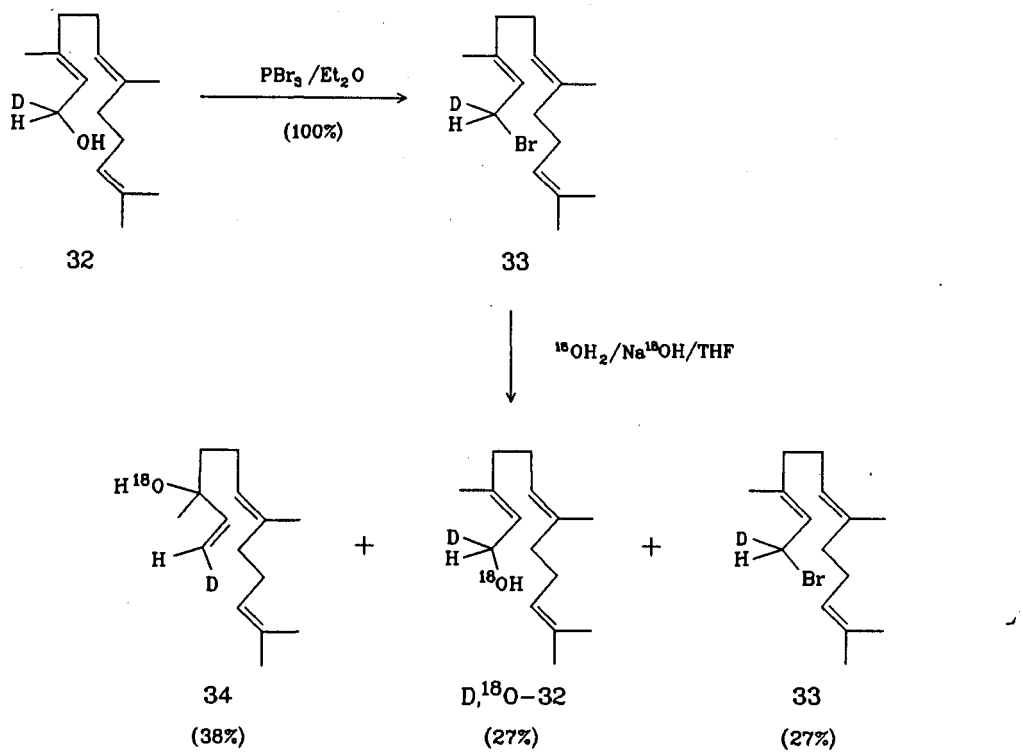
Deuterated *(E,E)*-farnesol (D-**32**) was synthesized from commercially available *(E,E)*-farnesol, according to Scheme 5. *(E,E)*-Farnesol was oxidized to the corresponding aldehyde (**31**) with DMSO-activated oxalyl chloride, using the method of Mancuso *et al.* (1978). The *(E,E)*-farnesal obtained was reduced with $NaBD_4$ to give D-**32** in an overall yield of 58% (96% pure by GC; 95 atom % D). Subsequent preparation of [1-D]-*(E,E)*-farnesol by this route gave quantitative yields of both products.



32 31 D-32

Scheme 5: Synthetic route to [1-D]-*(E,E)*-farnesol (D-32).

[1-D,1-^{18}O]-*(E,E)*-Farnesol (D,^{18}O-32) was prepared as outlined in Scheme 6, in 26% overall yield. [1-D]-*(E,E)*-Farnesol, prepared as described previously (quantitative yield, 97% enriched for D), was brominated with PBr_3 (quantitative yield). Bromide 33 was hydrolyzed with $Na^{18}OH/^{18}OH_2$ to give a 27% yield of the desired product (which was 84% enriched for ^{18}O by SIM-MS). Also isolated from the crude product were unreacted 33 (27%) and the major product (38%), identified as [1-D,3-^{18}O]-3,7,11-trimethyldodeca-1,6,10-trien-3-ol (34). This product was identified on the basis of its MS (which showed an M^+ peak of 225, the same as D,^{18}O-32), IR (which showed a broad peak at 3340 cm^{-1} indicative of a hydrogen bonded hydroxyl group) and its ^{1}H NMR spectrum. The latter was similar to that of 32 but possessed no signals attributable to the C-1 allylic methylene. A signal due to one of the vinyl methyl groups was absent, but a signal due to new methyl hydrogens (deshielded) appeared. A new signal due to a deshielded vinyl methine hydrogen also appeared. This information coupled with the knowledge that allylic bromides can react by S_N1' and S_N2' processes allowed assignment of the structure 34. All spectra were consistant with this assignment. It was surprising that 34 was the major product since it was anticipated that the steric hindrance



Scheme 6: Synthetic route to [1-D,1-^{18}O]-*(E,E)*-farnesol (D,^{18}O-32).

provided by the C-3 methyl group of farnesyl bromide would ensure that most displacement would proceed at C-1.

Incorporation of [1-D]-*(E,E)*-farnesol into cucujolide I

When *C. ferrugineus* was fed on oats coated with [1-D]-*(E,E)*-farnesol, the cucujolide **I** produced was enriched with deuterium (EF=18%, Table 17; see also Figure 31), indicating that [1-D]-*(E,E)*-farnesol served as a precursor.

In the absence of exogenously added substrate the farnesol used by the beetles to form cucujolide **I** is likely synthesized endogenously. No farnesol was detected in extracts of the oats (using *Methods 1* and *2*, described in the *Experimental*).

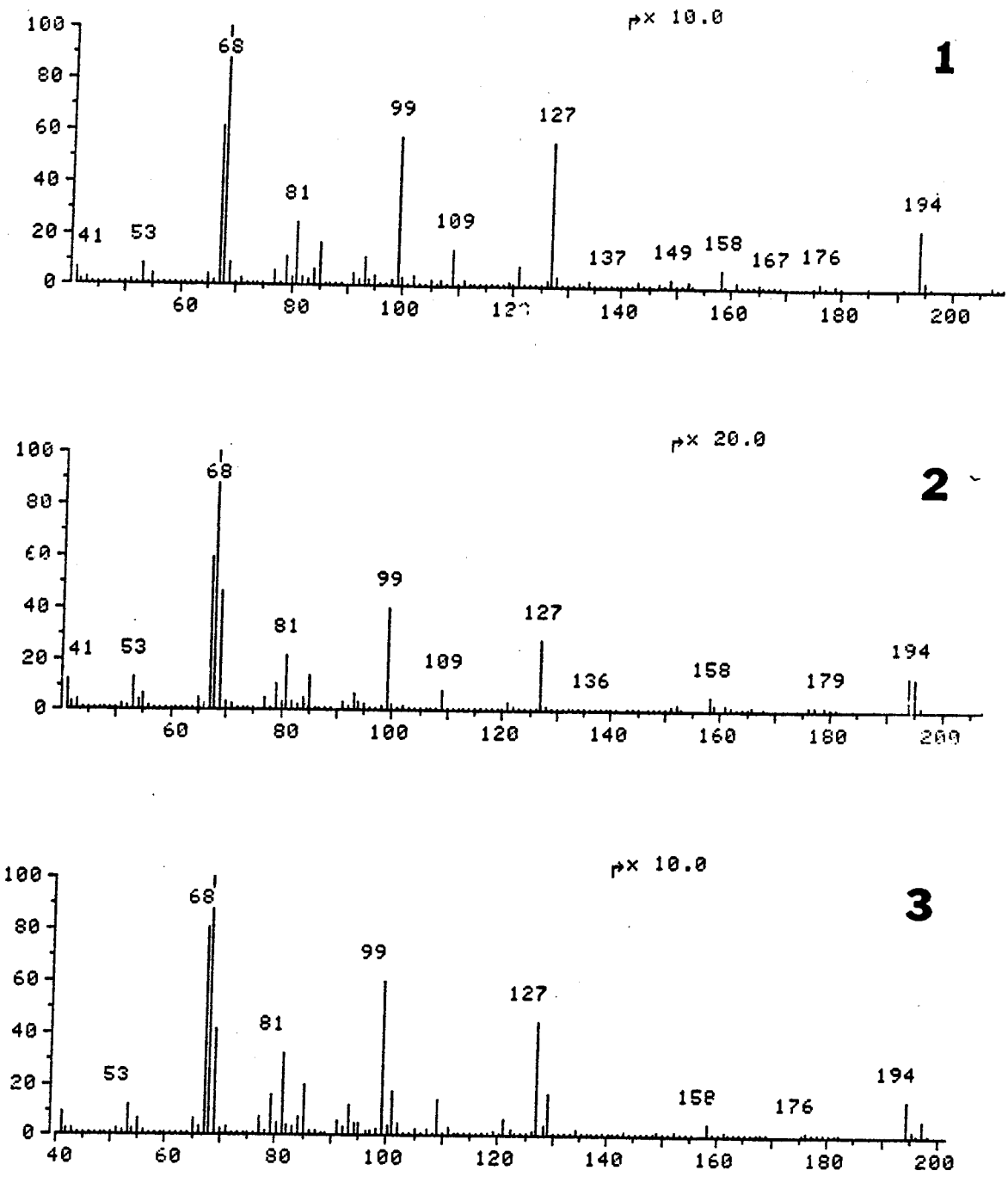


Figure 31: GC-MS (EI) of cucujolide I produced by *C. ferrugineus* feeding on: 1, untreated oats; 2 oats coated with D-32; and 3, oats coated with D, ^{18}O-32.

Table 17: Incorporation of [1-D]-*(E,E)*-farnesol (D-32) into cucujolides[1] by *C. ferrugineus*: relative abundances of GC/SIM-MS peaks of interest.

	M^+	$(M + 1)^+$	$(M + 2)^+$
Cucujolide I			
unlabelled:	100.0	12.7	1.2
D-32:	100.0	34.7	4.4
Cucujolide II			
unlabelled:	100.0	14.8	1.2
D-32:	100.0	16.2	1.4
Cucujolide III			
unlabelled:	100.0	15.6	1.6
D-32:	100.0	15.0	1.6

[1] 2.01 g of beetles were fed on 30 g of defatted oats, coated with 100 mg D-32.

When *C. ferrugineus* was fed on oats that had been coated with [1-D,1-^{18}O]-farnesol the cucujolide **I** produced was 18% enriched in deuterium (Table 18). Eighty-seven per cent of the deuterated cucujolide **I** produced was also enriched in ^{18}O. Since the D,^{18}O-**32** presented to the insects was 84% enriched in ^{18}O it is clear that the D,^{18}O-farnesol did not lose the ^{18}O-labelled hydroxyl group *en route* to cucujolide **I**. Thus, lactonization proceeds with retention of the hydroxyl oxygen and therefore cannot proceed through a mechanism involving loss of this oxygen (such as lactonization through the pyrophosphate intermediate shown in Figure 30).

Table 18: Incorporation of [1-D,1-^{18}O]-*(E,E)*-farnesol (D,^{18}O-32) into cucujolides[1] by *C. ferrugineus*: relative abundances of GC/SIM-MS peaks of interest.

	M^+	$(M + 1)^+$	$(M + 2)^+$	$(M + 3)^+$	$(M + 4)^+$
Cucujolide I					
unlabelled:	100.0	13.7	1.0	0.2	n.d.
D,^{18}O-32:	100.0	16.5	2.0	19.7	2.6
Cucujolide II					
unlabelled:	100.0	14.7	1.1	0.1	n.d.
D,^{18}O-32:	100.0	16.5	1.4	0.1	n.d.

[1] 2.02 g of beetles were fed on 30 g of defatted oats, coated with 100 mg D,^{18}O-32.

Biosynthesis of unknown E from exogenous farnesol

Beetles exposed to farnesol also produced relatively large quantities (68% - 86% the level of cucujolide **I**) of a previously unseen compound, unknown **E**, which was not present in the samples of labelled farnesol. MS (EI and CI) analyses indicated that unknown **E** had a molecular weight of 194. Formation from farnesol would thus involve a net loss of C_2H_4. The fragmentation pattern showed an M-18 peak of high relative abundance in the CI MS, indicating the presence of an alcohol, aldehyde or ketone. The IR showed a strong carbonyl absorption at 1710 cm^{-1}, confirming the presence of a saturated ketone. The ^{1}H NMR showed a singlet (3H) at δ 2.15, indicative of a methyl ketone. The most obvious methyl ketone that could be formed from farnesol with the loss of C_2H_4 is geranyl acetone (6,10-dimethylundeca-5,9-dien-2-one). All spectra were consistent with this assignment.

D. CONCLUSIONS

The results of this study support the hypothesis that cucujolide **I** is of terpenoid origin in *C. ferrugineus*. This insect was able to synthesize **I** either *de novo* from acetate and mevalonate, or through the conversion of exogenous farnesol.

The results of this study also support the hypothesis that cucujolides **II - V** are of fatty acid origin. These cucujolides were also synthesized *de novo* from acetate. The possibilty that symbiotic microorganisms might be involved in this *de novo* biosynthesis (particularly of cucujolides **IV** and **V**, derived from linoleic acid) was not ruled out.

Stable isotope-labelling studies indicated that lauric acid was not an effective precursor of **II** in *C. ferrugineus*. 11-Hydroxydodecanoic acid was not an effective precursor of **II** in either *C. ferrugineus* or *O. mercator*. In contrast, 3-dodecenoic acid and 11-hydroxy-3-dodecenoic acid (**26**) served as precursors of the cucujolide **II** *in vivo* in either *C. ferrugineus* or *O. mercator*. Although the insects were presented with racemic **26**, in each case the **II** produced was of the natural chirality. *C. ferrugineus*, which normally produces 98% *S*-**II**, was found to produce 95% (*S*)-**II** in the presence of **26**. *O. mercator* was found to produce the normal 97% (*R*)-**II**.

Dual-labelling studies with deuterium and ^{18}O indicate that the cyclization of **26** to cucujolide **II** in both *C. ferrugineus* and *O. mercator* proceeded with retention of the C-11 hydroxyl. Similarly, the conversion of farnesol to cucujolide **I** in *C. ferrugineus* proceeded with retention of the hydroxyl oxygen. Thus the lactonization reactions forming cucujolides **I** and **II** both proceeded as shown in Figure 32.

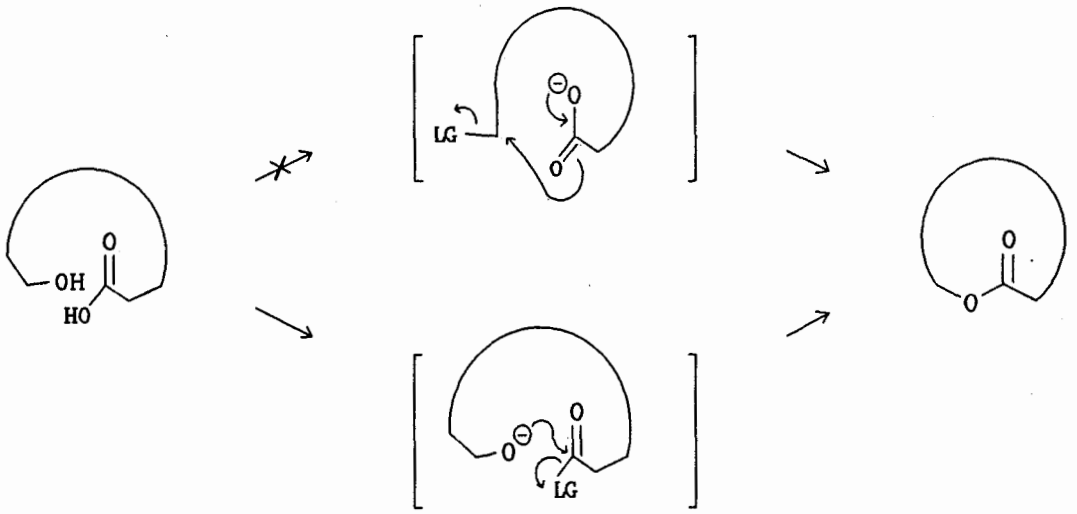


Figure 32: Generalized direction of lactonization of cucujolides I and II in *C. ferrugineus* and *O. mercator*. "LG" denotes a leaving group (such as pyrophosphate or CoA).

This study supports the suggestion by Pierce *et al.* (1986) that cucujolide biosynthesis is regulated by factors other than the presence or absence of dietary precursors, since it was found that cucujolides I - V could be synthesized *de novo*. The results of this study further indicate that, for cucujolide II biosynthesis in *O. mercator*, the regulated step is likely the ω-1 oxidation of 3-(*Z*)-dodecenoic acid.

CHAPTER III

BREVICOMIN BIOSYNTHESIS IN THE MOUNTAIN PINE BEETLE, *DENDROCTONUS PONDEROSAE*, AND THE BALSAM BARK BEETLE, *DRYOCOETES CONFUSUS* (COLEOPTERA: SCOLYTIDAE)

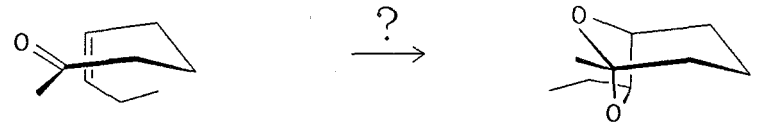
A) INTRODUCTION

Virtually nothing is known about the biosynthesis of cyclic ketal and acetal pheromones (Vanderwel and Oehlschlager, 1987; *Chapter I*). In the past, investigations of the biosynthesis of these pheromones were limited by the technical difficulties involved in working with small quantites of volatile compounds. As discussed in the previous chapter, these difficulties can be overcome by the use of stable isotope-labelled precursors, whose incorporation can be detected by GC/MS. This obviates requirements to purify product isolates, and makes study of the biosynthesis of cyclic ketal pheromones feasible.

The objectives of this study were twofold. The first was to determine if *(Z)*- and *(E)*-6-nonen-2-one could serve as precursors to *exo*- and *endo*-brevicomin, respectively, *in vivo* (Figure 33). These two compounds are logical precursors to the brevicomins and have, in fact, served as such in laboratory routes (Rodin *et al.*, 1971).

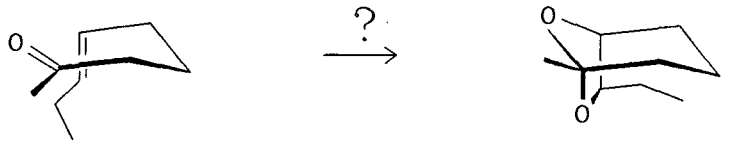
If *(Z)*- and *(E)*-6-nonen-2-one were found to serve as precursors to the brevicomins *in vivo*, the second objective of this study was to determine the mechanisms of these conversions.

The mountain pine beetle (MPB), *Dendroctonus ponderosae* Hopkins, and (when available) the western balsam bark beetle (BBB), *Dryocoetes confusus* Swaine, were used in this study. Newly emerged, adult male MPB and BBB produce both *exo*- and *endo*-brevicomin (Borden, 1985; Borden *et al.*, 1987a).



(Z)-6-nonen-2-one

exo-brevicomin



(E)-6-nonen-2-one

endo-brevicomin

Figure 33: Logical precursors to *exo*- and *endo*-brevicomin, *(Z)*- and *(E)*-6-nonen-2-one, respectively.

B) EXPERIMENTAL

1. Insects

Lodgepole pine, *Pinus contorta* var. *latifolia* Engelmann, infested with MPB was collected from a site near Princeton, BC. The cut ends of the logs were sealed with hot paraffin wax to prevent dessication. The logs were either used fresh or stored at 4 °C until use. The logs were kept in cages held at approximately 28 °C. Emergent beetles were collected daily, and their sex was immediately determined by the shape of the seventh abdominal tergite (Lyon, 1958). The insects were either used the same day, or were stored on moistened paper at 4°C in loosely-capped jars for a maximum of one week. Only healthy-appearing individuals were selected for experiments.

Subalpine fir, *Abies lasiocarpa* (Hook.) Nutt. infested with BBB was collected from sites in the interior of BC. The logs and beetles were treated as described for MPB. The sex of the emergent beetles was determined by the presence (females) or absence (males) of the setal brush on the frons (Bright, 1976). Insects were stored as described for MPB.

2. Treatment of Insects

For the initial tests for the incorporation of *(E)*- or *(Z)*-D_2-6-nonen-2-one into brevicomin, newly-emerged male MPB or BBB were individually placed into 3.5 mL screw-cap vials (described by Gries *et al.*, 1990a), into which either 0 (control), 0.1 (for MPB) or 0.05 (for BBB) μL of the labelled precursor was placed. The sealed vials were stored at RT in the dark. After 24 h, the insects were either: (1) extracted immediately (whole beetles were individually extracted with hexane); or (2) individually aerated. Purified, moisturized air was drawn over the beetles, and the volatiles emanating from the beetles were trapped on Porapak Q (50-80 mesh, Applied Science Laboratories). After 48 h, the insects were individually extracted. Captured volatiles were removed from the Porapak Q by flushing with diethyl ether.

a) Exposure to $^{18}O_2$

Tests for the incorporation of $^{18}O_2$ into brevicomin were performed in two ways. The gas could not be efficiently transferred in the usual way (using a high vacuum line, and condensing the gas into the flask of interest with liquid nitrogen), since oxygen does not condense in liquid nitrogen under high vacuum.

Method 1: Gradual exchange of $^{16}O_2/N_2$ with $^{18}O_2/N_2$

The first study was performed by placing the insects (usually 40) in glass round-bottomed-flasks equipped with glass beads (for the insects to crawl on and to prevent fighting) and serum stoppers. The void volumes of the flasks (filled with beads) were ca. 35 mL. Using gas-tight syringes, 10 mL of N_2 were introduced into each flask, while 10 mL were withdrawn. Then 3 mL of $^{18}O_2$ (>97% $^{18}O_2$, Isotec Inc.) were added while 3 mL were withdrawn (the volume in the original container of $^{18}O_2$ was replaced with deoxygenated water). Finally, 40 mL of $^{18}O_2/N_2$ (1/4) were introduced to the bottom of the flasks while 40 mL were withdrawn from the top. It was expected that after this procedure nitrogen would comprise about 80% of the atmosphere in the flask (to mimic the normal atmosphere); and that $^{18}O_2$ would comprise about 80% of the oxygen in the flasks. In trials involving treatment with precursors, 1 μL of (*E*)- or (*Z*)-6-nonen-2-one was added by syringe through the serum stoppers. The insects were held at RT in the dark. After a period of time (specified in the *Results and Discussion*), the insects were extracted as described previously. Samples usually consisted of 10 pooled males.

It was observed that after about 16 h under these conditions, the beetles in the flasks became comatose (likely due to a lack of oxygen). Therefore in subsequent experiments, fewer beetles were introduced per flask.

Method 2: Introduction of $^{18}O_2/N_2$ into evacuated flask

The process described in *Method 1* to replace the $^{16}O_2$ in the flasks with $^{18}O_2$ was fairly tedious. Therefore subsequent studies were performed using Schlenk tubes, equipped with glass beads and serum stoppers. First the void volumes of the flasks (with the beads) were measured (ca. 35 mL). Then flasks were evacuated under high vacuum (the vacuum line was freshly cleaned, to avoid contamination of the flasks with unknown compounds). The $^{18}O_2$ was added by gas-tight syringe to 1/5 atmosphere (calculated by volume, based on the void volume of the flask). Nitrogen was added to atmospheric pressure. Then the beetles (usually 12 per flask) were quickly introduced into each flask by carefully lifting off the septum, while $^{18}O_2/N_2$ (1/4, v/v) was gently blown into the bottom of the flask *via* a gas-tight syringe inserted through the septum. The $^{18}O_2/N_2$ was continually blown into the flask until the septum was replaced.

b) Exposure to $^{18}OH_2$

In order to label the beetles with $^{18}OH_2$, males were first held on dry paper in a small loosely capped jar for 24 h in the dark (to dehydrate partially), and then were held in a vial containing a piece of Kimwipe soaked in ca. 200 μL $^{18}OH_2$ (MSD, 97% ^{18}O) for 48 h. Beetles to be treated with *(Z)*-6-nonen-2-one were not first dehydrated: they were immediately treated with $^{18}OH_2$ for 48 hours, and then were exposed individually to the vapors of 0.1 μL *(Z)*-6-nonen-2-one in 3.5 mL vials for 24 h. Insects were extracted as described previously; samples were prepared from 10 males.

3. Analyses of *exo*- and *endo*-brevicomin in extracts:

Analyses by GC were performed on a Hewlett-Packard 5890 GC equipped with a capillary inlet system and an FID. Samples were introduced by splitless injection onto a DB-1 column (15 m x 0.25 mm I.D., 0.25 μm film thickness). The oven temperature program was: 50 °C for 1 min, then 4 °C/min to 80 °C for 10 min, followed by an increase of 25 °C/min to a

final temperature of 275 °C for 10 min. The brevicomins were identified by comparing their GC retention times and MS fragmentation patterns with known standards. Brevicomin content in the extracts was quantified using 3-octanol as an internal standard.

Mass spectra were obtained on a Hewlett-Packard 5985 GC/MS system by EI ionization (70 eV) or CI using isobutane as the ionizing gas.

4. Determination of the chirality of the exo-brevicomin produced

The chirality of the *exo*-brevicomin produced by MPB was determined by complexation chromatography as described by Schurig *et al.* (1983).

The coating on the chiral column was not stable in the presence of ketones; therefore prior to analyses it was necessary to purify the extracts by preparative GC. In order to maintain the integrity of the preparative GC column, nonvolatile residues were first removed from the extracts. The crude extracts from control (untreated) and [4,4-D_2]-(*Z*)-6-nonen-2-one-treated male and female MPBs (15 per treatment) were pooled and soaked onto a glass wool plug placed at one end of a 12" length of glass tubing (which had been bent into an "ω" shape, leaving the first 1" straight and bent to be horizontal). The bottom half of the ω-shaped tubing was cooled with liquid nitrogen, thus forming two traps. The crude beetle extract was volatilized by passing a gentle stream of nitrogen gas through the glass wool plug, which was finger-warmed through the glass. The volatiles were collected in the glass traps. The brevicomin samples thus trapped were concentrated to 30 μL and purified by preparative GC (*Chapter II*). The oven temperature program used was: 70 °C for 2 min, then 6 °C/min to 180 °C. Samples were collected in glass tubes (20 cm x 1.6 mm O.D.). The collected samples were washed into half-dram glass vials, with 50 μL diethyl ether. The chirality of the brevicomin produced by the insects was determined by comparison of the retention times with known standards on a 25m x 0.25 mm (I.D.) fused silica capillary column coated with nickel-*bis*(1*R*)-3-heptafluorobutyrylcamphorate (50 °C for 2

min, then 2.5 °C/min to 85 °C for 30 min) (Schurig *et al.*, 1983).

5. Statistical analyses

Data were analyzsed by Analysis of Varience (ANOVA). Where more than two treatments were used, means were separated by Tukeys multiple range test (P=0.05).

6. Purchased/donated chemicals:

$LiAlD_4$ (98 atom % D) and $NaBD_4$ (98 atom % D) were purchased from Sigma Chemical Company; deuterium oxide (D_2O, 99.9 atom % D) and ^{18}O-water ($^{18}OH_2$, 97 atom% ^{18}O) were purchased from MSD. Leaf alcohol [*(Z)*-3-hexen-1-ol] was purchased from Bedoukian Research Inc. Palladium on activated carbon (10%) was purchased from Strem Chemicals, Inc.

Pherotech, Inc generously donated *(E)*- and *(Z)*-6-nonen-2-one. *(E)*-6,7-D_2-6-Nonen-2-one was kindly synthesized for use by Dr. Mohan Singh*.

7. Synthesis of stable isotope labelled chemicals

a) Analyses:

^1H and ^{13}C NMR spectra were recorded at 400.13 MHz and 100.6 MHz, respectively, on a Bruker WM-400 Spectrometer. MS (EI and CI) and IR spectra were recorded as described in *Chapter II*.

b) Syntheses of chemicals:

Reactions were performed and worked up as described in *Chapter II*. Flash chromatography was performed as described in *Chapter II*.

Preparation of [4,4-D_2]-*(Z)*-6-nonen-2-one (Scheme 7):

(Z)-3-Hexenoic acid (35)

2.5 M Chromic acid was added dropwise to a soln of leaf alcohol

---

* Department of Chemistry, Simon Fraser University. Burnaby, BC, Canada. V5A 1S6.

[(*Z*)-3-hexenol, **36**, 60 mmol] in acetone (200 mL) at 0 °C, until the yellow color persisted (about 80 mmol). The soln was stirred for an additional 15 min at RT, then 2-propanol was added dropwise until the green color persisted. The reaction mixture was then filtered through Celite. The acetone was removed *in vacuo*. The remaining oil was dissolved in diethyl ether, washed with brine and dried over anhyd $MgSO_4$. Removal of the solvent *in vacuo* gave a green oil. Distillation under vacuum left a colorless oil (4.15 g, 61% yield), bp 104-110 °C (ca. 10 mm Hg), 79% pure by GC analysis. ^{1}H NMR ($CDCl_3$): δ 0.97 (3 H, t, 6-H); 2.04 (2 H, dt, 5-H); 3.10 (2 H, d, 2-H); 5.43-5.52 (1 H, m, 3-H); 5.54-5.63 (1 H, m, 4-H); 10.25-10.90 (br s, COOH). IR: 2950 (s); 2700 (m); 2650 (m); 1700 (s); 1450 (m); 1420 (s); 1290 (s); 1210 (s); 1170 (m); 930 (m); 720 (m) cm^{-1}. MS (EI), m/e (relative abundance): 114 (M^+, 56); 99 (15); 96 (20); 81 (10); 73 (36); 69 (55); 68 (82); 60 (58); 55 (100); 41 (90).

[1,1-D_2]-(Z)-3-Hexenol (D_2-36)

The acid **35** (26 mmol) was dissolved in anhyd diethyl ether (20 mL), and the soln was added dropwise to a cooled (0 °C) suspension of $LiAlD_4$ (21.7 mmol) in anhyd diethyl ether (50 mL). After the addition was complete, the reaction mixture was stirred at 0 °C for an additional 1 h. The reaction was quenched by pouring into ice-cold 2% HCl and worked up in the usual fashion to yield a colorless oil (2.21 g, 82% yield), 93% pure by GC analysis. ^{1}H NMR ($CDCl_3$): δ 0.96 (3 H, t, 6-H); 1.50 (1 H, br s, OH); 2.07 (2 H, p, 5-H); 2.30 (2 H, d, 2-H); 3.64 (0.3 H, t, 1-H); 5.27-5.37 (1 H, m, 4-H); 5.50-5.60 (1 H, m, 3-H). IR: 3340 (s); 2990 (s); 2950 (s); 2920 (s); 2860 (s); 2190 (w); 2080 (w); 1640 (w); 1450 (m); 1390 (m); 1360 (m); 1300 (w); 1280 (w); 1215 (w); 1115 (s); 1090 (s); 965 (s); 840 (w) cm^{-1}. MS (EI) m/e (relative abundance): 102 (M^+, 34); 84 (58); 70 (20); 69 (100); 68 (45); 67 (48); 55 (38); 41 (90).

[1,1-D_2]-1-Tosyl-(Z)-3-hexene (D_2-37)

A suspension of the alcohol D_2-**36** (20 mmol), tosyl chloride (22 mmol) and powdered KOH (210 mmol) in anhyd diethyl ether (20 mL) was stirred for 2 h at 0 °C. The reaction mixture was filtered, the solvent removed *in vacuo*, and the crude product flash chromatographed (CH_2Cl_2) to yield a colorless oil (3.09 g, 62% yield), 96% pure by GC analysis. ^1H NMR ($CDCl_3$): δ 0.91 (3 H, t, 6-H); 1.91-2.02 (2 H, m, 5-H); 2.36 (2 H, d, 2-H); 2.44 (3 H, s, tosyl methyl); 3.96-4.00 (0.2 H, m, 1'-H); 4.06-4.15 (0.2 H, m, 1-H); 5.13-5.22 (1 H, m, 4-H); 5.42-5.51 (1 H, m, 3-H); 7.33 (2 H, d, tosyl 3'-H and 5'-H); 7.76 (2 H, d, tosyl 2'-H and 6'-H). MS (EI) m/e (relative abundance): 173 (30); 155 (50); 112 (62); 91 (100); 83 (50); 70 (92); 65 (42); 57 (52); 41 (44).

[3,3-D_2]-Ethyl-2-aceto-5-octenoate (D_2-38)

The tosylate D_2-**37** (12 mmol) was slowly added to a stirred soln of sodium ethylacetoacetate (14 mmol) dissolved in anhyd ethanol (20 mL). The reaction mixture was heated at reflux for 17 h, during which time a heavy precipitate of sodium tosylate developed. The reaction mixture was cooled and neutralized with glacial acetic acid. The ethanol was removed *in vacuo*. The crude oil was taken up in hexane (200 mL) and extracted with water (1 x 50 mL). The organic layer was dried over anhyd $MgSO_4$. Removal of the solvent *in vacuo* yielded a yellow syrup (1.91 g, 76% yield), which was 49% pure by GC analysis. This product was carried through to the next step.

[4,4-D_2]-(Z)-6-Nonen-2-one [D_2-(Z)-39]

A mixture of the ketoester D_2-**38** as a crude syrup (1.91 g) and NaOH (18 mmol) in water (12 mL) was stirred for 24 h at RT. The reaction mixture was extracted with hexane (2 x 10 mL). The hexane layer contained unreacted **39** so the solvent was removed *in vacuo* and the remaining oil re-submitted to the reaction conditions. The aqueous phase was acidified

with conc sulfuric acid to pH 2, and stirred at 90-100 °C for 5 h. The aqueous phase was extracted with diethyl ether (3 x 30 ml). The organic layers were pooled, washed with 5% $NaHCO_3$ soln (1 x 20 mL) and then brine (1 x 20 mL), dried over anhyd $MgSO_4$, filtered, and concentrated *in vacuo* to yield a colorless oil (0.460 g, 28% yield from the tosylate 37), which was 96% pure by GC analysis. ^1H NMR ($CDCl_3$): δ 0.95 (3 H, dt, $J_{7,9}$=1 Hz, $J_{8,9}$=8 Hz, 9-H); 1.57-1.67 (0.5 H, m, 4-H); 1.96-2.07 (4 H, m, 5-H and 8-H); 2.12 (3 H, s, 1-H); 2.40 (2 H, br s, 3-H); 5.23-5.31 (1 H, m, $J_{7,8}$=1 Hz, 7-H); 5.35-5.44 (1 H, m, $J_{5,6}$=1 Hz, 6-H). MS (EI) m/e (relative abundance): 142 (M^+, 11); 127 (8); 113 (14); 99 (11); 85 (23); 84 (86); 83 (18); 82 (24); 73 (17); 69 (100); 68 (50); 67 (63); 58 (20); 57 (11); 55 (14); 43 (78); 42 (10); 41 (26). By GC/SIM-MS analysis the product was 88% enriched with two deuteriums and 12% unenriched.

Preparation of ^{18}O-*(Z)*-6-nonen-2-one (Scheme 8):

(Z)-6-Nonen-2-one 2',2'-dimethyl-propane ketal (40)

A toluene soln (60 mL) containing *(Z)*-6-nonen-2-one (10.7 mmol), 2,2-dimethyl-1,3-propandiol (11.3 mmol) and a catalytic quantity of *p*-toluenesulfonic acid (0.011 mmol) was refluxed overnight. Water was removed as it formed by Dean-Stark extraction. The soln was cooled and extracted with a satd soln of $NaHCO_3$. The usual workup (except that the organic extract was dried over anhyd K_2CO_3) and distillation under vacuum yielded a colorless oil (2.29 g, 95% yield), bp 89-92 °C (1.2 mm Hg), 96% pure by GC analysis. ^1H NMR ($CDCl_3$): δ 0.89 (3 H, d, $J_{CH2,CH3}$=1.2 Hz, ketal methyl); 0.95 (3 H, dt, $J_{8,9}$=7.5 Hz, $J_{7,9}$=1.2 Hz, 9-H); 1.00 (3 H, s, ketal methyl); 1.35 (3 H, d, J=2.9 Hz, 1-H); 1.42-1.51 (2 H, m, 4-H); 1.65-1.72 (2 H, m, 3-H); 1.99-2.08 (4 H, m, 5-H and 8-H); 3.43 (2 H, dd, J_{gem}=11 Hz, $J_{CH2,CH3}$=1.2 Hz, ketal methylenes); 3.53 (2 H, d, J_{gem}=11 Hz, ketal methylenes); 5.28-5.40 (2 H, m, 6-H and 7-H). MS (EI) m/e (relative intensity): 226 (M^+, 5); 211 (50); 129 (100); 82 (10); 69 (30).

^{18}O-(Z)-6-Nonen-2-one [^{18}O-(Z)-39]

Gaseous HCl was bubbled through a vigorously stirred mixture of hexane (200 μL, previously dried over Na wire) and $^{18}OH_2$ (200 μL) for ca. 10 sec. The ketal **40** (4.0 mmol) was added with cooling. The mixture was stirred at RT and the progress of the reaction was monitored periodically by GC. As required, more HCl was bubbled through the reaction mixture (with cooling). After about 4 h, the reaction was complete. Anhyd hexane (ca. 0.5 mL) was added, and the aqueous and organic phases were allowed to separate. The organic layer was passed through a mini-column of anhyd K_2CO_3. The aqueous phase was washed with anhyd hexane (6 x 50 μL); the washings were passed through the mini-column. The combined hexane extracts were distilled from K_2CO_3, using a 1" Vigreux column. The hexane was first removed at atmospheric pressure, then a vacuum was applied to distill the product. A colorless oil was obtained (0.32 g, 56% yield), 98% pure by GC analysis. ^1H NMR ($CDCl_3$): δ 1.05 (3 H, t, 9-H); 1.64-1.73 (2 H, m, 4-H); 1.96-2.07 (4 H, m, 5-H and 8-H); 2.12 (3 H, s, 1-H); 2.43 (2 H, t, 3-H); 5.23-5.31 (1 H, m, 7-H); 5.35-5.44 (1 H, m, 6-H). MS (EI) m/e (relative abundance): 142 (M^+, 18); 127 (12); 113 (20); 82 (86); 67 (100); 60 (12); 55 (14); 45 (50). The product was 94% ^{18}O by GC/SIM-MS analysis.

Preparation of ^{18}O-exo-brevicomin (Scheme 9):

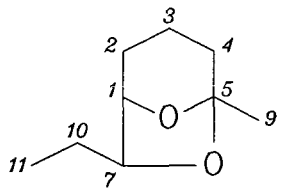
6,7-(Z)-Epoxynona-2-one 2',2'-dimethylpropane ketal (41)

A soln of the ketal **40** (2.2 mmol) in CH_2Cl_2 (20 mL) was added to a vigorously stirred soln of $NaHCO_3$ (3.6 mmol) in water (7 mL). *m*-Chloroperbenzoic acid (2.7 mmol) was added, and the biphasic system was stirred vigorously at RT overnight. The usual workup yielded a colorless oil (0.55 g, quant yield), 87% pure by GC analysis. ^1H NMR ($CDCl_3$): δ 0.88 (3 H, s, ketal methyl); 1.02 (3 H, s, ketal methyl); 1.04 (3 H, t, $J_{8,9}$=8 Hz, 9-H); 1.37 (3 H, s, 1-H); 1.47-1.78 (8 H, m, 3-H, 4-H, 5-H, 8-H); 2.84-2.90 (1 H, m, 7-H); 2.90-2.96 (1 H, m, 6-H); 3.42 (2 H, d, J_{gem}=12 Hz,

ketal methylenes); 3.56 (2 H, d, J_{gem}=12 Hz, ketal methylenes). MS (EI) m/e (relative intensity): 227 (75%); 129 (100); 69 (30).

^{18}O-Exo-brevicomin (^{18}O-exo-42)

The ketal-epoxide **41** (1.9 mmol) dissolved in anhyd diethyl ether (ca. 200 μL) was added to $^{18}OH_2$ (0.5 mL) through which gaseous HCl had been bubbled for ca. 10 sec. The reaction mixture was stirred vigorously at RT, and the reaction was monitored by GC. HCl gas was bubbled through periodically, as needed. After 12 h, the two phases were allowed to separate. The upper organic layer was removed. The aqueous phase was washed with anhyd diethyl ether (3 x 0.1 mL). A gentle Ar stream was blown over the combined ether extracts to remove most of the ether. Bulb-to-bulb distillation under vacuum yielded crude product (0.38 g) 70% pure by GC analysis. This product was purified by distillation under vacuum to give a colorless oil (0.30 g, 91% yield), 95% pure by GC analysis. ^{1}H NMR ($CDCl_3$) (according to numbering scheme shown): δ 0.91 (3 H, distorted t, $J_{10,11}$=7.5 Hz, 11-H);1.41 (3 H, s, 9-H); 1.43-1.95 (8 H, m, 2-H, 3-H, 4-H and 10-H); 3.93 (1 H,t, $J_{7,10}$=6.5 Hz, 7-H); 4.13 (1 H, br s, 1-H). ^{13}C NMR ($CDCl_3$): δ 9.610 (11-C); 17.15 (3-C); 25.00 (9-C); 28.00 and 28.58 (2-C and 10-C); 35.03 (4-C); 78.63 (1-C); 81.49 (7-C); 108.19 (5-C). MS (EI) m/e (relative intensity): 158 (M^+, 14); 129 (18); 116 (17); 114 (80); 101 (11); 100 (26); 88 (21); 87 (17); 85 (100); 81 (16); 72 (12); 68 (22); 67 (16); 45 (26); 43 (18).



The position of the ^{18}O label in the product was deduced from the ^{18}O-isotope-induced shifts of the ^{13}C NMR signals, after resolution enhancement of the ^{13}C NMR spectrum (line broadening, -0.5 Hz; Gaussian broadening, 0.3; 64 K data set). The results are presented in the *Results and Discussion*.

Preparation of [4,4-D_2]-(*Z*)-6-nonen-2-ol [D_2-(*Z*)-43]

D_2-(*Z*)-6-Nonen-2-one [D_2-(*Z*)-39, 0.7 mmol] was dissolved in 95% ethanol (1 mL). $NaBH_4$ (ca. 3 mmol) dissolved in 95% ethanol (1 mL) was added dropwise. The reaction mixture was stirred at RT for 1 h, before it was quenched with 2% HCl (10 mL). The usual workup yielded a colorless oil (86 mg, 85% yield), 98% pure by GC analysis. ^1H NMR ($CDCl_3$): δ 0.94 (3 H, t, $J_{8,9}$=7.5, 9-H); 1.18 (3 H, d, $J_{1,2}$=6.0, 1-H); 1.39-1.47 (2 H, m, 3-H); 1.50-1.54 (0.7 H, br s, 4-H); 1.97-2.07 (4 H, m, 5-H and 8-H); 3.74-3.83 (1 H, sx, $J_{1,2}$=$J_{2,3}$=6.0, 2-H); 5.27-5.41 (2 H, m, 7-H and 8-H). MS (EI) m/e (relative intensity): 144 (M^+, 10); 97 (25); 84 (100); 82 (28); 73 (53); 71 (14); 70 (11); 69 (79); 68 (60); 67 (44); 58 (16); 57 (12); 56 (11); 55 (15); 45 (30); 43 (15); 41 (21).

Preparation of [6,7-D_2]-(*E*)-6-nonen-2-ol [D_2-(*E*)-44]

The alcohol was prepared by reduction of [6,7-D_2]-(*E*)-6-nonen-2-one (D_2-(*E*)-39] with $NaBH_4$ as described above. The product was obtained in 90% yield, and was >99% pure by GC analysis. ^1H NMR ($CDCl_3$): δ 0.89 (3 H, t, $J_{8,9}$=7.5, 9-H); 1.16 (3 H, d, $J_{1,2}$=6.0, 1 H); 1.30-1.45 (4 H, m, 3-H and 4-H); 1.78 (1 H, br s, OH); 1.93-2.00 (4 H, m, 5-H and 8-H); 3.72-3.81 (1 H, sx, $J_{1,2}$=$J_{2,3}$=6.0, 1-H). MS (EI) m/e (relative intensity): 144 (M^+, 11); 111 (7); 97 (30); 85 (12); 84 (100); 83 (24); 82 (14); 81 (7); 72 (12); 71 (58); 70 (16); 69 (85); 68 (48); 67 (13); 58 (11); 57 (16); 56 (12); 55 (12); 45 (27); 43 (22); 42 (14); 41 (10).

Preparation of [6,7-D_2]-nonan-2-one (D_2-45)

Palladium (10%) on activated carbon (20 mg) was suspended in anhyd absolute ethanol (10 mL). H_2 gas was bubbled through the vigorously stirred suspension for 10 min, then [6,7-D_2]-(*E*)-6-nonen-2-one [D_2-(*E*)-39, 2.8 mmol] dissolved in anhyd absolute ethanol (3 mL) was added in one portion. The reaction mixture was stirred vigorously at RT while H_2 gas was continually bubbled through. After 4 h, $MgSO_4$ was added to dry the

reaction mixture. The mixture was filtered, and the precipitate was rinsed thoroughly with anhyd diethyl ether. The filtrate and washes were combined, and the solvent removed by distillation through a 12" Vigreux column. The residue was subjected to bulb-to-bulb distillation (2 mm Hg with gentle warming) and yielded a colorless oil (322 mg, 79% yield), 98% pure by GC analyisis. ^{1}H NMR ($CDCl_3$): δ 0.82 (3 H, t, 9-H); 1.11-1.31 (6 H, br s, 5-H to 8-H); 1.46-1.56 (2 H, m, 4-H); 2.08 (3 H, s, 1-H); 2.38 (2 H, t, 3-H). MS (EI) m/e (relative intensity): 147 (1.6); 146 (4.8); 145 (10.4); 144 (M^+, 13.2); 143, (7.9); 142 (2.3); 130 (3.4); 129 (5.9); 128 (3.8); 102 (1.8); 101 (3.4); 100 (3.4); 99 (1.5); 87 (4.6); 86 (7.5); 85 (7.4); 84 (3.4); 83 (3.8); 82 (3.2); 73 (6.2); 72 (16.2); 71 (28.4); 70 (4.6); 60 (16); 59 (59); 58 (99); 57 (27); 56 (16); 55 (21); 45 (17); 44 (50); 43 (100); 42 (89); 41 (78). MS (CI) m/e (relative intensity): 148 (1.9); 147 (10.5); 146 (44.4); 145 (M+1, 100); 144 (61.9); 143 (18.0); 142 (0.9).

C) RESULTS AND DISCUSSION

The male MPB used in this study produced about ten times more *exo*-brevicomin than *endo*-brevicomin. Initial efforts were therefore directed towards the study of the biosynthesis of the *exo*-isomer and will be discussed first.

1. Biosynthesis of *exo*-brevicomin

a) Is (*Z*)-6-nonen-2-one a precursor of *exo*-brevicomin *in vivo*?

The first objective of this study was to determine if (*Z*)-6-nonen-2-one could serve as a precursor of *exo*-brevicomin *in vivo*. The approach taken was to expose insects to vapors of a deuterated analogue of (*Z*)-6-nonen-2-one (namely, [4,4-D_2]-(*Z*)-6-nonen-2-one) and to check for the incorporation of deuterium into the brevicomins produced by the insects (by GC-MS analysis). Incorporation of the precursor into *exo*-brevicomin was expected to proceed as shown in Figure 34.

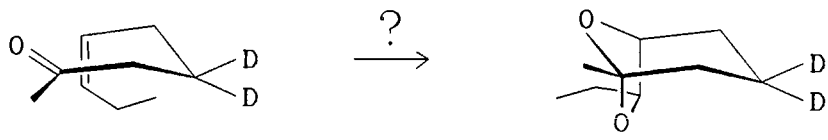
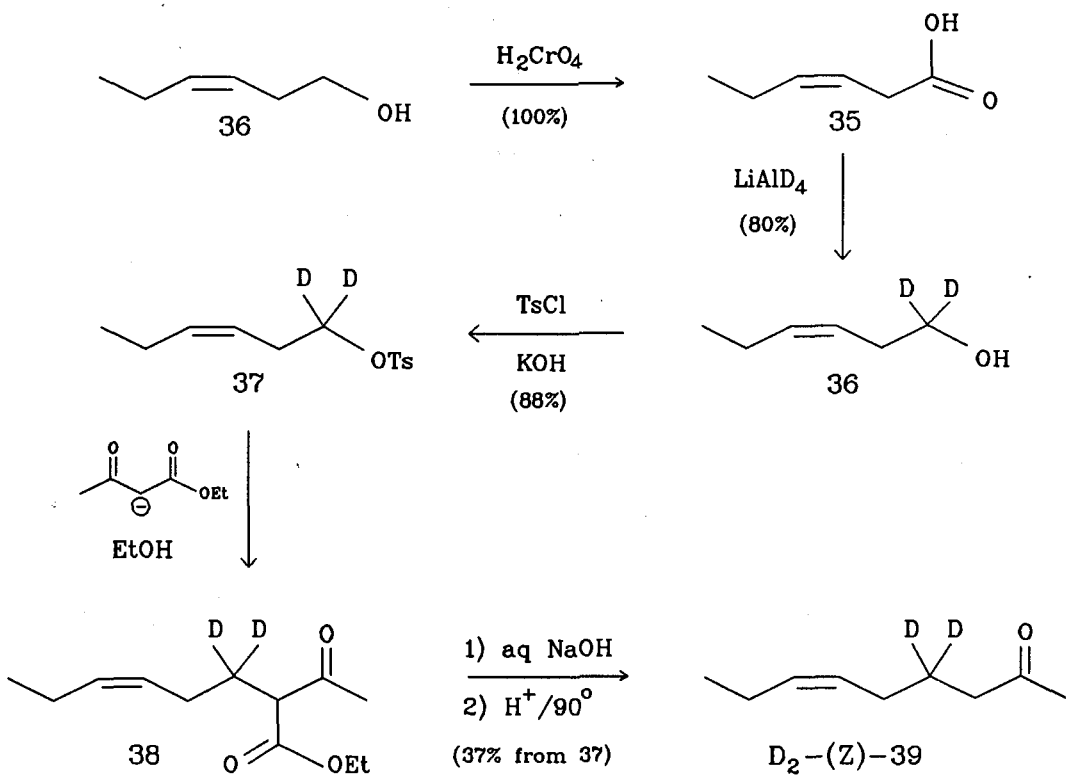


Figure 34: Expected pattern of deuterium incorporation into *exo*-brevicomin synthesized from the deuterated analogue of (*Z*)-6-nonen-2-one.

Synthesis of labelled (*Z*)-6-nonen-2-one

[4,4-D_2]-(*Z*)-6-Nonen-2-one [D_2-(*Z*)-**39**] was synthesized from [1,1-D_2]-3-hexen-1-ol (D_2-leaf alcohol, **36**) as shown in Scheme 7. Deuterated leaf alcohol was prepared by a Jones oxidation of commercially available leaf alcohol (quantitative yield), followed by reduction of the acid with $LiAlD_4$ (80% yield after distillation). The remainder of the synthetic route (summarized in Scheme 7) followed the method of Rodin *et al.* (1971), and produced [4,4-D_2]-(*Z*)-6-nonen-2-one (96% pure) in an overall yield of



Scheme 7: Synthetic route to [4,4-D_2]-(*Z*)-6-nonen-2-one, D_2-(*Z*)-39.

26%. The product was 88% enriched with two deuteriums and 12% unenriched, as judged by SIM-MS analysis.

Incorporation of (*Z*)-6-nonen-2-one into *exo*-brevicomin *in vivo*

Male MPBs and BBBs exposed to vapors of [4,4-D_2]-(*Z*)-6-nonen-2-one produced *exo*-brevicomin that was clearly enriched in two deuteriums. In Figure 35 the MS of *exo*-brevicomin extracted from unexposed MPB males is compared to that from males exposed to the precursor for 24 hours. The mass spectra of deuterium-enriched *exo*-brevicomin isolated from MPBs and BBBs were indistinguishable and, therefore, only one is presented in the Figure.

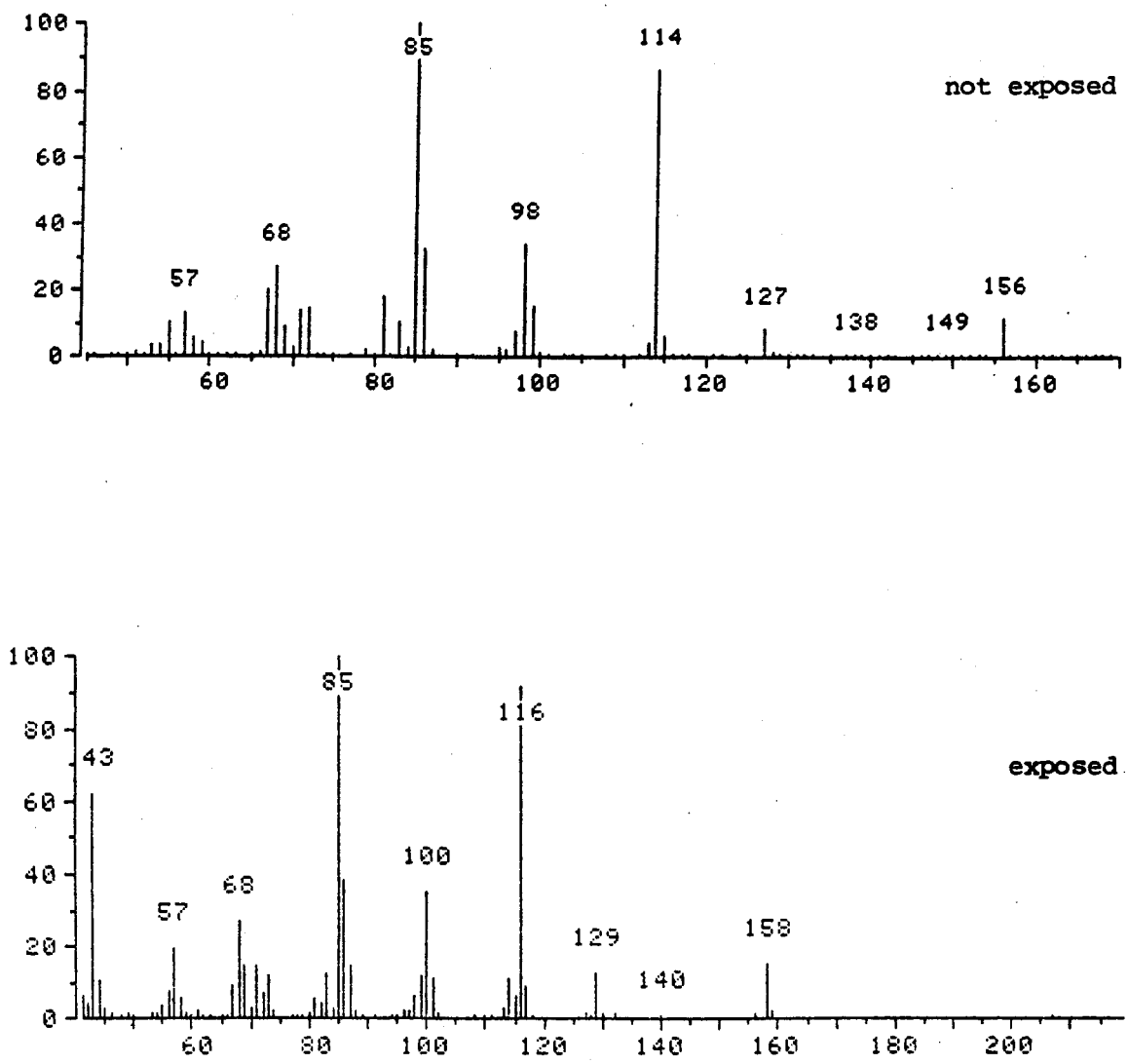


Figure 35: MS (EI) of *exo*-brevicomin extracted from unexposed male MPBs and from males exposed to [4,4-D_2]-(*Z*)-6-nonen-2-one.

It is not clear if female MPBs and BBBs normally produce *exo*-brevicomin. Pitman *et al.* (1969) reported that emergent and fed female MPBs contained trace amounts of *exo*-brevicomin, but Pierce *et al.* (1987b) failed to detect *exo*-brevicomin in fed females. To my knowledge the production of *exo*-brevicomin by female BBBs has not been investigated. In this study, emergent unfed female MPBs and BBBs did not produce detectable levels of *exo*-brevicomin unless they were exposed to [4,4-D_2]-(*Z*)-6-nonen-2-one. Although the quantity of *exo*-brevicomin produced varied from experiment to experiment*, female MPBs and BBBs exposed to (*Z*)-6-nonen-2-one always produced less *exo*-brevicomin than exposed males (although this difference was not always statistically significant, Table 19).

The *exo*-brevicomin produced by female MPBs exposed to [4,4-D_2]-(*Z*)-6-nonen-2-one was enriched in two deuteriums. The *exo*-brevicomin produced by female BBBs was identified on the basis of the comparison of GC retention time (R.T.) with known standards. Unfortunately the samples evaporated before MS analyses could be performed.

Male MPBs continued to emit *exo*-brevicomin, after the initial 24 hour exposure period. Male and female beetles were held individually in glass vials for 24 hours, while exposed (or not exposed) to vapors of (*Z*)-6-nonen-2-one. After 24 hours the beetles were either: (1) extracted immediately (to give a "whole beetle extract", WBE-1); or (2) aerated for 48 hours while the volatiles emanating from the beetles were collected on Porapak Q. After aeration for 48 hours the insects were individually extracted (to give the second "whole beetle extract", WBE-2). Captured volatiles were removed from the Porapak Q by flushing with diethyl ether (to provide the "Porapak Q extract", PQE). The quantity of *exo*-brevicomin that was present in each extract is presented in Figure 36.

---

* This variation was likely due to such factors as the health of the beetles, temperature and atmospheric conditions (Professor J. H. Borden, personal communication; personal observations). Professor Borden's address is: Dept. of Biological Sciences, Simon Fraser University, Burnaby, BC, Canada. V5A 1S6.

Table 19: *Exo*-brevicomin production by male or female MPB or BBB[1], exposed (or not exposed) to (*Z*)-6-nonen-2-one.

Species	treatment	*Exo*-brevicomin production[1] (ng/beetle, $\bar{x}$ ± SE) males	females
MPB:			
Trial 1	Unexposed	70 ± 14 (8)[a]	n.d.
	Exposed	246 ± 30 (21)[b]	59 ± 12 (10)[a]
Trial 2	Unexposed	93 ± 18 (4)[a]	n.d.
	Exposed	714 ± 163 (7)[b]	451 ± 142 (7)[b]
Trial 3	Unexposed (16 h)	20.4 ± 2 (4)[a]	n.d.
	(24 h)	47.0 ± 7 (9)[a]	n.d.
	Exposed (16 h)	71.9 ± 4 (4)[a]	14.3 ± 2 (4)[a]
	(24h)	398 ± 136 (4)[b]	19.6 (1)[a]
BBB:	Unexposed	n.d.	n.d.
	Exposed	237 ± 57 (4)[a]	50 ± 3 (2)[b]

[1] The number of samples for each treatment is shown in brackets. Numbers (within a trial) that are not significantly different from one another ($P < 0.05$) have the same letter superscripts. Each sample was usually prepared from one beetle.

As can be seen in Figure 36, control and exposed males continued to emit relatively large quantities of *exo*-brevicomin during the aeration period. In contrast, the females emitted relatively small quantities of *exo*-brevicomin during the aeration period.

Table 20 reveals that the *exo*-brevicomin produced by exposed male MPB over the 48 hour aeration period (PQE) was as enriched in two deuteriums as the WBE-1, while the enrichment seemed to be somewhat less in the WBE-2 extract. Likely during the 48 hours following the exposure, the exogenously supplied labelled precursor dropped to low enough levels to be significantly diluted by endogenously-produced unlabelled precursor.

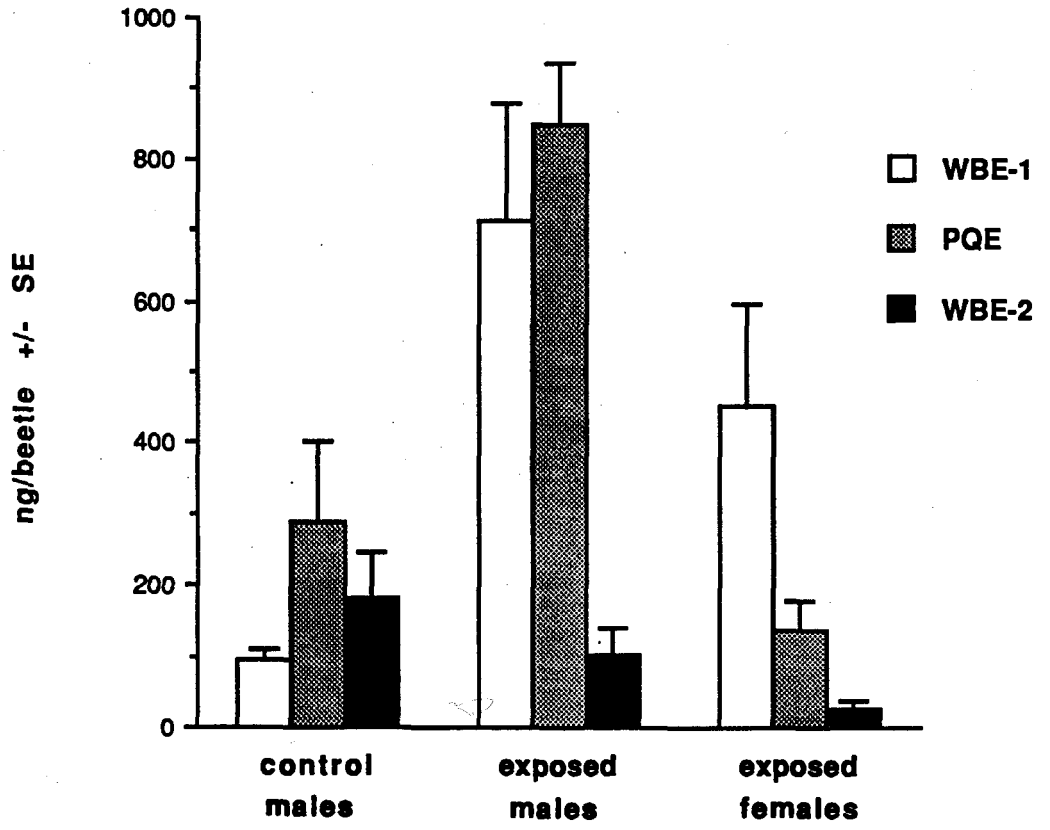


Figure 36: Content of *exo*-brevicomin in extracts of male and female MPB, exposed (or not exposed) to (*Z*)-6-nonen-2-one. (WBE-1, whole beetle extract prepared after the 24 hour exposure period; PQE, Porapak Q extract; WBE-2, whole beetle extract prepared after the aeration period.)

The volatiles extracted from beetles exposed to vapors of (*Z*)-6-nonen-2-one included unused precursor, as well as (*Z*)-6-nonen-2-ol which the insects likely produced through reduction of the ketone. The identities of these two compounds were determined by comparison of their GC-R.T.s and GC-MS fragmentation patterns with those of authentic standards (prepared as described in the *Experimental* section). The quantities of each compound in the WBE-1, WBE-2, and PQE of male and female MPB are presented in Figure 37.

Table 20: Enhancement of the $(M+2)^+$ peak in *exo*-brevicomin isolated from various extracts of male BBB or MPB, exposed (or not exposed) to [4,4-D_2]-(*Z*)-6-nonen-2-one. The relative abundance of the $(M+2)^+$ peak in the MS of the putative precursor [4,4-D_2]-(*Z*)-6-nonen-2-one is provided for comparison.

Treatment[1]	Relative abundance ($\bar{x}$ ± SE) M^+	$(M+2)^+$
Unexposed MPB:		
WBE-1 (4)	100.0	1.0 ± 0.04
PQE (4)	100.0	2.0 ± 0.5
WBE-2 (3)	100.0	0.8 ± 0.0
Exposed MPB:		
WBE-1 (2)	21.9 ± 1	100.0
PQE (7)	20.8 ± 0.4	100.0
WBE-2 (7)	28.5 ± 4	100.0
Exposed BBB[2]:		
WBE-1 (4)	18.0 ± 0.5	100.0
Precursor:		
D_2-(*Z*)-6-nonen-2-one	13.9	100.0

[1] The number of samples (each prepared from 1 beetle) is indicated in brackets; [2] *exo*-brevicomin was not detected in extracts of unexposed BBBs.

Endo-brevicomin enriched in deuterium was also formed when the insects were exposed to [4,4-D_2]-(*Z*)-6-nonen-2-one (Table 21). No (*E*)-isomer was detectable in this precursor by GC analysis. However, each beetle was exposed to roughly 8 x 10^5 ng of [4,4-D_2]-(*Z*)-6-nonen-2-one, and produced up to 33 ng of D_2-*endo*-brevicomin (0.004%). Thus the observed D_2-*endo*-brevicomin production could be due to extremely small quantities (not detected by GC or NMR analyses) of the (*E*)-isomer present as an impurity in the (*Z*)-6-nonen-2-one.

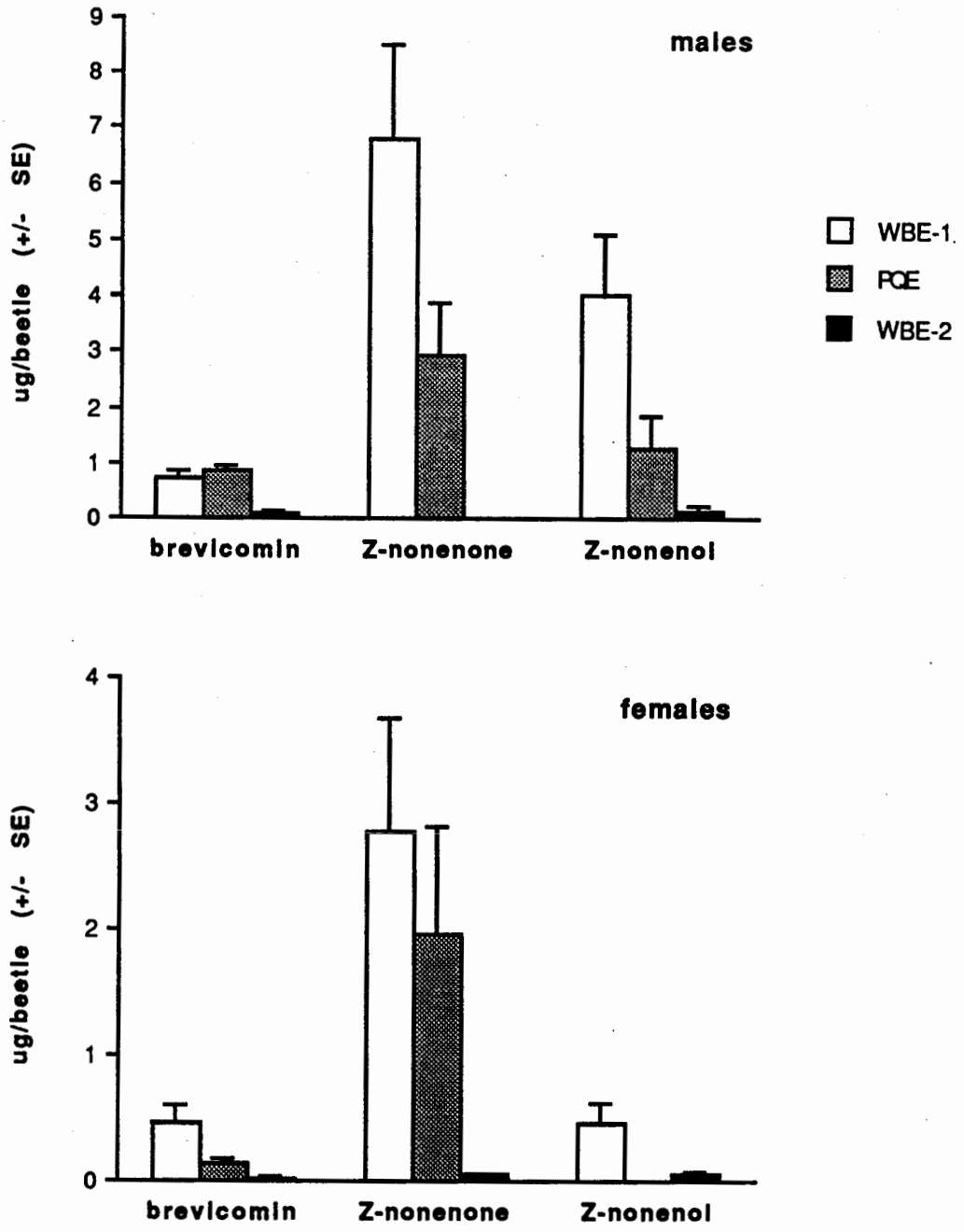


Figure 37: Content of *exo*-brevicomin, (*Z*)-6-nonen-2-one and (*Z*)-6-nonen-2-ol in extracts of male or female MPB exposed to (*Z*)-6-nonen-2-one. (Note the different scales).

Table 21: Relative abundance of peaks of interest in MS (IBCI)[1] of the brevicomins produced by male MPB after exposure to [4,4-D_2]-(*Z*)-6-nonen-2-one for 24 hours.

Treatment[2]	Relative abundance ($\bar{x}$ ± SE) P^+	$(P+1)^+$	$(P+2)^+$	$(P+3)^+$
unexposed (2)				
exo-brevicomin:	100.0	9.9 ± 0.1	1.1 ± 0.03	0.1 ± 0.0
+ D_2-(*Z*)-nonenone (1)				
exo-brevicomin:	21.7	7.8	100.0	9.9
endo-brevicomin:	43.1	14.1	100.0	14.4

[1] The MS of brevicomin obtained by chemical ionization (rather than electron impact) exhibits the parent peak as m/e = $(M+1)^+$. To avoid confusion, therefore, the peak representing the $(M+1)^+$ peak of the unlabelled compound is represented as P^+. Molecules enriched for one deuterium would exhibit an $(M+2)^+$ peak, represented here as $(P+1)^+$; [2] the number of samples [each prepared from 3 (control) or 7 (exposed) beetles] is indicated in brackets.

Incorporation of (Z)-6-nonen-2-one into exo-brevicomin: biologically relevant?

One might suggest that the observed conversion of (*Z*)-6-nonen-2-one into *exo*-brevicomin is not relevant to normal *in vivo* brevicomin biosynthesis. A plausible mechanism for this is non-specific epoxidation of the keto-alkene by poly-substrate mono-oxidases (which are known to epoxidize alkenes) followed by the cyclization of the thus-formed keto-epoxide. The latter process is known to occur spontaneously in the laboratory under acidic conditions (Bellas *et al.*, 1969), or neutral conditions if heat is applied (Wasserman and Barber, 1969). However, I believe that the incorporation of (*Z*)-6-nonen-2-one into *exo*-brevicomin is biologically relevant for the following reasons:

1. Female MPBs and BBBs exposed to the precursor under the same conditions as the males always produced less *exo*-brevicomin (Table 19). The female MPBs used in this study weighed, on average, about 1.5 times as much as the males, and would be expected to have PSMOs at **least** as active as the males since they are the first attacking sex. For these reasons I would expect the females to far out-produce the males, if the conversion of *(Z)*-6-nonen-2-one to *exo*-brevicomin was non-specific.

2. Unlike the males, female MPB did not continue to emit large quantities of *exo*-brevicomin during the 48 hour aeration period after the initial exposure (Figure 36).

3. When male and female *Ips pini*, which are not known to produce exo-brevicomin, were exposed to the precursor under identical conditions as the MPB, very little (if any) *exo*-brevicomin was produced. A peak which co-eluted on the GC with *exo*-brevicomin was produced at a level of about 2% of the *exo*-brevicomin produced by male MPB (the *I. pini* used in this experiment weighed half as much as male MPB, so production by male MPB was compared to twice as many *I. pini*). Unfortunately the samples evaporated before the identity of this peak could be determined by GC-MS.

4. The *exo*-brevicomin produced by male and female MPBs exposed to the precursor was found to be of the natural (+)-chirality by complexation chromatography, using the method of Schurig *et al.* (1983).

5. Dr. Gerhard Gries* (personal communication) has observed that male MPBs and BBBs both emit *(Z)*-6-nonen-2-one. The volatiles of the insects were trapped on Porapak Q, and analyzed by GC-MS. The putative *(Z)*-6-nonen-2-one peak had the same GC-R.T. as an authentic sample, and exhibited the same MS fragmentation pattern.

---

* Department of Biological Sciences, Simon Fraser University, Burnaby, BC, Canada. V5A-1S6.

b) Elucidation of the mechanism of conversion of *Z*-6-nonen-2-one into *exo*-brevicomin:

One could envision several intermediates that might be involved in the conversion of (*Z*)-6-nonen-2-one to *exo*-brevicomin (Figure 38). The first step likely involves the epoxidation of the double bond. The resulting keto-epoxide (**46**) could cyclize to brevicomin directly, or could first be acted upon by an epoxide hydrolase. The resulting keto-diol intermediate (**47**), in turn, could either cyclize to brevicomin directly or could first be derivatized. One of the alcohol groups could be converted to a leaving group (LG), such as pyrophosphate, and one of the derivatized intermediates **48** or **49** might then cyclize to form brevicomin.

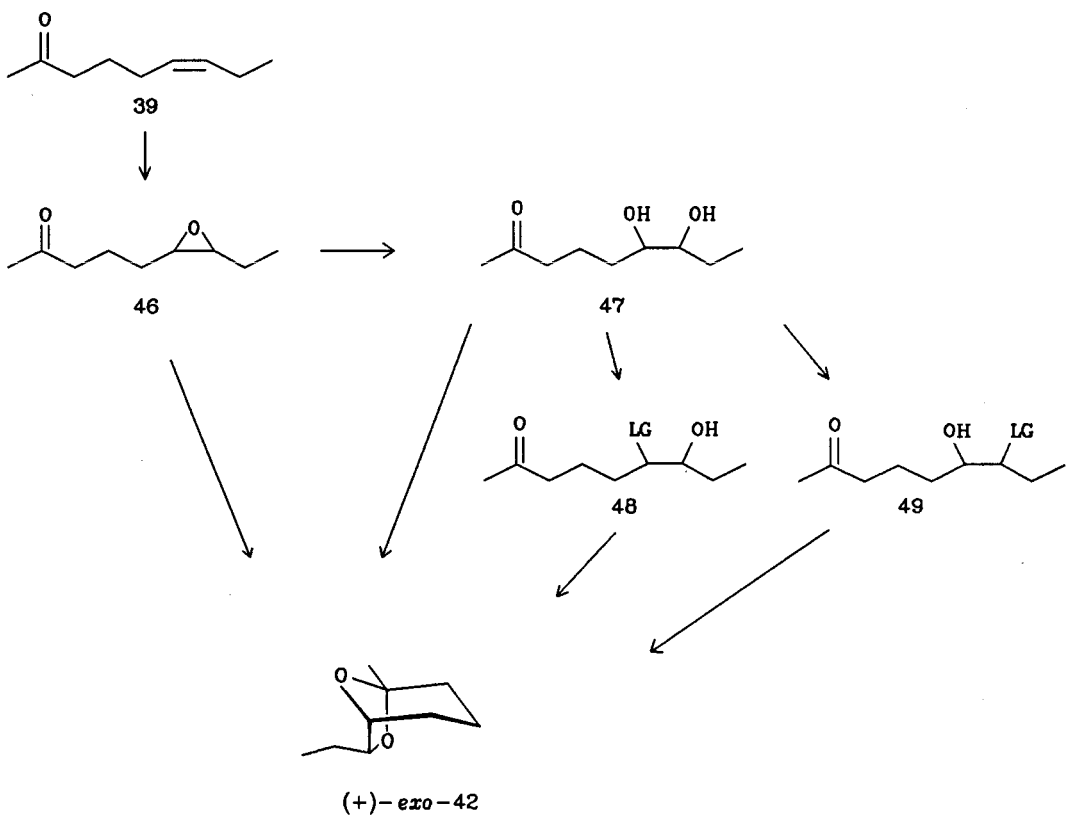


Figure 38: Possible intermediates in the conversion of (*Z*)-6-nonen-2-one to (+)-*exo*-brevicomin, **42**. ("LG" denotes "leaving group").

It should be possible to distinguish between these different paths experimentally. One method would be to allow the insects to synthesize brevicomin from ^{18}O-labelled (*Z*)-6-nonen-2-one. Depending on the intermediate involved and the mechanism of attack, quite different results with respect to the ^{18}O content and stereochemistry of the brevicomin product should be obtained.

For example, there exist two enantiomers of 6,7-epoxynonan-2-one (**46**). Each enantiomer could cyclize in one of two ways [by attack at either *a* or *b*, as shown in Figure 39 for the (6*R*,7*S*)-enantiomer]. In this Figure, the cyclization is represented as proceeding stepwise, through discrete intermediates. It is possible that some of these steps are concerted, but this should not affect the stereochemical outcome of the overall reaction.

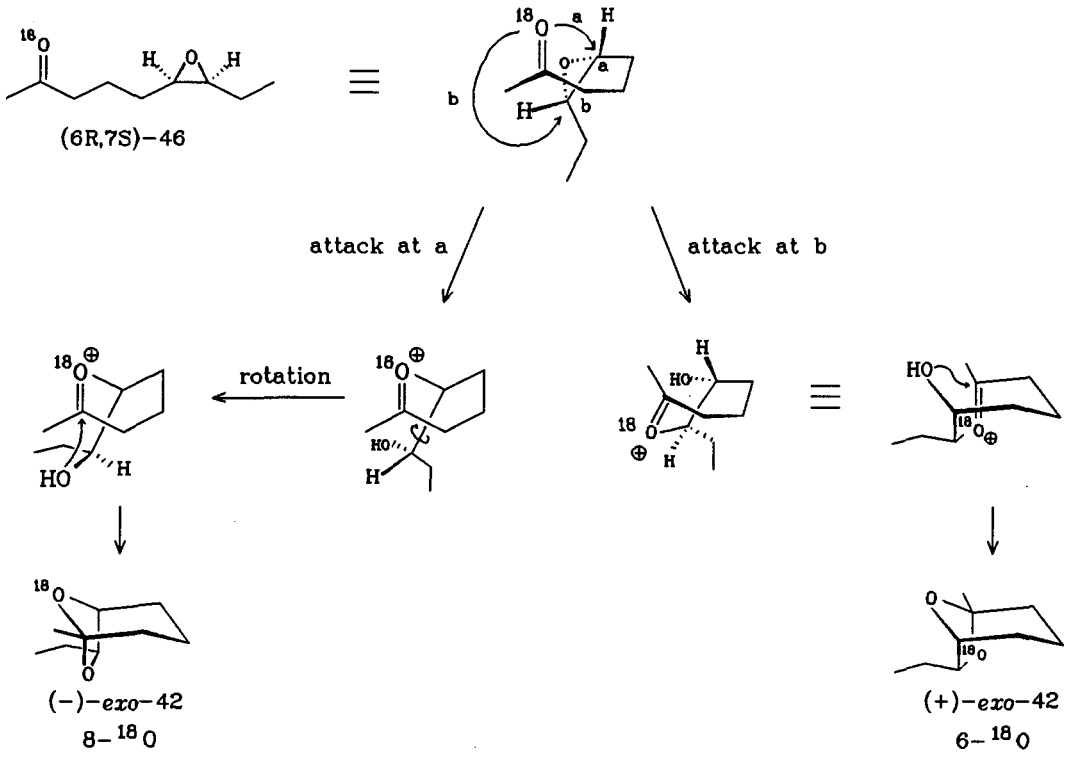


Figure 39: Stereochemical outcome of the cyclization of [2-^{18}O]-(6*R*,7*S*)-epoxynonan-2-one, **46**, to *exo*-brevicomin, **42**.

In a similar fashion, one can derive the identity of the products formed through the cyclization of the keto-diol **47**. All epoxide hydrolases studied to date catalyze the *trans* addition of water across the oxirane ring, with inversion of configuration at the oxirane carbon being attacked (Armstrong, 1987; Meijer and DePierre, 1988). To my knowledge, the *cis* addition of water has never been reported. Thus only the *threo* isomers of **47** will be considered in this discussion. There are two *threo* enantiomers of 2-keto-6,7-nonandiol. Each enantiomer could cyclize in one of two ways (by attack by one or the other of the alcohol groups, as shown in Figure 40 for one enantiomer).

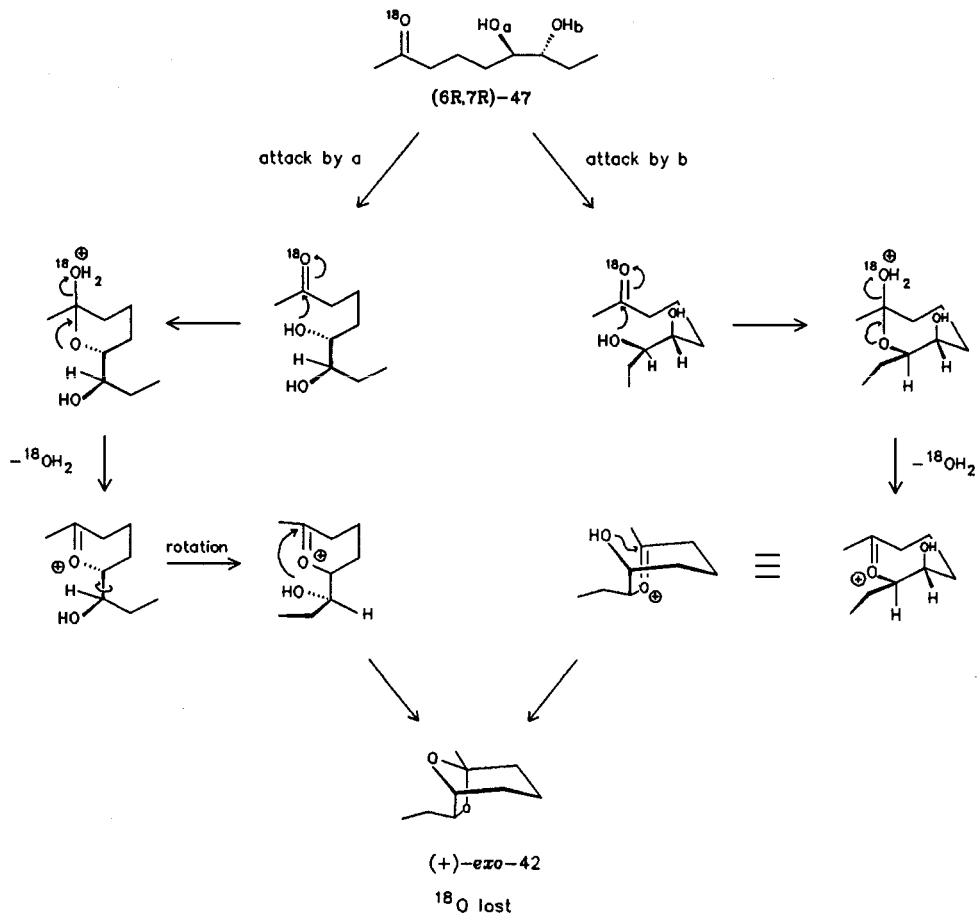


Figure 40: Stereochemical outcome of the cyclization of [2-^{18}O]-2-keto-(6*R*,7*R*)-nonandiol, **47**, to *exo*-brevicomin, **42**.

If the two keto-diol enantiomers are derivatized at one of the alcohols prior to cylization, the cyclization could proceed with expulsion of the leaving group. This is shown in Figure 41 for derivative **49**.

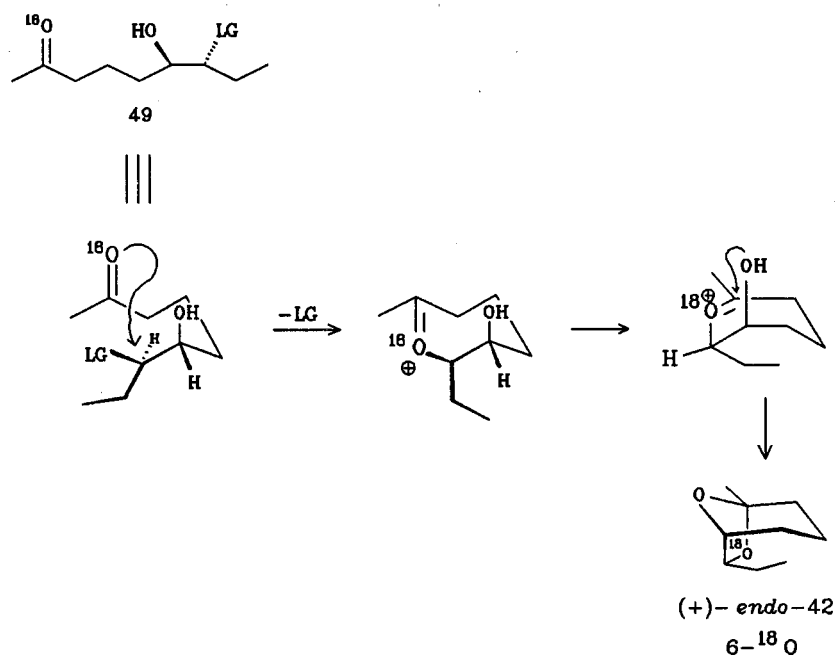
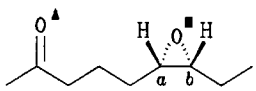
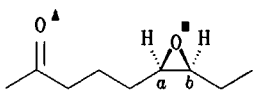
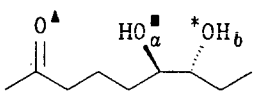
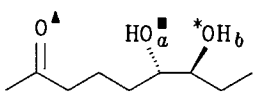


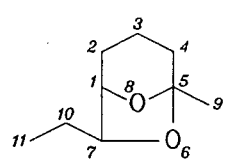
Figure 41: Stereochemical outcome of the cyclization of **49**, a putative biosynthetic derivative of ^{18}O-(*Z*)-6-nonen-2-one, to *endo*-brevicomin, **42**. (LG = "leaving group").

Summarized in Table 22 are the possible intermediates ultimately involved in the conversion of (*Z*)-6-nonen-2-one to brevicomin, the stereochemical outcome of the reactions, and the fate of each oxygen atom in each intermediate. Only mechanisms leading to the formation of *exo*- or *endo*-brevicomin are considered in the Table. Since the keto-diol derivatives **47** produce the same product regardless of which alcohol group is drawn to attack "first" (Figure 40), the two mechanisms are not differentiated.

Table 22: Isomers of brevicomin formed (and fate of oxygens) through the cyclization of putative biosynthetic derivatives of (*Z*)-6-nonen-2-one.

Intermediate	Mechanism	#	Brevicomin product			
			isomer	O position[1] 6	8	lost
	attack at *a*	*1*	(+)-*exo*	■	▲	
	attack at *b*	*2*	(−)-*exo*	▲	■	
	attack at *a*	*3*	(−)-*exo*	■	▲	
	attack at *b*	*4*	(+)-*exo*	▲	■	
	through diol	*5*	(+)-*exo*	*	■	▲
	derivatized at *a*	*6*	(−)-*endo*	*	▲	■
	derivatized at *b*	*7*	(+)-*endo*	▲	■	*
	through diol	*8*	(−)-*exo*	*	■	▲
	derivatized at *a*	*9*	(+)-*endo*	*	▲	■
	derivatized at *b*	*10*	(−)-*endo*	▲	■	*

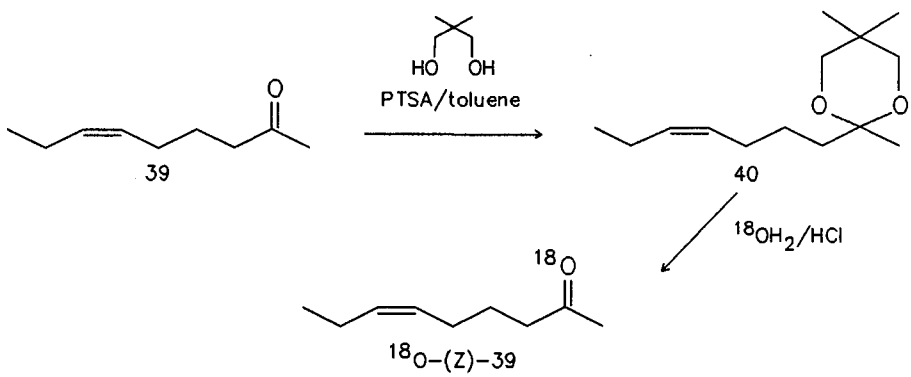
[1] The numbering scheme is as shown:



Since MPB was found to produce (+)-*exo*-brevicomin, mechanisms leading to the other isomers may be ruled out immediately. Thus, all mechanisms involving cyclization of the derivatized keto-diol intermediates **48** and **49** (mechanisms #6, 7, 9 and 10, Table 22) may be eliminated from consideration since they all lead to *endo*-brevicomin. Similarly, mechanisms #2, 3 and 8 may be ruled out since they lead to the production of (-)-*exo*-brevicomin. Only mechanisms #1, 4 and 5, which lead to (+)-*exo*-brevicomin, may be considered as viable options. As summarized in Table 22, these mechanisms can be distinguished if one oxygen of the precursor is labelled as ^{18}O, by the stereochemistry and the ^{18}O-labelling pattern of the brevicomin product. Thus MPBs were exposed to ^{18}O-(*Z*)-6-nonen-2-one and the fate of the ^{18}O label in the final (+)-*exo*-brevicomin product was examined.

Synthesis of ^{18}O-(*Z*)-6-nonen-2-one

The ^{18}O-label was introduced into (*Z*)-6-nonen-2-one (**40**) with acid in the presence of ^{18}O-water, as summarized in Scheme 8. The ^{18}O-(*Z*)-6-nonen-2-one was synthesized in 25% overall yield (98% pure by GC analysis; 95% ^{18}O by GC/SIM-MS analysis).



Scheme 8: Synthetic route to ^{18}O-(*Z*)-6-nonen-2-one, ^{18}O-(*Z*)-**39**.

Incorporation of ^{18}O-(*Z*)-6-nonen-2-one *in vivo*

Male MPBs were exposed to vapors of ^{18}O-(*Z*)-6-nonen-2-one, and the incorporation of ^{18}O was determined by GC/SIM-MS analysis. Inspection of the results in Table 23 reveals that the (+)-*exo*-brevicomin produced by MPB exposed to ^{18}O-(*Z*)-6-nonen-2-one was not enriched in ^{18}O.

Table 23: Enhancement of the $(M+2)^+$ peak in *exo*-brevicomin isolated from male MPB exposed (or not exposed) to ^{18}O-(*Z*)-6-nonen-2-one.

Treatment[1]	Relative abundance ($\bar{x}$ ± SE) M^+	$(M+2)^+$
Unexposed (4)	100.0	1.0 ± 0.04
Exposed (5)	100.0	0.9 ± 0.2

[1] Each sample was prepared from 1 beetle.

The lack of ^{18}O incorporation would seem to indicate that the cyclization proceeds with loss of the carbonyl oxygen. If so, the conversion of (*Z*)-6-nonen-2-one to (+)-*exo*-brevicomin likely involves a keto-diol intermediate (Table 22). However, it is possible that the ^{18}O label was simply washed out before it was incorporated into *exo*-brevicomin. Carbonyl oxygens are known to undergo acid-catalyzed exchange with the oxygens of water. Evidence that this process occurred in this system is that the (*Z*)-6-nonen-2-one isolated from the insects after the 24 hour exposure to ^{18}O-(*Z*)-6-nonen-2-one no longer had detectable levels of ^{18}O. The corresponding alcohol recovered from the beetles was only about 38% enriched for ^{18}O. If the conversion of the precursor to *exo*-brevicomin occurred on a time scale comparable to that of the reduction of the precursor to the alcohol, it would seem that the *exo*-brevicomin should have been significantly enriched in ^{18}O, unless the conversion had

proceeded through a keto-diol intermediate. Alternatively, the label may have washed out before the precursor was converted to *exo*-brevicomin.

Alternative approach to the investigation of the reaction mechanism

An alternate approach to the study of the conversion of (*Z*)-6-nonen-2-one to (+)-*exo*-brevicomin is to study the incorporation of ^{18}O supplied by $^{18}O_2$ or $^{18}OH_2$. If the results obtained in the study of the incorporation of ^{18}O-(*Z*)-2-nonen-6-one are not misleading, and the cyclization does proceed through a keto-diol intermediate with loss of the carbonyl carbon, then one of the oxygens of *exo*-brevicomin should be derived from molecular oxygen (which is introduced when the double bond is epoxidized), while the other oxygen should be derived from water (when the epoxide is opened up to form the diol). Thus, *exo*-brevicomin synthesized in the presence of $^{18}O_2$, should ultimately contain one ^{18}O-labelled oxygen (Figure 42).

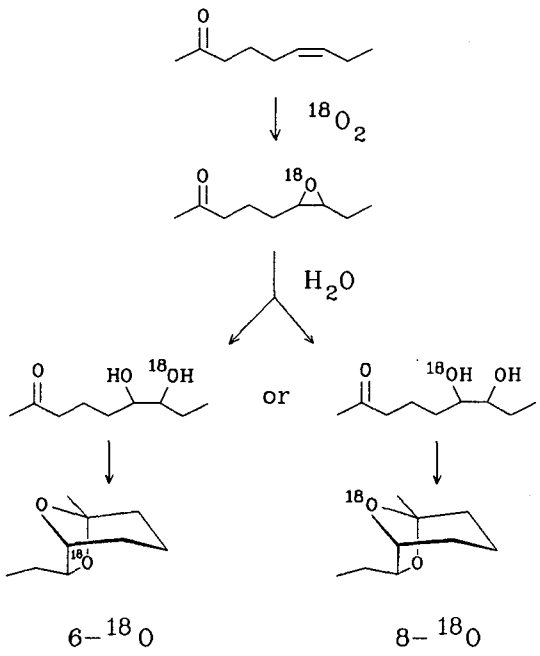


Figure 42: Expected ^{18}O-labelling pattern in (+)-*exo*-brevicomin produced from 6-nonen-2-one *in vivo* in the presence of $^{18}O_2$ (if the conversion proceeds through a keto-diol intermediate).

Similarly, in the presence of $^{18}OH_2$, only one oxygen (introduced when the epoxide is opened up) should be labelled (Figure 43). In this case, the carbonyl oxygen would probably also exchange with the water and could become ^{18}O-labelled. However, if the cyclization proceeds through a keto-diol intermediate, the carbonyl oxygen would be lost and thus would not serve as a source of ^{18}O label in the final product.

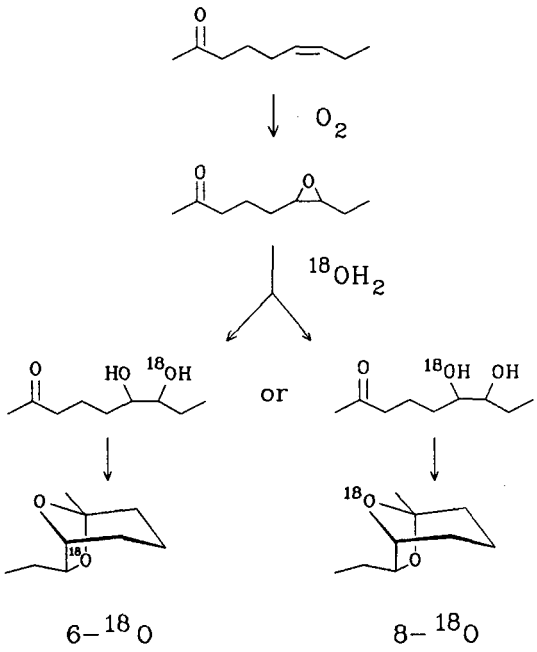


Figure 43: Expected ^{18}O-labelling pattern in (+)-*exo*-brevicomin produced from 6-nonen-2-one *in vivo* in the presence of $^{18}OH_2$ (if the conversion proceeds through a keto-diol intermediate).

Incorporation of $^{18}O_2$ and $^{18}OH_2$ *in vivo*

Exo-brevicomin synthesized by male MPBs in the presence of $^{18}O_2$ (but not exposed to the precursor (*Z*)-6-nonen-2-one) was significantly enriched in ^{18}O (Table 24). After 24 hours exposure to $^{18}O_2$, roughly one fifth of the *exo*-brevicomin molecules were enriched for two atoms of ^{18}O. This proportion increased to over half by 48 hours. This clearly indicates that both oxygens of *exo*-brevicomin were derived from molecular oxygen. This prohibits cyclization through a keto-diol intermediate (mechanism #5,

Table 24: Incorporation of ^{18}O into *exo*-brevicomin[1] by male and female MPBs exposed to $^{18}O_2$.

Treatment[2]	Relative abundance ($\bar{x}$ ± SE) M^+	$(M+2)^+$	$(M+4)^+$
$^{16}O_2$			
males, 24 h (2)	100.0	0.7 ± 0.1	n.d.
$^{18}O_2$			
males, 24 h (4)	100.0	66 ± 12	43 ± 13
48 h (1)	100.0	262.3	463.1
$^{18}O_2$ + (*Z*)-6-nonen-2-one			
males, 24 h (7)	100.0	307 ± 15	27 ± 2
females, 24 h (5)	100.0	278 ± 70	2 ± 1

[1] The (*Z*)-6-nonen-2-one and (*Z*)-6-nonen-2-ol isolated from the beetles were not enriched in ^{18}O; [2] each sample was prepared from 1-10 beetles.

Table 22): cyclization must proceed through a keto-epoxide intermediate (mechanism # 1 or 4, Table 22; see also Figure 42).

A relatively high proportion of the *exo*-brevicomin synthesized by MPBs in the presence of $^{18}O_2$ was enriched in one ^{18}O (Table 24). This indicates that both oxygen atoms were not derived from the same molecule of oxygen. (The $^{18}O_2$ gas used was >97% ^{18}O). The oxygens must have been introduced singly. *Exo*-brevicomin that was singly-enriched for ^{18}O may have been synthesized from precursor that already had one (unlabelled) oxygen inserted before the experiment was initiated; or may have been synthesized using one ^{18}O and one of the unlabelled oxygens that were unvoidably present during the experiment.

Relatively little of the (+)-*exo*-brevicomin synthesized by male and female MPBs exposed to both $^{18}O_2$ and vapors of (*Z*)-6-nonen-2-one was doubly

enriched with ^{18}O* (Table 24). This is consistent with cyclization proceeding through a keto-epoxide intermediate, since one oxygen was already present in the (*Z*)-6-nonen-2-one. This (unlabelled) oxygen would have been retained if cyclization proceeded through a keto-epoxide intermediate.

Exo-brevicomin synthesized by male MPB [not exposed to (*Z*)-6-nonen-2-one] in the presence of $^{18}OH_2$ was enriched for one ^{18}O atom/molecule of *exo*-brevicomin (Table 25). This result would initially appear to contradict the results of the $^{18}O_2$-labelling experiment, which indicated that both oxygens were derived from molecular oxygen. However as discussed previously, carbonyl oxygens can exchange with the oxygen atoms of water. Thus, the ^{18}O may have been introduced through exchange with endogenous precursor. The *exo*-brevicomin synthesized by male MPBs in the presence of both $^{18}OH_2$ and (*Z*)-6-nonen-2-one was greatly enriched in one ^{18}O atom/molecule of brevicomin. The (*Z*)-6-nonen-2-one and (*Z*)-6-nonen-2-ol that were isolated from the beetles were significantly enriched in ^{18}O (EFs = 50% and 38%, respectively). These observations support the hypothesis that the introduction of label into the *exo*-brevicomin was *via* label exchange into the carbonyl oxygen, and not through cyclization of a keto-diol intermediate. It is noteworthy that, even in one case in which the *exo*-brevicomin was 51% enriched in one ^{18}O, there was no detectable enrichment for two ^{18}O atoms/molecule of *exo*-brevicomin.

---

* The $(M+4)^+$ peak of the *exo*-brevicomin produced by the females is 0.7 ± 0.5% the abundance of the $(M+2)^+$ peak. This is largely due to the natural abundance of heavy isotopes in *exo*-brevicomin molecules singly enriched for ^{18}O, rather than enrichment for two ^{18}O atoms/molecule of *exo*-brevicomin.

Table 25: Incorporation of ^{18}O into *exo*-brevicomin by male and female MPBs exposed to $^{18}OH_2$ for 24 h: relative abundance of peaks of interest (GC/SIM-MS).

Treatment[1]	Relative abundance ($\bar{x}$ ± SE) M^+	$(M+2)^+$	$(M+4)^+$
$^{16}OH_2$, males (2)	100.0	0.7 ± 0.1	n.d.
$^{18}OH_2$, males (2)	100.0	17 ± 2	n.d.
$^{18}OH_2$ + (*Z*)-6-nonen-2-one			
males (3)	100.0	65 ± 35	n.d.
females (1)	100.0	25	n.d.

[1] Each sample was usually prepared from 5 beetles.

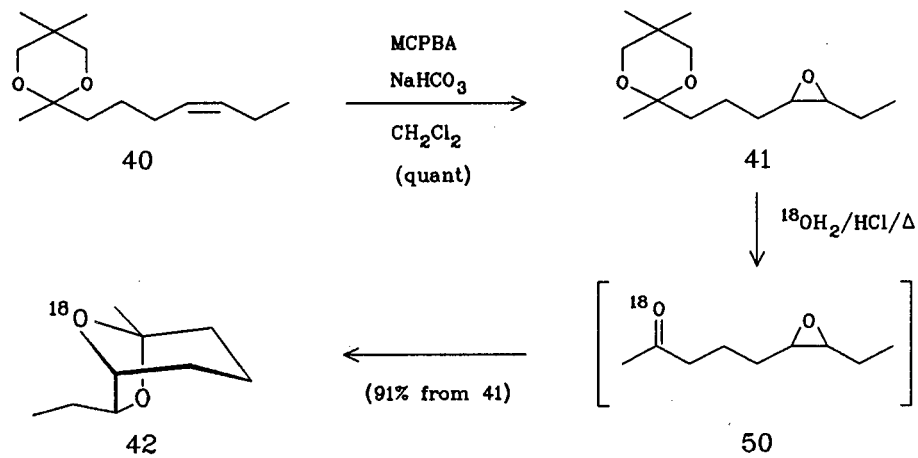
c) Stereochemistry of the cyclization of the keto-epoxide intermediate.

As shown in Table 22, it is possible to determine both the isomer of keto-epoxide intermediate cyclized and the site of epoxide attack, by examination of the position of the ^{18}O label in the (+)-*exo*-brevicomin product. The identity of the oxygen of brevicomin labelled with ^{18}O can be discerned through examination of the MS fragmentation pattern. Accordingly, [8-^{18}O]-*exo*-brevicomin was synthesized and its MS fragmentation pattern was compared to that of unlabelled *exo*-brevicomin. The fragments most suitable for analysis were then determined.

Synthesis of [8-^{18}O]-exo-brevicomin

The ^{18}O-labelled *exo*-brevicomin was synthesized according to Scheme 9. Ketal-epoxide **41** was prepared through the epoxidation of ketal-alkene **40** under alkaline conditions, using the method of Anderson and Veysoglu (1973). Hydrolysis of the ketal in the presence of $^{18}OH_2$ then gave the ^{18}O-keto epoxide intermediate **50**. To prevent loss of the ^{18}O label this intermediate was not isolated, but rather was cyclized directly

by treatment with heat and acid. The MS (EI), ^{1}H and ^{13}C NMR spectra of the product **42** were in agreement with those previously published for *exo*-brevicomin (Gore *et al.*, 1976; Silverstein *et al.*, 1968 and Pearce *et al*, 1977, respectively). The product was 96% enriched for ^{18}O as judged by GC/SIM-MS analysis.



Scheme 9: Synthetic route to ^{18}O-*exo*-brevicomin, **42**.

The ^{18}O label in the *exo*-brevicomin was unambiguously determined to be in position 8 by ^{13}C NMR spectroscopy. The resonances of carbons α to ^{18}O exhibit an upfield shift, on the order of about 0.010 to 0.035 ppm for singly bonded oxygen atoms (Vederas, 1987). This small difference is difficult to detect by comparison of the ^{13}C NMR spectra of labelled and unlabelled compounds. For this reason, synthetic samples of unlabelled- and ^{18}O-*exo*-brevicomin were mixed in a 2:1 ratio (respectively). The ^{13}C NMR absorptions of the carbons of interest [*i.e.*, those α to the two oxygens, assigned according to Pearce *et al* (1977)] in the 2:1 mix are represented in Figure 44. The absorption due to C-7 was not shifted in the ^{18}O-*exo*-brevicomin. However, those of C-1 and C-5 were shifted upfield 0.03 and 0.04 ppm, respectively. Thus the latter carbons must be α to the ^{18}O, which must be in position 8.

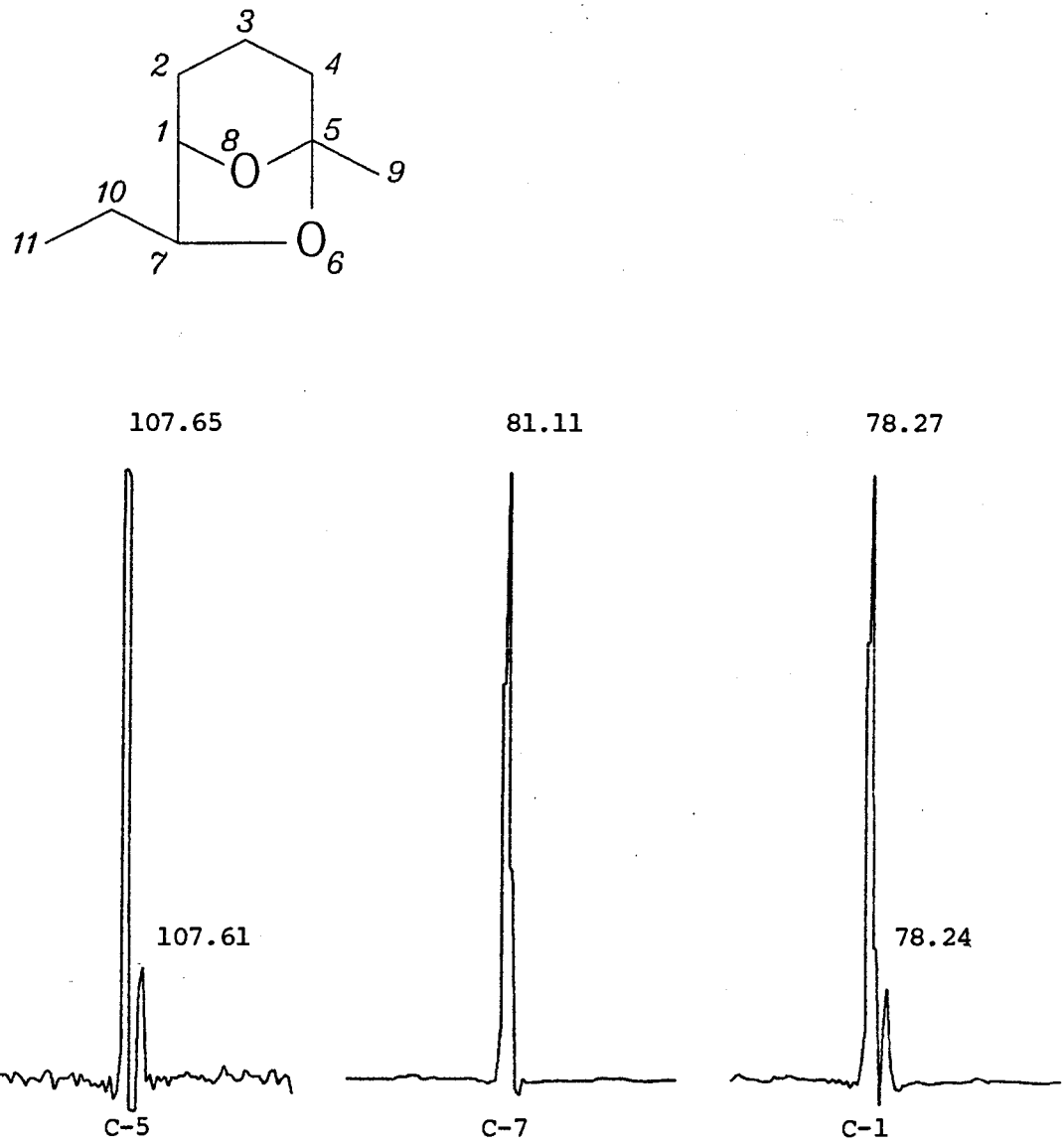


Figure 44: ^{13}C NMR resonances of C-1, C-5 and C-7 of unlabelled and ^{18}O-exo-brevicomin, mixed in a 2:1 ratio (respectively).

Comparison of MS (EI) fragmentation patterns of unlabelled and of [8-^{18}O]-*exo*-brevicomin:

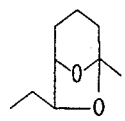
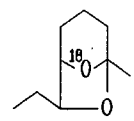
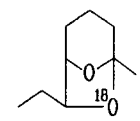
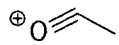
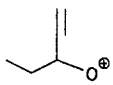
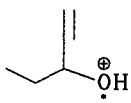
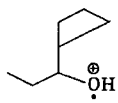
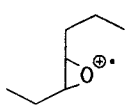
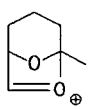
Comparisons of the MS (EI) of [8-^{18}O]-*exo*-brevicomin to that of unlabelled *exo*-brevicomin are summarized in Tables 26 and 27. Table 26 compares the observed relative abundances of the major MS (EI) fragments in the two compounds (the actual MS (EI) of unlabelled and ^{18}O-*exo*-brevicomin are presented in Figure 45). Table 27 summarizes the identity of the fragments and their expected m/e values in the two compounds (based on mechanistic information in Gore *et al.*, 1976).

The usefulness of the major MS (EI) fragments for determination of the position of the ^{18}O label in the *exo*-brevicomin synthesized by MPB was evaluated by comparison of the expected fragmentations of the unlabelled and ^{18}O-labelled analogues to those observed (Table 27).

Table 26: Comparison of relative abundances of important MS (EI) fragments in unlabelled- and [8-^{18}O]-*exo*-brevicomin.

Fragments	ratio of relative abundances of fragments in brevicomins	
F^+ / $(F+2)^+$	unlabelled	[8-^{18}O]
43 / 45	100.0 / n.d.	68.1 / 100.0
114 / 116	100.0 / n.d.	100.0 / 21.8
86 / 88	100.0 / n.d.	83.0 / 100.0
85 / 87	100.0 / 14.8	100.0 / 16.6
98 / 100	100.0 / 3.9	24.2 / 100.0
127 / 129	100.0 / 29.7	2.8 / 100.0

Table 27: Major fragment ions by MS (EI) of unlabelled and ^{18}O-*exo*-brevicomin.

Fragment	Expected m/e of fragments 	 [8-^{18}O]	 [6-^{18}O]	Comments
	43	45	43	not clearcut: there is a large m/e=43 in [8-^{18}O]-*exo*-brevicomin
	85	85	87	
	86	86	88	not clearcut: there is a large m/e=88 in [8-^{18}O]-*exo*-brevicomin
	98	100	98	
	114	114	116	
	114	114	116	
	127	129	129	not useful: both oxygens are retained

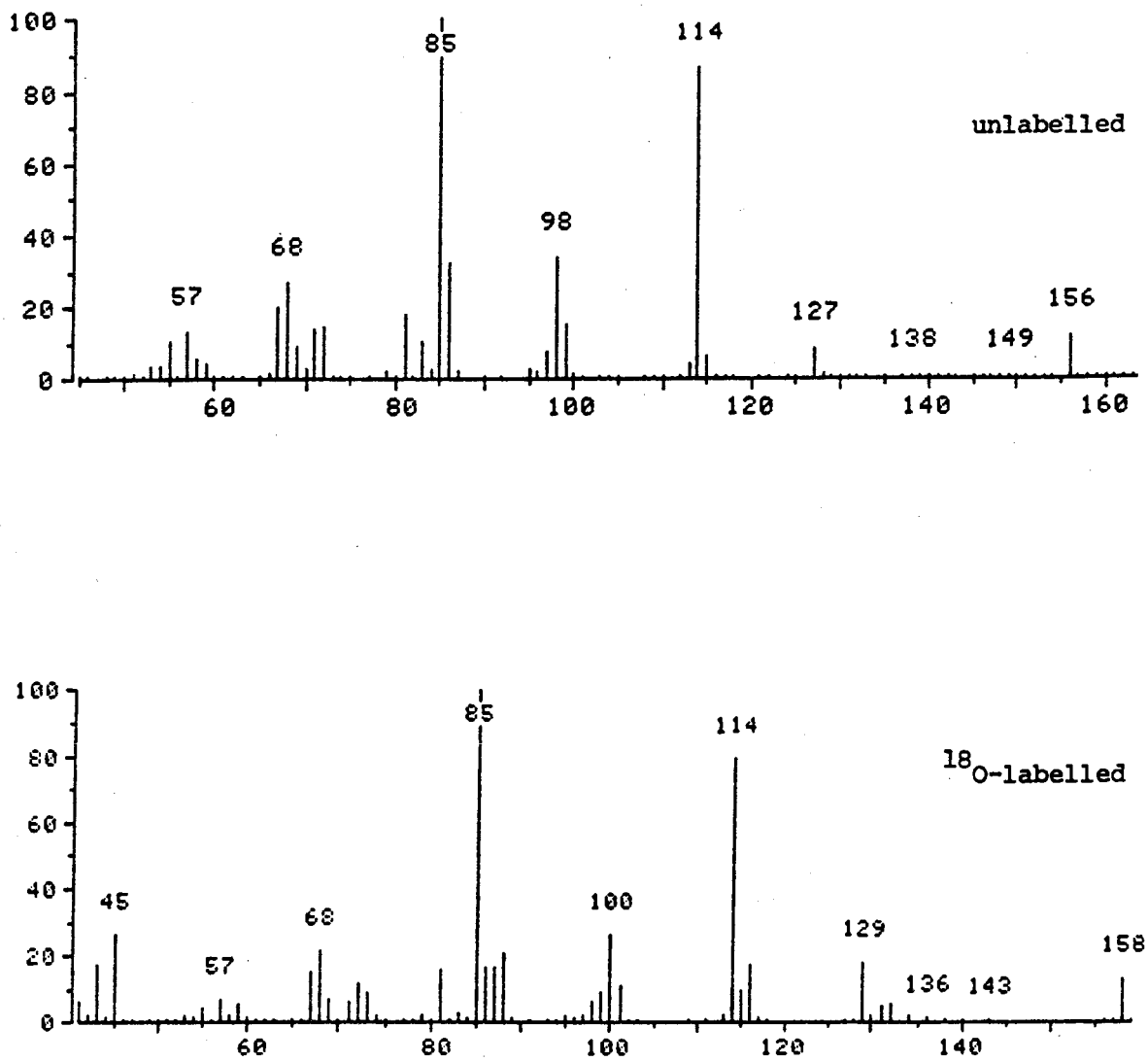


Figure 45: MS (EI) of unlabelled and [8-^{18}O-]-*exo*-brevicomin.

None of the fragmentations could be interpreted completely unambiguously. Fragments of m/e=98, 85, and 114 (in unlabelled *exo*-brevicomin) looked most promising, and were therefore selected for further study.

Choice of fragments for GC/SIM-MS analyses

Male and female MPBs were exposed to $^{18}O_2$ and, in some cases, the precursor (*Z*)-6-nonen-2-one. The relative abundance of the fragments of interest are presented in Table 28. By way of example, the interpretation of the data for one of the samples will be discussed in the following paragraphs. Those of the other samples are summarized in Table 29.

Exo-brevicomin produced by the male MPB exposed to $^{18}O_2$, sample **A** (Table 28), was 18% enriched for two ^{18}O atoms per molecule of *exo*-brevicomin, 34% enriched for one ^{18}O atom, and 48% unenriched. The relative abundances of the m/e = 114 and 116 fragments indicate that this sample was 36% enhanced for *exo*-brevicomin molecules containing ^{18}O in position 6 (this includes [6-^{18}O]- and [6,8-$^{18}O_2$]-*exo*-brevicomin). Since the relative abundance of the m/e=160 peak indicated that this sample was 18% (36% minus 18%) enriched for the doubly labelled brevicomin, this implies that about 18% was singly labelled in position 6. (Note: this is only a rough estimate, since it was shown in Table 26 that [8-^{18}O]-*exo*-brevicomin exhibits an m/e=116 fragment of about 22% the relative abundance of the m/e=114 fragment). Furthermore, since the relative abundance of the m/e=158 fragment indicated that the sample was 34% enriched for one atom of ^{18}O, of which about 18% was in position 6, the sample must be about 16% [8-^{18}O]-*exo*-brevicomin.

Using similar reasoning, the relative abundances of the fragments of m/e=98 and 100 indicated that this sample was at least 32% enriched for ^{18}O in position 8. (The sample likely contained more [8-^{18}O]-, since a portion of the relative abundance of the m/e=98 fragment is also due to [8-^{18}O]-exo-brevicomin). Since about 18% of the [8-^{18}O]- was due to doubly

Table 28: Relative abundance of fragments of interest [GC/SIM-MS (EI)] of *exo*-brevicomin (**42**) (synthetic or isolated from male or female MPB[1]).

Sample	relative abundances of fragment groups[2]			
	$F^+/(F+2)^+$			$M^+/(M+2)^+/(M+4)^+$
	85/87	98/100	114/116	156/158/160
exo-**42**	100.0/14.8	100.0/3.9	100.0/n.d.	100/n.d./n.d.
[8-^{18}O]-*exo*-**42**	100.0/16.6	24.2/100	100.0/21.8	4.4/100/n.d.
male MPB + $^{18}O_2$				
A	100/64	100/53	100/57	100/70/37
B	100/23	100/23	100/24	100/24/20
C	100/58	100/54	100/56	100/70/46
D	100/49	100/39	100/59	100/41/41
male MPB + $^{18}O_2$ + (*Z*)-**39**				
A	83/100	100/37	93/100	53/100/12
B	88/100	100/37	95/100	44/100/13
C	100/99	100/35	100/94	46/100/11
D	92/100	100/39	100/100	38/100/9
female MPB + $^{18}O_2$ + (*Z*)-**39**				
A	100/38	100/91	100/37	42/100/n.d.
B	100/41	100/79	100/36	53/100/n.d.
C	100/32	100/91	100/36	36/100/n.d.
D	100/34	97/100	100/28	43/100/n.d.

[1] Beetles were exposed to (*Z*)-6-nonen-2-one, **39**, and/or $^{18}O_2$ for 16 hours. Letters (**A** through **D**) represent different samples (replicates), each prepared from 10 beetles; [2] the abundance of each fragment shown is relative to the others in the group.

labelled brevicomin, the remainder (about 14%) was due to brevicomin molecules singly labelled in position 8. This indicates that the sample was about 20% [6-^{18}O]-*exo*-brevicomin.

The relative abundances of the m/e=85 and 87 fragments indicate that the sample was about 30% enriced for [6-^{18}O]-*exo*-brevicomin. By arguments similar to those above, this indicates that the sample was about 12% enriched for singly labelled [8-^{18}O]-*exo*-brevicomin (and was thus 22% [6-^{18}O]-*exo*-brevicomin).

These numbers for the ratios of [6-^{18}O]- as compared to [8-^{18}O]-*exo*-brevicomin (namely, 18/16, 20/14, and 22/12) are only estimates. However, they are in fair agreement and are useful to indicate trends. All numbers indicate that the *exo*-brevicomin in sample **A** contained both [6-^{18}O]- and [8-^{18}O]-*exo*-brevicomin in roughly equal quantities (with slightly more of the former).

In order to maximize sensitivity, it is desirable to scan the least possible numbers of ions during GC/SIM-MS analyses. It was clearly necessary to determine the relative abundance of the ions of m/e = M^+, $(M+2)^+$ and $(M+4)^+$ in order to determine how many atoms of ^{18}O were incorporated per molecule of *exo*-brevicomin. However it did not seem necessary to scan all three pairs of fragment ions. I decided to choose one of the three pairs to scan in future experiments. The m/e=85/87 pair was the first that I eliminated, since the $(F+1)^+$ peak of m/e=86 (which is about 10% of the relative abundance of the F^+, due to the natural abundance of heavy isotopes) might affect the abundance of the m/e=87 peak in complicated ways. In order to determine which of the remaining pairs (m/e=98/100 or 114/116) should be eliminated, the results obtained from calculations using the relative abundances (obtained by GC/SIM-MS) of each were compared. In these calculations, it was attempted to correct for the fact that the fragmentations did not appear to go cleanly as expected (for example, [8-^{18}O]-*exo*-brevicomin exhibited an m/e=116 peak that was about 22% the abundance of the m/e=114 peak). Samples of these calculations are

supplied in Appendix I; the results are presented in Table 29. Those obtained using the m/e=98/100 pair seemed to agree best with the overall result obtained by the reasoning process previously described. The results using the relative abundances of the m/e=114/116 pair appeared to become skewed in some cases (most notably, in the *exo*-brevicomin samples isolated from male MPBs exposed to $^{18}O_2$). Therefore m/e=98/100 pair was selected for future studies.

Table 29: Ratios of unlabelled, [6-^{18}O]-, [8-^{18}O]-, and [6,8-$^{18}O_2$]-*exo*-brevicomin produced by MPBs as calculated from results of GC/SIM-MS: ratios calculated from both the 98/100 and the 114/116 pairs. (Original data presented in Table 28).

	Ratios of isomers of *exo*-brevicomin (%)							
	98/100				114/116			
Sample[1]	^{16}O	6-^{18}O	8-^{18}O	6,8-$^{18}O_2$	^{16}O	6-^{18}O	8-^{18}O	6,8-$^{18}O_2$
male MPB + $^{18}O_2$								
A	49	18	15	18	49	7	26	18
B	74	9	6	12	74	0	15	12
C	53	18	9	20	53	3	24	20
D	55	19	4	22	55	0	24	22
male MPB + $^{18}O_2$ + (*Z*)-**39**								
A	30	47	17	5.2	30	42	23	5.2
B	27	50	18	4.7	27	40	29	4.7
C	31	43	19	6.3	31	35	28	6.3
D	26	47	20	6.6	26	37	30	6.6
female MPB + $^{18}O_2$ + (*Z*)-**39**								
A	34	10	56	n.d.	34	15	51	n.d.
B	31	22	47	n.d.	31	12	57	n.d.
C	24	28	48	n.d.	24	11	65	n.d.
D	38	4.7	58	n.d.	38	8.4	54	n.d.

[1] Letters (**A** through **D**) represent different samples (replicates), each prepared from 10 beetles.

Incorporation of ^{18}O from $^{18}O_2$ into *exo*-brevicomin: ratios of production of [6-^{18}O]- to [8-^{18}O]-*exo*-brevicomin and implications for the mechanism of formation

Male MPBs exposed to $^{18}O_2$ produced *exo*-brevicomin that was singly labelled with oxygen in both the 6- and the 8- positions (Table 29). Since the proportions of the *exo*-brevicomin that were unlabelled and doubly labelled with $^{18}O_2$ are not relevant to this discussion, the data from Table 29 are re-presented in Table 30, showing only the ratios of [6-^{18}O]- to [8-^{18}O]-*exo*-brevicomin (Trial 1, Table 30). Similar results were obtained when this experiment was repeated (Trial 2, Table 30).

When the beetles were exposed to both $^{18}O_2$ and (*Z*)-6-nonen-2-one, the ^{18}O label was incorporated mainly *via* the epoxide oxygen (since the keto-oxygen was already in place, see Figure 41). According to Table 22, incorporation of label into both positions 6 and 8 of *exo*-brevicomin indicates that cyclization proceeds through both enantiomers of 6,7-epoxynonan-2-one, **46**, according to the mechanisms shown in Figure 46.

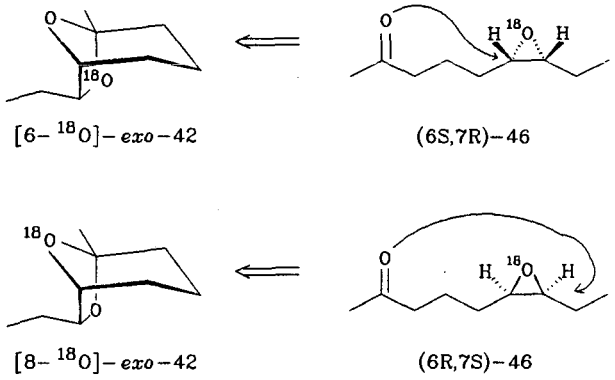


Figure 46: Production of (+)-*exo*-brevicomin from (6*S*,7*R*)- and (6*R*,7*S*)-epoxynonan-2-one, **46**, labelled with ^{18}O from $^{18}O_2$.

Cyclization through the (6*R*,7*S*)-epoxide appears to be favored in males. Interestingly, the opposite mechanism of cyclization appears to be dominant in the females: cyclization appears to proceed largely through the (6*S*,7*R*)-epoxide (Table 30).

Table 30: Incorporation of ^{18}O from $^{18}O_2$ into *exo*-brevicomin by male and female MPB exposed (or not) to (*Z*)-6-nonen-2-one, (*Z*)-**39**: ratio of [6-^{18}O]- to [8-^{18}O]-*exo*-brevicomin. (Results of two experiments, labelled Trials 1 and 2[1]).

Sample	Ratios ($\bar{x}$ ± SE) [6-^{18}O]-	[8-^{18}O]-
Trial 1[2]:		
male MPB + $^{18}O_2$ (4)	2.4 ± 0.7	1.0
male MPB + $^{18}O_2$ + (*Z*)-**39** (4)	2.5 ± 0.1	1.0
female MPB + $^{18}O_2$ + (*Z*)-**39** (4)	1.0	5.4 ± 2.1
Trial 2[3]:		
male MPB + $^{18}O_2$ (2)	1.6 ± 0.0	1.0
male MPB + $^{18}O_2$ + (*Z*)-**39** (2)	1.5 ± 0.2	1.0
female MPB + $^{18}O_2$ + (*Z*)-**39** (2)	1.0	2.0 ± 0.3
Trial 2 (single beetles)[4]:		
male MPB + $^{18}O_2$ (2)	3.6 ± 1.1	1.0
male MPB + $^{18}O_2$ + (*Z*)-**39** (5)	2.6 ± 0.3	1.0
female MPB + $^{18}O_2$ + (*Z*)-**39** (3)	1.0	3.5 ± 0.6

[1] Raw data from Trials 2 and 3 presented in Table 40, *Appendix II*; [2] each sample prepared from 10 beetles; [3] each sample prepared from 20, 5, or 10 beetles, respectively; [4] each sample prepared from 1 beetle.

All of the samples discussed so far were prepared from several beetles. In order to determine if some individuals synthesize the *exo*-brevicomin by one route, while other beetles use the other route, the *exo*-brevicomin extracted from single beetles exposed to $^{18}O_2$ was analyzed. Both [6-^{18}O]- and [8-^{18}O]-*exo*-brevicomin were found in the extracts of single beetles (Table 30).

The observation that cyclization proceeds through both routes even in males that were not exposed to vapors of (*Z*)-6-nonen-2-one argues

against the result being an artifact. The fact that the trend was not altered by the presence of (*Z*)-6-nonen-2-one is yet another indication that this is a biologically relevant intermediate in *exo*-brevicomin synthesis.

Incorporation of ^{18}O from $^{18}OH_2$ into *exo*-brevicomin: ratios of production of [6-^{18}O]- to [8-^{18}O]-*exo*-brevicomin and implications for the mechanism of formation

When MPB was exposed to $^{18}OH_2$, the labelled oxygen exchanged into the keto-oxygen of (*Z*)-6-nonen-2-one and was thus incorporated into the *exo*-brevicomin produced by the beetles (Table 25). This is equivalent to the experiment designed to determine the mechanism of the conversion of (*Z*)-6-nonen-2-one to *exo*-brevicomin. In that instance, no meaningful results were obtained since the ^{18}O label exchanged out of ^{18}O-(*Z*)-6-nonen-2-one, into the water. In this instance, the label exchanged into the originally unlabelled (*Z*)-6-nonen-2-one from the labelled water.

The *exo*-brevicomin produced by MPB exposed to $^{18}OH_2$ was labelled with ^{18}O in both positions (Table 31). Most of the label in *exo*-brevicomin produced by males was in position 8; in contrast, most of the label in that produced by the females was in position 6. These distribution trends are opposite to those in the $^{18}O_2$ experiment, as expected (Figure 47).

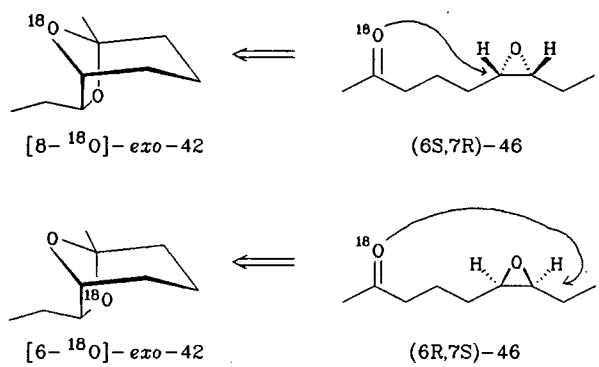


Figure 47: Production of (+)-*exo*-brevicomin from (6*S*,7*R*)- and (6*R*,7*S*)-epoxynonan-2-one, **46**, labelled with ^{18}O from $^{18}OH_2$.

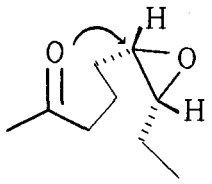
Table 31: Incorporation of ^{18}O from $^{18}OH_2$ into *exo*-brevicomin by MPB: ratio of [6-^{18}O]- to [8-^{18}O]-*exo*-brevicomin[1].

Sample	Ratios ($\bar{x}$ ± SE) [6-^{18}O]-	[8-^{18}O]-
male MPB + $^{18}OH_2$ (1)[2]	1.0	1.1
male MPB + $^{18}OH_2$ + (*Z*)-6-nonen-2-one (2)[3]	1.0	4.6 ± 0.5
female MPB + $^{18}OH_2$ + (*Z*)-6-nonen-2-one (1)[4]	1.8	1.0

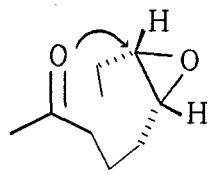
[1] Raw data presented in Table 41, *Appendix II*; [2] sample prepared from 6 beetles; [3] each sample prepared from 5 beetles; [4] sample prepared from 5 beetles.

Interpretation of results

The data presented in this study support the conclusion that, in MPB, both (6*S*,7*R*)- and (6*R*,7*S*)-epoxynonan-2-one cyclize to the (+)- isomer of *exo*-brevicomin, with the initial attack of the oxirane ring by the carbonyl oxygen proceeding as shown in Figures 45 and 46. This situation seems analogous to that reported by Prestwich *et al.* (1989) regarding the hydrolysis of disparlure, the epoxide pheromone of the gypsy moth, by enzymes located in the antennae of the male. The hydrolysis of both (+)- and (-)-disparlure led to the same (7*R*,8*R*)-*threo*-diol. As shown in Figure 48, the initial steps of the cyclizations of (6*S*,7*R*)- and (6*R*,7*S*)-epoxynonan-2-one could, similarly, be imagined to be catalyzed by a single enzyme. In the Figure, the active site of the enzyme is envisioned to lock the oxygens of the two isomers into the same relative positions. The hydrogens are pictured to rise out of the plane of the paper, while the hydrocarbon chains are envisioned to fold into a hydrophobic pocket in the enzyme below the plane. The carbonyl oxygens are envisioned to attack the carbons of the oxirane rings in the same relative position.



(6S,7R)-46



(6R,7S)-46

Figure 48: Example of possible stereochemistry of the initial steps in the cyclization of (6*S*,7*R*)- and (6*R*,7*S*)-epoxynonan-2-one, **46**, ultimately leading to (+)-*exo*-brevicomin.

The mechanisms shown in Figure 48 are the same as those in Figures 45 and 46, and thus lead to (+)-*exo*-brevicomin.

2. Biosynthesis of *endo*-brevicomin

The objective of this portion of the project was to determine if (*E*)-6-nonen-2-one would serve as a precursor of *endo*-brevicomin *in vivo*. The approach used was to expose insects to vapors of a deuterated analogue of (*E*)-6-nonen-2-one, namely [6,7-D_2]-(*E*)-6-nonen-2-one, and to determine if deuterium was incorporated into the *endo*-brevicomin produced by the beetles by GC/SIM-MS. Incorporation of the precursor into *endo*-brevicomin was expected to proceed as shown in Figure 49.

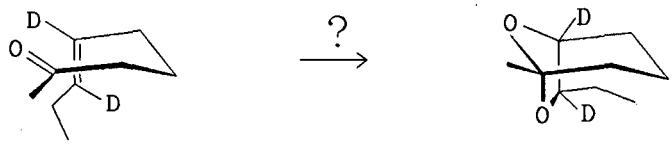
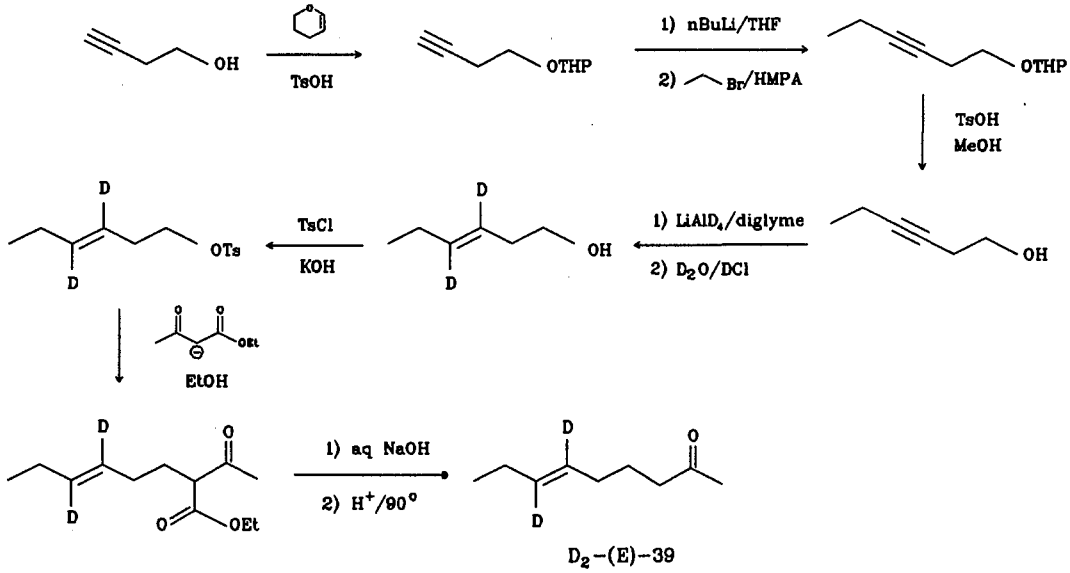


Figure 49: Expected pattern of deuterium incorporation into *endo*-brevicomin synthesized from the deuterated analogue of (*E*)-6-nonen-2-one.

a) Synthesis of labelled (*E*)-6-nonen-2-one

The [6,7-D_2]-(*E*)-6-nonen-2-one required for this study was kindly

synthesized by Dr. Mohan Singh* according to Scheme 10. The product was >99% pure by GC analysis and >98% enriched in two deuteriums as judged by GC/SIM-MS analysis.



Scheme 10: Synthetic route to [6,7-D_2]-(*E*)-6-nonen-2-one, D_2-(*E*)-39. (Designed and executed by Dr. Mohan Singh).

b) Incorporation of (*E*)-6-nonen-2-one into *endo*-brevicomin *in vivo*

The *endo*-brevicomin produced by both MPB and BBB in the presence of D_2-(*E*)-6-nonen-2-one was enriched for two deuteriums (Figure 50, Table 32). *Endo*-brevicomin production was significantly enhanced in male and female MPBs exposed to the precursor (Table 33). The chirality of the *endo*-brevicomin produced by the beetles was not determined. Analogous to the situation with (*Z*)-6-nonen-2-one, the extracts of both MPB and BBB exposed to (*E*)-6-nonen-2-one contained excess precursor [(*E*)-6-nonen-2-one] and the corresponding alcohol [(*E*)-6-nonen-2-ol]. The identities of these compounds were determined by comparison of the GC R.T.s and the GC/MS fragmentation patterns with those of authentic samples.

---

* Department of Chemistry, Simon Fraser University. Burnaby, BC, Canada. V5A 1S6.

Table 32: Enhancement of the $(M+2)^+$ peak in *endo*-brevicomin isolated from male BBB or MPB, exposed (or not exposed) to D_2-(*E*)-6-nonen-2-one.

Treatment	Relative abundance ($\bar{x}$ ± SE) M^+	$(M + 2)^+$
Exposed MPB (4)	n.d.	100.0
Exposed BBB (2)	0.6 ± 0.4	100.0

Table 33: *Endo*-brevicomin content in extracts of male and female MPB and BBB, exposed (or not exposed) to (*Z*)- or (*E*)-2-nonen-6-one for 24 hours.

Treatment	*Endo*-brevicomin[1] (ng/beetle, $\bar{x}$ ± SE) males	females
MPB, unexposed	7.3 ± 1.4 (8)[a]	n.d.
MPB, exposed to:		
(*Z*)-6-nonen-2-one	11 ± 5 (21)[a]	n.d.
(*E*)-6-nonen-2-one	33 ± 5 (19)[b]	4.2 ± 0.9 (11)[a]
BBB, unexposed	n.d.	n.d.
BBB, exposed to:		
(*Z*)-6-nonen-2-one	n.d.	n.d.
(*E*)-6-nonen-2-one	139 ± 76 (3)[a]	27.9 (1)[b]

[1] Numbers (within an experiment) that are not significantly different from one another ($P < 0.05$) have the same letter superscripts. Each sample was normally prepared from 3 beetles.

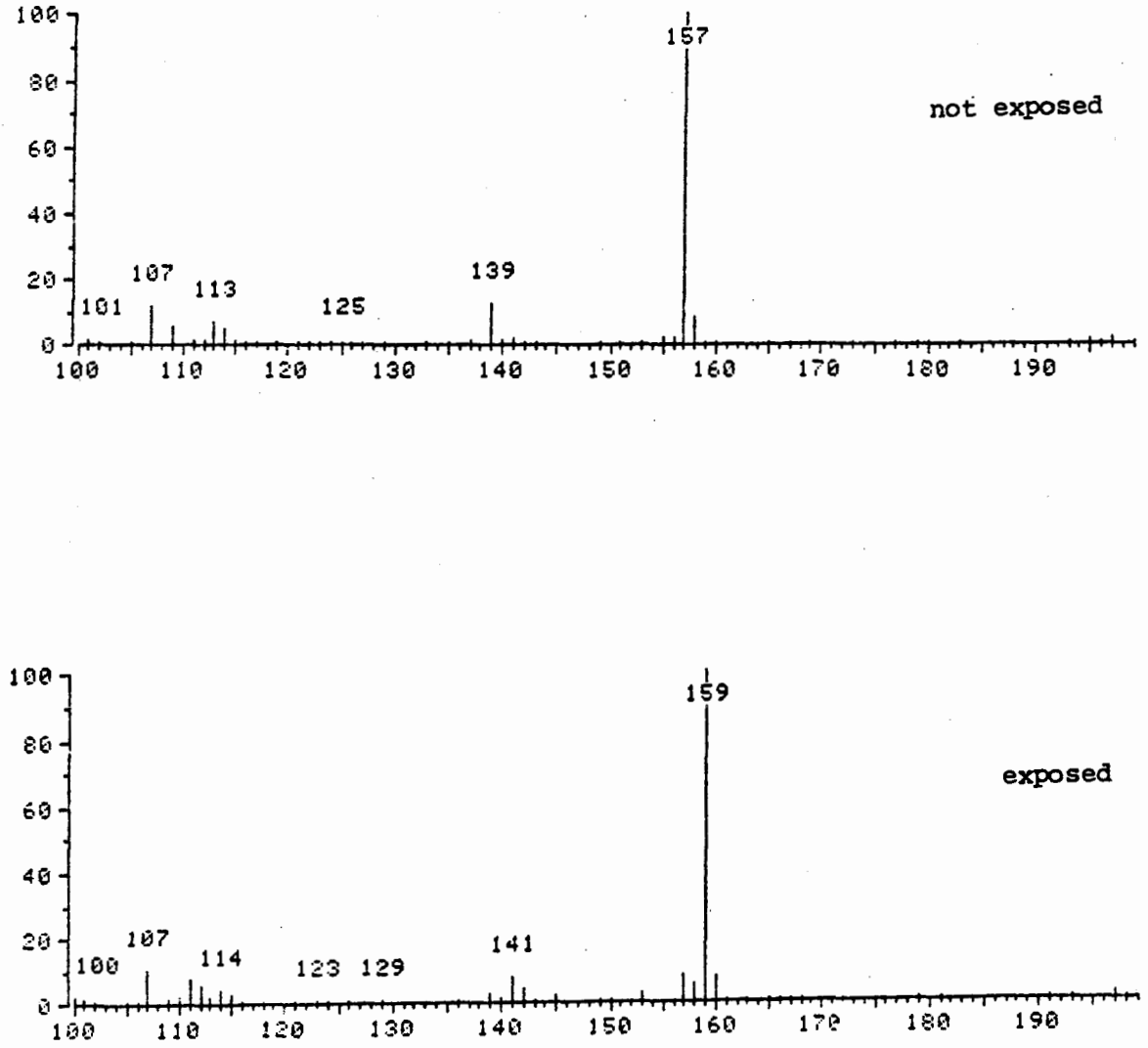


Figure 50: MS (CI) of *endo*-brevicomin produced by male MPBs exposed (or not exposed) to (*E*)-6-nonen-2-one.

Table 34: Relative abundance of peaks of interest in MS (IBCI) of the brevicomins produced by male MPB after exposure to D_2-(*E*)-6-nonen-2-one for 24 hours.

Treatment[1]	Relative abundance ($\bar{x}$ ± SE) P^+	$(P + 1)^+$	$(P + 2)^+$	$(P + 3)^+$
unexposed (2)				
exo-brevicomin:	100.0	9.9 ± 0.1	1.1 ± 0.03	0.1 ± 0.0
+ D_2-(*E*)-nonenone (1)				
exo-brevicomin:	100.0	13.8	8.9	1.0
endo-brevicomin:	7.3	9.6	100.0	7.6

[1] Each sample was prepared from 3 (unexposed) or 7 (exposed) beetles.

The production of *exo*-brevicomin by male MPB was sharply inhibited in the presence of (*E*)-6-nonen-2-one. The analogous inhibition of *endo*-brevicomin production in the presence of (*Z*)-6-nonen-2-one was not observed (Table 33). This phenomenon is very interesting and worthy of further study.

Exo-brevicomin enriched in deuterium was present in extracts of male MPBs that had been exposed to D_2-(*E*)-6-nonen-2-one (Table 34). By GC, the (*E*)-6-nonen-2-one precursor contained no detectable *Z* isomer. However, each beetle was exposed to roughly 8 x 10^5 ng of D_2-(*E*)-6-nonen-2-one and produced less than 2 ng of exo-brevicomin. Thus, the observed D_2-*exo*-brevicomin could have been produced from as little as 0.0003% of a D_2-(*E*)-6-nonen-2-one contaminant. This small quantity would not have been detectable by GC under the conditions that the analysis was performed.

c) Incorporation of ^{18}O from $^{18}O_2$ into *endo*-brevicomin: preliminary study

At least one atom of ^{18}O was incorporated into *endo*-brevicomin by male MPBs exposed to $^{18}O_2$ (Table 35). Male and female MPBs exposed to $^{18}O_2$

and (*E*)-6-nonen-2-one (either unlabelled or [6,7-D_2]) produced *endo*-brevicomin enriched with one atom of ^{18}O/molecule. There was not enough *endo*-brevicomin in the sample to support or to rule out the possibility that a significant proportion of the molecules was enriched with two atoms of ^{18}O. Thus these data alone are not enough to determine if the conversion of (*E*)-6-nonen-2-one to *endo*-brevicomin proceeds through a keto-epoxide or a keto-diol intermediate. However, by analogy with the situation with (*Z*)-6-nonen-2-one, the cyclization of the *E*-isomer through a keto-diol intermediate, in which one of the hydroxyl groups is converted to a leaving group, can be eliminated since this mechanism would lead to *exo*-brevicomin. The biosynthesis of *endo*-brevicomin was not further investigated.

Table 35: Incorporation of ^{18}O from $^{18}O_2$ into *endo*-brevicomin by MPBs exposed (or not) to (*E*)-6-nonen-2-one, (*E*)-**39**.

Treatment	Relative abundance ($\bar{x}$ ± SE)		
	M^+	$(M+2)^+$	$(M+4)^+$
$^{16}O_2$			
males (1)[1]	100.0	n.d.	n.d.
$^{18}O_2$			
males (2)[2]	100.0	37 ± 6	n.d.
$^{18}O_2$ + (*E*)-**39**			
males (1)[2]	100.0	137	n.d.
females (2)[3]	100.0	57 ± 7	n.d.
$^{18}O_2$ + D_2-(*E*)-**39**			
males (1)[2]	n.d.	100	175
females (1)[3]	n.d.	100	266

[1] Sample was prepared from 20 beetles; [2] each sample prepared from 5 beetles; [3] each sample prepared from 10 beetles.

3. Origin of (*Z*)-6-nonen-2-one

The biosynthetic origin of (*Z*)-6-nonen-2-one is unknown. It is possible that the beetles synthesize this compound themselves; or the compound (or a precursor) might be supplied by either the host tree or by fungi associated with the beetles. It is well known that scolytids often use compounds supplied by the host tree as pheromone precursors. Microorganisms are suspected to be involved in the production of some beetle "pheromones" (Borden, 1985; Vanderwel and Oehlschlager, 1987; *Chapter I* of this thesis).

Attempts to detect (*Z*)-6-nonen-2-one by analytical GC in diethyl ether extracts of the phloem of lodgepole pine, or in ether extracts of fungi scraped from the galleries of MPB in lodgepole pine, failed. Some possible biosynthetic precursors to (*Z*)-6-nonen-2-one are presented in Figure 51. It is possible that the beetles synthesize the compound *de novo* from simple precursors such as acetate. Alternatively, it is conceivable that the beetles derive (*Z*)-6-nonen-2-one from other compounds which contain a double bond in the ω-2 position. Two such compounds are leaf alcohol (which could be derived form the host) and linolenic acid.

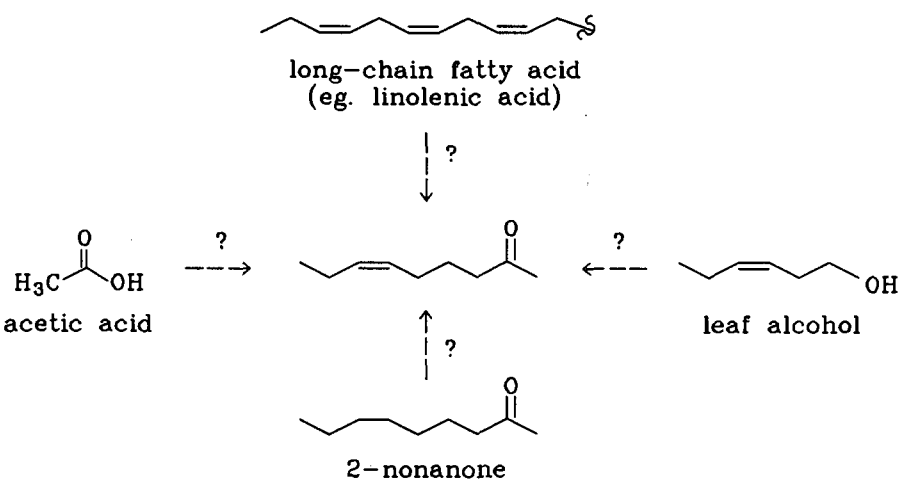


Figure 51: Putative precursors of (*Z*)-6-nonen-2-one.

The possibility that (*Z*)-6-nonen-2-one is derived from fatty acids was not examined. Male MPBs exposed to ^{13}C-labelled acetate did not produce *exo*-brevicomin enriched in ^{13}C in detectable levels (Table 36). Likewise, the *exo*-brevicomin produced by MPB males exposed to D_2-leaf alcohol was not detectably enriched in deuterium (Table 36). As the incorporation of these precursors into *exo*-brevicomin is very inefficient (or non-existent), a better way to approach this problem would be to expose the insects to radiolabelled precursors, and to check for incorporation by radio-HPLC or radio-GC analysis.

Table 36: Incorporation of [2-^{13}C]-sodium acetate and [1,1-D_2]-leaf alcohol into *exo*-brevicomin by male MPB[1]: relative abundances of peaks of interest (GC/SIM-MS).

		Relative abundance		
Putative precursor:	Exposure time:	M^+	$(M + 1)^+$	$(M + 2)^+$
^{13}C-acetate	48 h	100.0	8.4	1.3
	72 h	100.0	9.2	1.4
D_2-leaf alcohol	24 h	100.0	11.6	n.d.
		100.0	10.9	0.9
	48 h	100.0	9.6	1.1
	72 h	100.0	9.3	1.0
		100.0	8.8	0.8

[1] Each sample was prepared from 1 beetle.

The ability of 2-nonanone to serve as a precursor to the brevicomins *in vivo* was also examined. The D_2-labelled analogue was readily synthesized through the reduction of [6,7-D_2]-(*E*)-6-nonen-2-one with hydrogen gas over palladium on activated carbon. The final product was

98% pure and did not contain any detectable starting material (by GC analysis). As mentioned in *Chapter II*, this method of hydrogenation causes some hydrogen exchange. Some scrambling of the deuterium label in the keto-alkane product was observed. The "D_2"-2-nonanone was actually 8% unenriched; 28% enriched in one deuterium; 44% enriched in two deuteriums; 15% enriched in three deuteriums, and 5% enriched for four deuteriums (as judged by GC/SIM-MS analysis).

When male MPBs were exposed to vapors of D_2-nonanone for 24 hours, the *exo*- and *endo*-brevicomin produced by the beetles were enriched for one, two and three deuteriums (Table 37). The GC-R.T.s of the two peaks were identical to those of *exo*- and *endo*-brevicomin, and the two compounds were produced in about the same quantity as *exo*- and *endo*-brevicomin produced in unexposed beetles (Table 38).

Table 37: Relative abundance of peaks of interest in GC/SIM-MS (IBCI)[1] of the brevicomins produced by male MPB after exposure to D_2-2-nonanone for 24 hours.

	Relative abundance ($\bar{x}$ ± SE)			
Treatment[2]	P^+	$(P+1)^+$	$(P+2)^+$	$(P+3)^+$
unexposed (2)				
exo-brevicomin	100.0	9.9 ± 0.1	1.1 ± 0.03	0.1 ± 0.0
+ D_2-nonanone (2)				
exo-brevicomin	100.0	36 ± 4	17 ± 3	6.3 ± 1.3
endo-brevicomin	100.0	62 ± 13	26 ± 12	3 ± 2

[1] The peak representing the $(M+1)^+$ peak of the unlabelled compound is represented as P^+. Molecules enriched for one deuterium would exhibit an $(M+2)^+$ peak, represented here as $(P+1)^+$; [2] each sample was prepared from three MPBs.

Table 38: Production of *exo*- and *endo*-brevicomin by male MPB exposed to vapors of D_2-2-nonanone.

Treatment[1]	Brevicomin production (ng/beetle, $\bar{x}$ ± SE)	
	exo-	*endo*-
unexposed (3)	68 ± 15	5.4 ± 1.3
+ D_2-2-nonanone (2)	60 ± 26	11 ± 2

[1] Each sample was prepared from three MPBs.

In the process of the conversion of D_2-nonanone to *exo*- and *endo*-brevicomin, some deuterium was lost (Table 39). Since deuterium was located mainly in the 6 and 7 positions, exactly where the double bond would be inserted during the production of 6-nonen-2-one, some deuterium would be lost *en route* to the brevicomins.

Table 39: Comparison of the deuterium content in the brevicomins produced by male MPBs after exposure to D_2-2-nonanone with that in the original D_2-2-nonanone sample: percentage of the molecules enriched for one, two or three deuteriums[1].

Sample	% D	% D_2	% D_3
D_2-nonanone	32	51	17
MPB + D_2-nonanone			
exo-brevicomin:	60	30	11
endo-brevicomin:	73	27	1

[1] This is a re-presentation of the data in Table 37. The percentages are calculated using only the abundances of the molecules enriched for deuterium: the data was calculated to exempt unenriched molecules, and those with a higher molecular weight due to the natural abundance of heavy isotopes.

D) CONCLUSIONS

MPBs and BBBs exposed to [4,4-D_2]-(*Z*)-6-nonen-2-one produced *exo*-brevicomin enriched with two deuterium atoms/molecule, indicating that (*Z*)-6-nonen-2-one is a precursor of this pheromone. Evidence in support of biological relevance of this observation include: (1) the *exo*-brevicomin produced by MPB was shown to be of the natural (+)- chirality by complexation chromatography; (2) Dr. Gerhard Gries has found (*Z*)-6-nonen-2-one in the volatiles of male MPBs and BBBs; (3) female BBBs and MPBs (which are not normally known to produce *exo*-brevicomin) produced significantly less *exo*-brevicomin when exposed to the precursor than did the males; and (4) *Ips pini*, a bark beetle not known to produce *exo*-brevicomin, produced little or no *exo*-brevicomin when exposed to (*Z*)-6-nonen-2-one.

MPBs exposed to $^{18}O_2$ produce *exo*-brevicomin that was enriched in two atoms of ^{18}O/molecule, indicating that both oxygens of *exo*-brevicomin were derived from molecular oxygen. A significant portion of the *exo*-brevicomin molecules were enriched in only one atom of ^{18}O, indicating that the oxygen atoms did not arise from the same molecule of oxygen. These results indicated that the conversion of (*Z*)-6-nonen-2-one to (+)-*exo*-brevicomin proceeded through a keto-epoxide intermediate, and that the epoxide was not converted to a diol prior to the cyclization.

Labelling studies with $^{18}O_2$ and $^{18}OH_2$ indicated that the conversion of the keto-epoxide to (+)-*exo*-brevicomin in MPB proceeded through both keto-epoxide derivatives of (*Z*)-6-nonenen-2-one, (6*S*,7*R*)- and (6*R*,7*S*)-epoxynonan-2-one. Though the route through the (6*S*,7*R*)-epoxide was favored in males, while that through the (6*R*,7*S*)-epoxide was favored in females, both routes operated in individuals of either sex. The initial steps in the cyclizations of the ketoepoxide precursors was envisioned to proceed as shown in Figure 48.

Attempts to demonstrate the incorporation of ^{13}C-acetate and D_2-leaf alcohol into *exo*-brevicomin by male MPB failed. However, male MPBs

exposed to D_2-nonanone produced *exo*- and *endo*-brevicomin that was enriched in deuterium.

(E)-6-Nonen-2-one was shown to serve as a precursor of *endo*-brevicomin in MPB and BBB. At least one of the oxygens of *endo*-brevicomin produced by male MPBs *in vivo* is derived from molecular oxygen. Not enough data was collected in this study to determine if the conversion of *(E)*-6-nonen-2-one to *endo*-brevicomin ultimately proceeds through a keto-epoxide or a keto-diol intermediate.

The production of *exo*-brevicomin by male MPB was repressed when the insects were exposed to *(E)*-6-nonen-2-one.

APPENDIX I

Example of calculations for ratios of [6-^{18}O]:[8-^{18}O]-*exo*-brevicomin produced by male MPB + $^{18}O_2$, sample A (Table 29)

m/e	114	116	156	158	160
relative abundance	100	55.3	100	69 .8	37.4

m/e=114 fragment was due to unlabelled (*U*) and [8-^{18}O]- (*y*) brevicomin
m/e=116 fragment was due to doubly labelled (*D*) and [6-^{18}O]- (*x*) brevicomin
m/e=156 ion was due to *U*
m/e=158 ion was due to singly labelled brevicomins (*x* + *y*)
m/e=160 ion was due to *D*

Step 1:
Attempt to correct values for natural abundance of heavy isotopes, *etc.*
a) There was a natural abundance of the m/e=158 peak in unlabelled brevicomin, equal to 0.7% of the abundance of the m/e=156 peak. Therefore
rel. abun. of singly labelled = 69.8 - 0.7 = 69.1 = *x* + *y*
Since the 0.7% represents unlabelled brevicomin, it was added to the relative abundance of the m/e=156 peak.
b) The [8-^{18}O]-*exo*-brevicomin standard exhibited an m/e=116 peak equal to 21.8% of the abundance of the m/e=114 peak. Therefore the abundance of m/e=116 peak was corrected to attempt to give a truer estimate of the relative abundance of brevicomins labelled in the 6 position:
55.3 - 21.8 = 33.5 = *D* + *x*

Step 2:
Set up equation as follows:
rel. abun. m/e=114 / corrected rel. abun. m/e=116
= *U* + *y* / *D* + *x*
= rel. abun. m/e=156 + *y* / rel. abun. m/e=160 + *x*
Since 69.1 = *x* + *y*; *y* = 69.1 - *x*.
Substituting in the values, the equation becomes:
100 / 33.5 = [100.7 + (69.1 - *x*)] / (37.4 + *x*)

Step 3:
Solve equation using simple algebra. Then solve for *y*, as *y* = 69.1 - *x*.

By a similar reasoning process, the calculation was repeated using the data of the m/e=99 and 100 pair.

m/e	98	100	156	158	160
relative abundance	100	49.4	100	69 .8	37.4

m/e=98 fragment was due to unlabelled (*U*) and [6-^{18}O]- (*x*) brevicomin
m/e=100 fragment was due to doubly labelled (*D*) and [8-^{18}O]- (*y*) brevicomin
m/e=156 ion was due to *U*
m/e=158 ion was due to singly labelled brevicomins (*x* + *y*)
m/e=160 ion was due to *D*

Step 1:
Attempt to correct values for natural abundance of heavy isotopes, *etc.*
a) Correct the relative abundance of singly labelled brevicomin as described previously.

rel. abun. of singly labelled = 69.1 = *x* + *y*

And relative abundance of unlabelled brevicomin becomes 100.7%.
b) The unlabelled standard brevicomin exhibited an m/e=100 peak equal to 5.2% of the abundance of the m/e=98 peak. Therefore correct as:

49.4 - 5.2 = 44.2

c) The [8-^{18}O]-*exo*-brevicomin standard exhibited an m/e=98 peak equal to 24.2% of the abundance of the m/e=100 peak. Therefore the abundance of m/e=98 peak was corrected to attempt to give a truer estimate of the relative abundance of brevicomins unlabelled in the 8 position. Therefore, first set the m/e=100 peak = 100. This makes the m/e=98 peak have a relative abundance of 226 (100/44.2 x 100). Then correct as

226 - 24.2 = 202 = *U* + *x*

Step 2:
Set up equations, using similar reasoning as described previously:

rel. abun. m/e=98 / rel. abun. m/e=100
= *U* + *x* / *D* + *y*
= rel. abun. m/e=156 + *x* / rel. abun. m/e=160 + *y*

When the values are substituted in, this becomes:

202 / 100 = (100.7 + *x*) / [37.4 + (69.1 - *x*)]

APPENDIX II

Table 40: Relative abundance of fragments of interest [GC/SIM-MS (EI)] of various *exo*-brevicomin (**42**) samples extracted from male or female MPB[1]. (Raw Data of Trials 1 and 2, Table 30).

	relative abundance of fragment groups[2]	
	$F^+/(F+2)^+$	$M^+/(M+2)^+/(M+4)^+$
Sample	98/100	156/158/160
male MPB + $^{18}O_2$		
A (20)	100.0/39.5	100.0/46.5/30.7
B (20)	100.0/45.4	100.0/56.0/38.9
C (1)	100.0/21.2	100.0/52.3/16.4
D (1)	100.0/66.5	100.0/107.4/86.9
male MPB + $^{18}O_2$ + (*Z*)-**39**		
A (5)	100.0/46.4	100.0/260.6/24.2
B (5)	100.0/55.4	100.0/280.8/22.1
C (1)	100.0/41.4	100.0/341.1/25.9
D (1)	100.0/45.7	100.0/292.8/25.2
E (1)	100.0/35.7	100.0/327.3/27.4
F (1)	100.0/33.7	100.0/272.2/29.1
G (1)	100.0/36.9	100.0/377.0/37.5
female MPB + $^{18}O_2$ + (*Z*)-**39**		
A (10)	100.0/62.7	100.0/181.6/5.6
B (10)	100.0/84.4	100.0/183.6/n.d.
C (1)	100.0/87.4	100.0/289.5/n.d.
D (1)	100.0/105.7	100.0/215.5/4.6
E (1)	100.0/95.2	100.0/218.6/n.d.

[1] Beetles were exposed to (*Z*)-6-nonen-2-one, **39**, and/or $^{18}O_2$ for 24 hours. Letters represent different samples (replicates), each prepared from the number of beetles shown in brackets; [2] the abundance of each fragment shown is relative to the others in the group.

Table 41: Relative abundance of fragments of interest [GC/SIM-MS (EI)] of various *exo*-brevicomin (**42**) samples extracted from male or female MPB[1]. (Raw Data of Table 31).

	relative abundance of fragment groups[2]	
	$F^+/(F+2)^+$	$M^+/(M+2)^+/(M+4)^+$
Sample	98/100	156/158/160
male MPB + $^{18}OH_2$		
A (6)	100.0/14.0	100.0/19.1/n.d.
male MPB + $^{18}OH_2$ + (*Z*)-**39**		
A (5)	100.0/20.3	100.0/20.0/n.d.
B (5)	100.0/22.4	100.0/24.8/n.d.
female MPB + $^{18}OH_2$ + (*Z*)-**39**		
A (10)	100.0/12.6	100.0/25.1/n.d.

[1] Beetles were exposed to (*Z*)-6-nonen-2-one [(*Z*)-**39**] and/or $^{18}OH_2$ for 24 hours. Letters represent different samples (replicates), each prepared from the number of beetles shown in brackets; [2] the abundance of each fragment shown is relative to the others in the group.

CHAPTER IV

IPSDIENOL BIOSYNTHESIS IN *DENDROCTONUS PONDEROSAE*, *IPS PARACONFUSUS*, AND TWO POPULATIONS OF *IPS PINI*

A) INTRODUCTION

Ipsdienol is used as an aggregation pheromone by several species of bark beetles (Borden, 1985). One mechanism by which attraction is limited to conspecifics is through variation of the chirality of the ipsdienol used (Borden, 1985). Interpopulational variation of ipsdienol chirality has been noted in the pine engraver, *Ips pini*. Populations of *I. pini* from Idaho (Plummer *et al.*, 1976), California (Birch *et al.*, 1980), and southeastern B.C. (Miller *et al.*, 1989) produce primarily (*R*)-(-)-ipsdienol. In contrast, populations from New York (Lanier *et al.*, 1980; Miller *et al.*, 1989) and southwestern B.C. (Miller *et al.*, 1989) produce primarily (*S*)-(+)-ipsdienol. Slessor *et al.* (1985) and Miller *et al.* (1989) reported significant differences in the chirality of ipsdienol produced by different individuals within an *I. pini* population.

The biosynthetic mechanism(s) by which closely-related organisms, presumably with similar biosynthetic pathways, can manipulate the chirality of their pheromone is not well understood.

Two possible biochemical mechanisms by which the chirality of the ipsdienol might be controlled are readily apparent. Different individuals might simply oxidize myrcene to different ratios of (*S*):(*R*)-ipsdienol: *i.e.*, the ratio of enantiomers that is initially produced is the ratio of enantiomers that is ultimately emitted by the insect. Alternatively, the oxidation of myrcene might NOT be enantioselective, but one enantiomer might be selectively removed by degradation, rearrangement, *etc.* For example in *I. aminitinus*, which uses only (*R*)-ipsdienol as a pheromone and produces only traces of ipsenol, it has been suggested that the reduction of (*R*)-ipsdienol is blocked, and that (*S*)-ipsdienol is enantioselectively rearranged to amitinol (Francke and Vité, 1983). Fish *et al.* (1984) have

suggested that an enantioselective interconversion between ipsdienol and ipsdienone might be involved in "fine-tuning" the chirality of the ipsdienol emitted by *I. paraconfusus*.

1. Evidence for ipsdienol-ipsdienone interconversion

Fish *et al.* (1979) originally suggested that an alternate pathway to (*S*)-ipsenol, involving an oxidation-reduction equilibrium between ipsdienol and its ketone, ipsdienone, was present in *I. paraconfusus* to account for the loss of deuterium observed when male beetles were exposed to racemic deuterated ipsdienol (64% D, labeled at the carbinol carbon). The deuterium contents of the ipsdienol and ipsenol recovered from the beetles were reduced to 59% and 25%, respectively (Fish *et al.*, 1979). These results were interpreted to mean that (*S*)-(-)-ipsenol could be generated indirectly, through the oxidation of ipsdienol to ipsdienone, reduction back to both (*S*)- and (*R*)-ipsdienol and, finally, through the reduction of the thus formed (*R*)-ipsdienol (Figure 52).

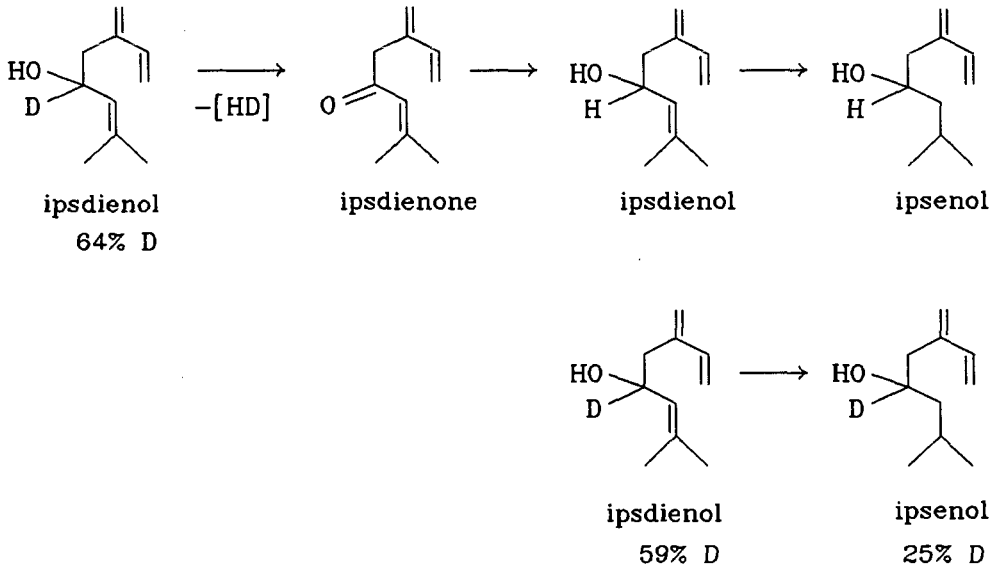


Figure 52: Interpretation of results by Fish *et al.* (1979): involvement of an ipsdienone intermediate in the biosynthesis of ipsenol.

In subsequent experiments, male beetles converted exogenous ipsdienone to (*R*)-ipsdienol [28% enantiomeric excess (ee)] and (*S*)-ipsenol (86% ee) (Fish *et al.*, 1984, Figure 53).

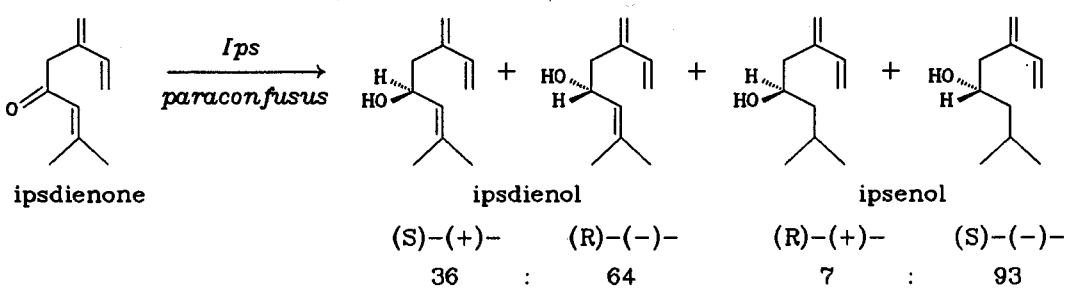


Figure 53: Products of the reduction of ipsdienone by *Ips paraconfusus* (Fish *et al.*, 1984).

Fish *et al.* (1984) believed that ipsenone, the ketone analogue of ipsenol, was not involved, since no ipsenol was detected in the males after they were exposed to ipsenone. Furthermore, although not directly demonstrated, the oxidation of ipsdienol was apparently enantioselective, in that only the (*R*)-enantiomer of ipsdienol was converted to ipsdienone. If this were not the case, beetles exposed to (*S*)-ipsdienol could form (*S*)-ipsenol through ipsdienone. This, in fact, does not occur (Fish *et al.*, 1979).

2. Implications of the ipsdienol-ipsdienone interconversion

Thus according to Fish *et al.* (1984), in *I. paraconfusus* only the (*R*)- enantiomer of ipsdienol is oxidized to ipsdienone, while both the (*S*)- and (*R*)-enantiomers of ipsdienol are formed upon reduction (Figure 54).

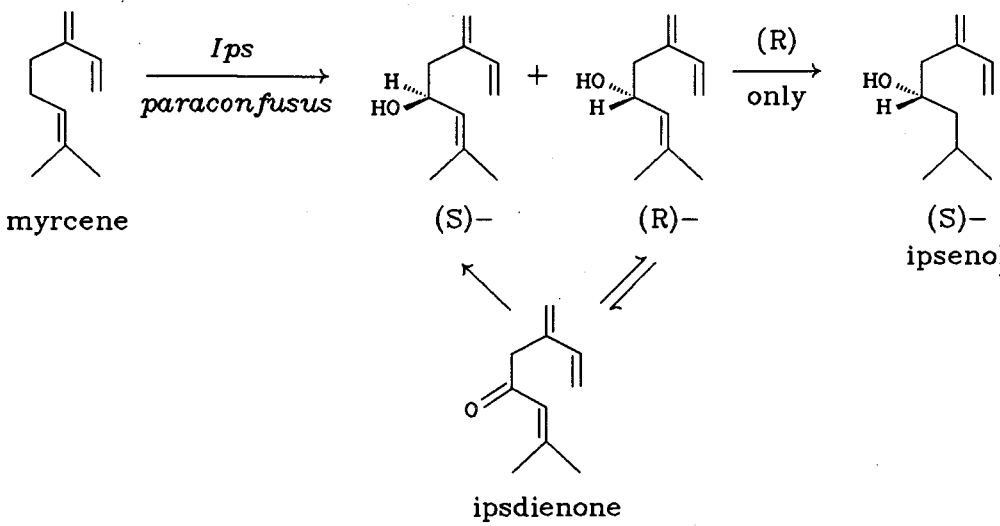


Figure 54: Enantioselective interconversion of ipsdienol and ipsdienone, according to Fish *et al.* (1984).

Such an equilibrium between ipsdienol and ipsdienone could affect the chirality of the ipsdienol emitted: the greater the extent of the interconversion, the more (*S*)-ipsdienol that would be formed (at the expense of the (*R*)-enantiomer) (Fish *et al.*, 1984).

This suggestion is very interesting, and provokes several questions. Why is the reaction apparently catalyzed in both directions for (*R*)-ipsdienol, but in only one direction for (*S*)-ipsdienol? How is (are) the enzyme(s) biased to use only one substrate in one direction, but both in the other? Why was the deuterium content in the recovered ipsenol (25%) so much lower than that in the recovered ipsdienol (59%), if ipsdienol is the precursor to ipsenol? Is this interconversion between ipsdienol and ipsdienone really involved in determining the chirality of the ipsdienol ultimately emitted by *I. paraconfusus*?

These questions seemed worthy of further study. The objectives of this project were to:

1. attempt to duplicate the results of Fish *et al.* (1979, 1984) which indicate that ipsdienol deuterated at the carbinol carbon

loses deuterium after exposure to *I. paraconfusus*; and that ipsdienone is converted to ipsdienol by *I. paraconfusus*;

2. determine if other scolytids are able to perform these conversions;

3. confirm that the ipsdienol-ipsdienone equilibrium occurs *in vivo* in *I. paraconfusus*;

4. determine if the enantioselective oxidation of (*R*)-ipsdienol to ipsdienone, and the reduction of ipsdienone to both (*R*)- and (*S*)-ipsdienol, is involved in the biological resolution of ipsdienol in *I. paraconfusus* and other scolytids.

The bark beetles used in this study were: *Dendroctonus ponderosae* Hopkins, reported to produce (*S*)-(+)-ipsdienol (97% ee) (Hunt, 1987); *Ips paraconfusus* Lanier, reported to produce (*S*)-ipsdienol [87.6% ee, Fish *et al.* (1984)]; and two populations of *Ips pini* (Say). The population from Princeton, in southwestern British Columbia (Canada) is reported to produce (*S*)-ipsdienol (32% ee, Miller *et al.* (1989)] while the population from Kimberly, in southeastern British Columbia is reported to produce (*R*)-ipsdienol [88% and 78% ee, according to Slessor *et al.* (1985) and Miller *et al.* (1989), respectively].

B) EXPERIMENTAL

1. Insects

Mountain pine beetle (MPB), *D. ponderosae*, was collected and reared as described in *Chapter III*.

Adult *I. paraconfusus* from infested ponderosae pine, *Pinus ponderosa*, in the Blodgett Research Forest, El Dorado Co., CA., were generously supplied by Steve J. Seybold*. After receipt the colony was maintained in the laboratory. Fifty to one hundred males were released in a cage (held at approximately 28 °C) which contained one to three unwaxed ponderosa pine logs (aged for 2 weeks to one month before use**). After the beetles started to bore into the logs (about two hours), a number of females roughly equal to the number of males were released in the cage. Two more batches of females were released in the cage at twelve hour intervals. Adult beetles began to emerge from the logs about four weeks after innoculation. Emergent beetles were collected daily and their sex was determined by the presence (males) or absence (females) of a major median frontal tubercle (Wood, 1982). Beetles were stored as described for MPB in *Chapter III*.

The two populations of pine engraver, *I. pini*, were obtained from infested lodgepole pine forests near Kimberly (in southeastern British Columbia) and near Princeton (in southwestern British Columbia). Infested bolts were waxed and treated as described for MPB in *Chapter III*. Colonies were maintained year-round as described above for *I. paraconfusus* (except that waxed lodgepole pine logs were used in place of ponderosa pine logs). Emergent adults were collected daily, their sex was determined by the presence (males) or absence (females) of an enlarged third spine (Wood, 1982), and stored as described for MPB in *Chapter III*.

---

* Department of Entomology, University of California, 201 Wellman Hall. Berkeley, CA. 94720.

** The beetles were sometimes pitched out from fresh logs. When the beetles were presented with both fresh and aged logs in the same cage, much more boring dust was observed around the aged logs.

2. Treatment of insects

a) Exposure to deuterium-labelled precursors

For the deuterium-labelling experiments, newly-emerged male *D. ponderosae*, *I. paraconfusus*, and *I. pini* were individually placed into 3.5 mL screw-cap vials, equipped with teflon-lined lids (Gries *et al.*, 1990a). The chemicals (quantities summarized in Table 42) were added, and the sealed vials were stored at RT in the dark. After the specified time period, the insects were removed from the vials and extracted.

Table 42: Exposure of beetles to precursors: quantities used and exposure times.

precursor and concentration	conditions of this study: quantity precursor used		time	conditions used in other studies
	µL/3.5 mL	µL/100 mL	(h)	
ipsdienol (0.100 g/mL, or 117 µL/mL)[1]	0.80 (soln) (0.094)[2]	2.7[2]	48	2.5-12.5 µL/100 mL for 24 h[3]
ipsdienone (0.500 g/mL, or 588 µL/mL)[1]	0.15 (soln) (0.088)[2]	2.5[2]	24	5 µL/100 mL for 40 h[4] 2.5-12.5 µL/100 mL for 24 h[3]
ipsenone (0.500 g/mL, or 588 µL/mL)[1]	0.15 (soln) (0.088)[2]	2.5[2]	24	5000 µL/100 mL for 24 h[4,5]
myrcene (neat)	0.10 - 0.18	2.9 - 5.1	24-48	50 µL/100 mL for 18 h[6]

[1] solution in pentane; [2] of actual precursor; [3] Fish *et al.* (1979); [4] Fish *et al.* (1984); [5] this quantity seems unreasonable high: perhaps there was a typographical error in the paper--more reasonably, the authors may have intended to indicate that they used 1 µL/20 mL (rather than 1 mL/20 mL), which would give 5 µL/100 mL; [6] Hendry (1980); Hunt (1987).

b) Exposure to ^{18}O-labelled water

Insects were treated sequentially, first by exposure to ^{18}O-water

and then by exposure to D_2-myrcene. Newly emerged male *I. paraconfusus* were first placed on a strip of Kimwipe (ca. 5 mm x 40 mm) soaked in 150 μL $^{18}OH_2$, in a loosely-capped 12 mL screw-cap vial with a teflon-lined lid. After 24 h at RT in the dark, the beetles were removed from the $^{18}OH_2$, and treated with D_2-myrcene as described previously.

c) Exposure to $^{18}O_2$

Insects were treated simultaneously with $^{18}O_2$ and D_2-myrcene. A Schlenck tube equipped with a serum stopper was filled wtih $^{18}O_2$ as described in *Chapter III* of this thesis. Insects were introduced as described previously, then D_2-myrcene was introduced by syringe through the septum. The insects were held in the dark at RT for 24 h.

3. Insect extraction

For each sample, one to three insects were transferred to a one mL glass vial containing 50 or 100 uL of hexane (containing 10 μg/mL racemic 3-octanol as an internal standard), which was cooled in dry ice. The insects were macerated with a blunt-edged microspatula. The extract was filtered through a mini-column of anhyd $MgSO_4$ (made in a Pasteur pipette with a plug of glass-fiber filter paper), into a 1 mL glass culture tube. The remains in the glass vial were washed twice with 100 μL of hexane. These washes were passed through the mini-column and combined with the first extract.

The samples were analyzed on a Hewlett-Packard 5890 GC equipped with a capillary inlet system and an FID. Samples were introduced by splitless injection onto a DB-1 column (15 m x 0.25 mm I.D.; 0.25 μm film thickness). The oven temperature program was: 50 °C for 1 min, then 5 °C/min to 85 °C where the temperature was held for 15 min, followed by an increase at 25 °C/min to 265 °C for a 12 min bake.

4. Derivatization of extracts

The insects were extracted as described in the previous section, except that 100 μL of CH_2Cl_2 (containing 10 μg/mL racemic 3-octanol) was used in place of hexane, and ca. 50 mg anhyd $NaSO_4$ was added to the extract prior to filtration through the mini-column of anhyd $MgSO_4$ to help to ensure that the extracts were dry. The washes were with CH_2Cl_2 instead of hexane.

The acetyl-(*S*)-lactyl derivatives were prepared by slight modifications of the procedure described by Slessor *et al.* (1985). One drop (ca. 10 μL) of pyridine (dried over KOH pellets) and 6 drops (ca. 60 μL) of acetyl *(S)*-lactyl chloride soln were added to each insect extract, and the solns well mixed. The tubes were sealed with a serum stopper, and left to stand overnight at RT.

In the morning the extracts were washed sequentially with ca. 0.2 mL each of distilled water, 25% $NaHCO_3$ soln, and brine. At each step the organic and aqueous phases were well mixed, allowed to separate, and the upper aqueous phase carefully removed with a Pasteur pipette. After the final wash, the organic extracts were forced through a mini-column of anhyd $MgSO_4$. Two pentane washes (each ca. 100 μL) of the aqueous phase were passed through the mini-column and combined with the organic extract.

The proportion of each diastereomer was determined by analytical GC. The oven temperature program was: 70 °C for 1 min, then 5 °C/min to 150 °C, followed by 30 °C/min to a final temperature of 265 °C with a 9 min bake. The (*S*)-isomer was assumed to elute first, as reported by Slessor *et al.* (1987). Samples containing more than a few percent of unreacted ipsdienol or ipsenol were resubjected to the derivatization procedure.

4. MS analyses

Mass spectra were recorded on a Hewlett-Packard 5985B GC/MS system as described in *Chapter II*, by either EI ionization or by CI using isobutane as the ionizing gas. Prior to GC/SIM-MS analyses, the

identities of peaks with the same GC R.T.s as ipsdienol, ipsenol, or the acetyl-(*S*)-lactyl derivatives of ipsdienol or ipsenol were confirmed by their MS fragmentation patterns.

The $(M+1)^+$ peak of ipsenol (m/e=155) was readily observable by GC/MS (CI) (Figure 55). Therefore for SIM analyses of ipsenol, the $(M+1)^+$ ion and those corresponding to deuterium incorporation were scanned. In contrast, the $(M+1)^+$ ion of ipsdienol (m/e=153) was very weak by GC/MS (CI) (Figure 55). The major fragment ion corresponded to that due to the loss of water from the $(M+1)^+$ ion. Therefore for the SIM analyses of ipsdienol, the fragments of m/e=135 (and the appropriate ions corresponding to incorporation of deuterium) were scanned.

The $(M+1)^+$ ions of the acetyl-(*S*)-lactyl derivatives of ipsdienol were difficult to discern by GC/MS (CI) due to loss of the acetyl-(*S*)-lactyl groups (Figure 56). The $(M+1)^+$ ions of the derivatives of ipsenol was observable, but again the major fragment ion corresponded to the loss of the acetyl-(*S*)-lactyl group (Figure 56). Therefore for SIM analyses the m/e=135 ion for the ipsdienol derivatives, and the m/e=137 ion for the ipsenol derivatives (as well as the appropriate ions corresponding to the incorporation of deuterium), were scanned.

5. Statistical analyses

Data were analyzed by one-way Analysis of Variance (ANOVA). Where more than two treatments were used, means were separated by Tukey's multiple range test (P = 0.05). Pearson's correlation coeeficients were calculated.

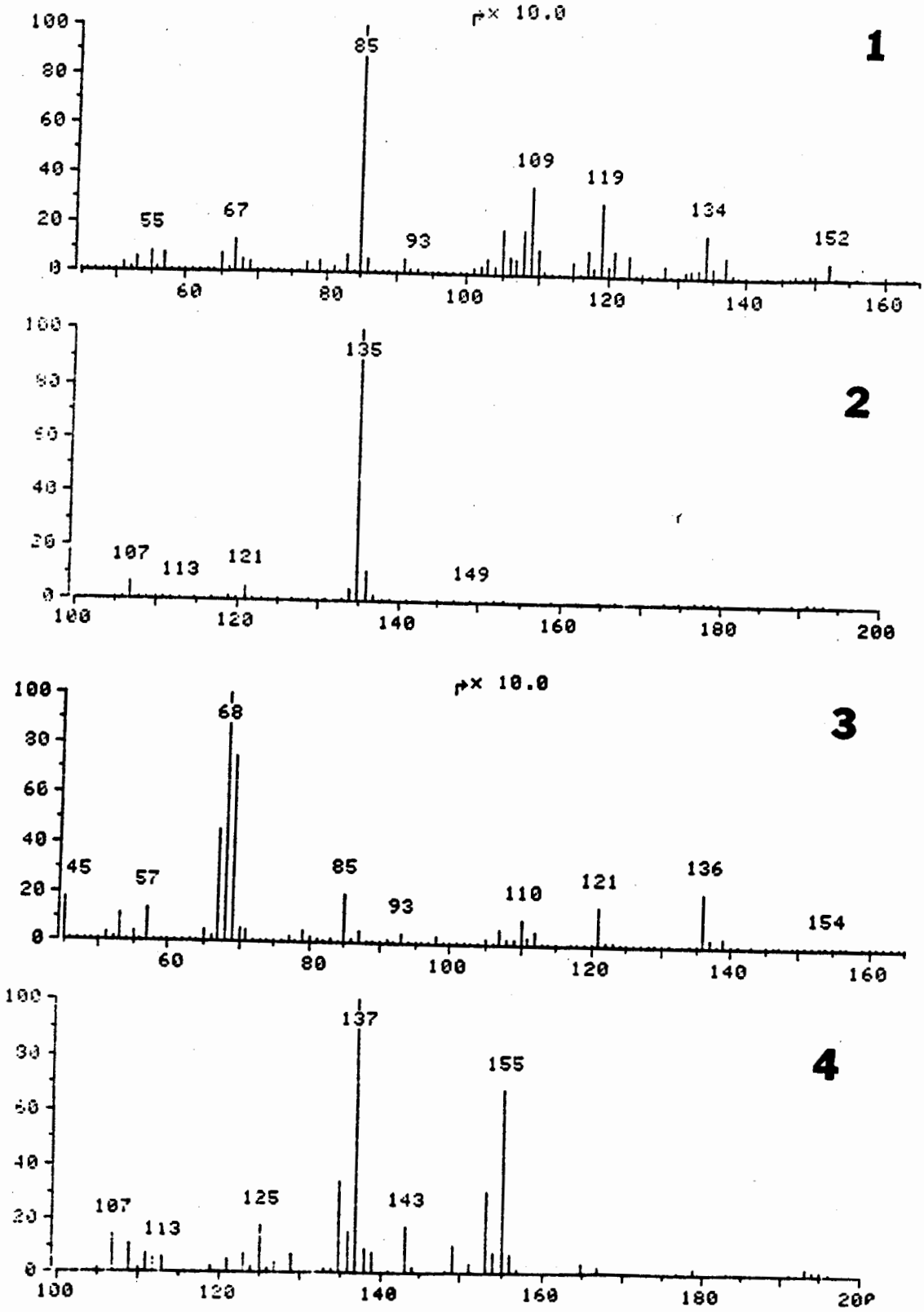
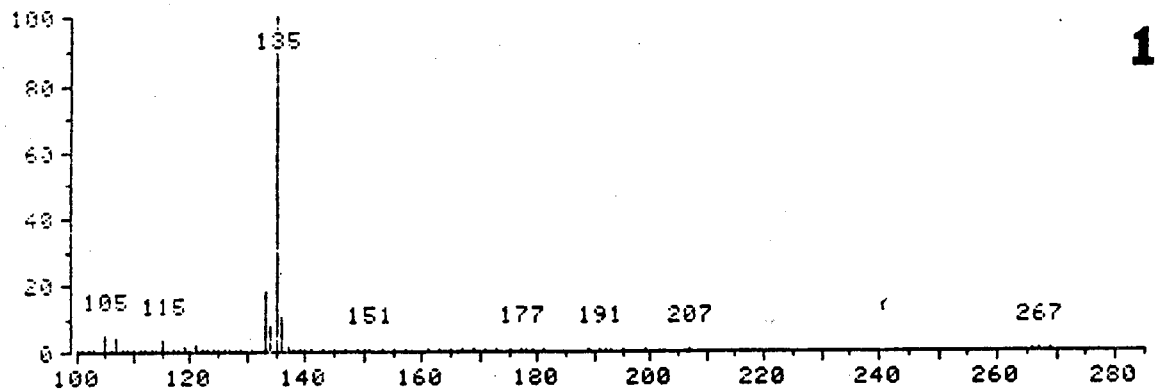


Figure 55: MS ipsdienol (1, EI; 2, CI) and ipsenol (3, EI; 4, CI).



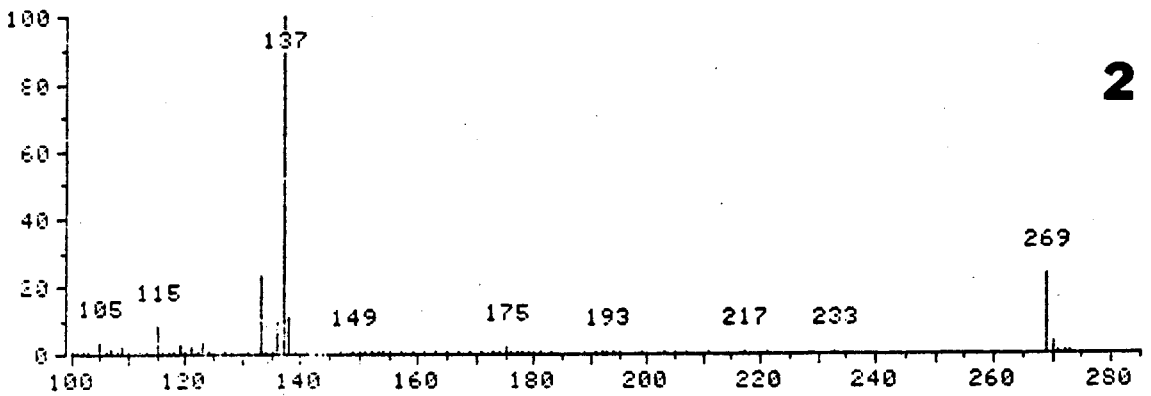


Figure 56: MS (EI) of the acetyl-(*S*)-lactyl derivatives of ipsdienol, **1**, and ipsenol, **2**.

6. Synthesis of stable isotope-labelled chemicals

a) Analyses:

^{1}H NMR , MS and IR spectra were recorded as described in *Chapter II*.

b) Syntheses of chemicals:

All reactions were performed and worked up as described in *Chapter II*, with the exception that solvent was usually removed by distillation through a 12" Vigreux column. Remaining traces of solvent were removed by blowing a gentle stream of Ar or N_2 gas over the product. All flash column chromatography was performed as described in *Chapter II*.

Anhyd diethyl ether was used as purchased. Absolute ethanol was stored over activated molecular sieves (4 Å). CH_2Cl_2 was distilled from CaH_2, and stored over activated molecular sieves (4 Å). 3-Methyl-3-buten-1-yne (Farcham) was distilled and stored over activated molecular sieves (4 Å). THF was always freshly distilled from sodium or potassium metal under Ar. Potassium t-butoxide, $LiAlD_4$, and $CuBr \cdot Me_2S$ were handled in a glove bag under N_2. LiBr was dried by heating to 100 °C under high vacuum for 24 h, and handled in a glove bag under N_2.

Lippa oil containing ipsdienone (60% by GC) was generously donated by Pherotech, Inc.

Preparation of acetyl (*S*)-lactyl chloride:

The reagent was prepared as described by Slessor *et al.* (1985) and in *Chapter II* of this thesis.

Preparation of D_2-ipsdienone and of D_3-ipsdienol (Scheme 11)

2-Methyl-6-methylene-2,7-octadien-4-one (Ipsdienone) (**50**)

Lippa oil containing ipsdienone (60% by GC) was subjected to flash chromatography (pentane/diethyl ether, 90/10) to yield ipsdienone, 95% pure by GC analysis. ^{1}H NMR ($CDCl_3$): δ 1.88 (3 H, s, methyl-H); 2.14 (3 H, s, methyl-H); 3.29 (2 H, s, 5-H); 5.06-5.25 (4 H, m, 8-H and

6-methylene H's); 6.15 (1 H, s, 3-H); 6.35-6.45 (1 H, dd, 7-H). MS (EI) m/e (relative abundance): 150 (M^+, 30); 135 (8); 122 (27); 83 (100); 55 (28)..

D_2-Ipsdienone (D_2-**50**)

Ipsdienone (12.7 mmol) dissolved in pentane (1 mL) was added to 20 mL of NaOD in D_2O (prepared by dissolving 2.5 mmol Na metal in 20 mL D_2O). The reaction was stirred vigorously at RT. The extent of the deuterium exchange was monitored periodically by working up a small sample and analyzing by NMR. After 20 h, the ipsdienone was extracted from the reaction mixture as usual. The crude oil (93% pure by GC analysis) was flash chromatographed (pentane/diethyl ether, 90/10) to yield 1.69 g (89% yield) of product 97% pure by GC analysis. ^1H NMR ($CDCl_3$): δ 1.88 (3 H, s, methyl-H); 2.14 (3 H, s, methyl-H); 3.29 (0.1 H, s, 5-H); 5.06-5.25 (4 H, m, 8-H and 6-methylene H's); 6.15 (1 H, p, $J_{1,3}=J_{2,3\text{-methyl}}=1.5$, 3-H); 6.35-6.45 (1 H, dd, $J_{7,8}=J_{6\text{-methylene},7}=17.5$, 7-H). MS (EI) m/e (relative abundance): 152 (M^+, 3); 124 (2); 84 (5); 83 (100); 55 (26). The product was stored as an 0.5 g/mL soln in pentane, under Ar gas, at -20 °C.

[4,5,5-D_3]-2-methyl-6-methylene-1,6-octadien-4-ol (D_3-Ipsdienol) (D_2-**51**)

D_2-Ipsdienone (3.29 mmol) dissolved in anhyd diethyl ether (3 mL) was added dropwise to a slurry of $LiAlD_4$ (1.23 mmol) in anhyd diethyl ether (15 mL) cooled to 0 °C. After 1 h stirring at 0 °C, the reaction was quenched by addition to 10% tartaric acid (15 mL). The usual workup yielded a quantitative yield of product, 96% pure by GC analysis. This was flash chromatographed (pentane/diethyl ether, 60/40) to yield 0.50 g (97% yield) of product 99.8% pure by GC analysis. ^1H NMR ($CDCl_3$): δ 1.67 (3 H, d, J=1.5, methyl-H); 1.73 (3 H, d, J=1.5, methyl-H); 5.07-5.15 (3 H, m, 6-methylene-H's and 8-H); 5.09 (1 H, br s, 3-H); 6.34-6.42 (1 H, dd, $J_{7,8}=J_{6\text{-methylene},7}=17.5$). MS (EI) m/e (relative abundance): 155 (M^+, 1); 86 (100); 68 (8); 58 (6); 55 (5); 43 (7); 42 (12); 41 (8). The product was

stored as an 0.1 g/mL soln in pentane under Ar gas at -20 °C.

Preparation of D_2-myrcene (Scheme12)

3-Methyl-2-butenoic acid chloride (3,3-dimethylacrylic acid chloride) (**52**)

Thionyl chloride (193 mmol) was added to 3,3-dimethylacrylic acid (140 mmol). The soln was stirred at RT for 3 h, then excess thionyl chloride was blown off with a gentle stream of N_2 gas. The crude product was distilled (52 °C, 50 mm Hg) to yield a colorless oil (14.6 g, 89% yield), 99% pure by GC analysis. ^{1}H NMR ($CDCl_3$): δ 1.96 (3 H, s, methyl-H); 2.14 (3 H, s, methyl-H); 6.03 (1 H, s, 2-H). MS (EI), m/e (relative abundance): 83 (100); 55 (32). IR: 3000 (m); 2960 (m); 2930 (m); 1770 (s); 1615 (s); 1445 (s); 1380 (s); 1210 (m); 1010 (m); 840 (s); 760 (s) cm^{-1}.

[1,1-D_2]-3-Methyl-2-butenol (D_2-**53**)

The acid chloride **52** (121 mmol) dissolved in anhyd diethyl ether (20 mL) was added dropwise to a slurry of $LiAlD_4$ (61 mmol) in anhyd diethyl ether (40 mL) cooled to -50 °C. The slurry was stirred at 4 °C for 1 h, then the reaction was quenched by addition to cold 3% HCl (aqueous). The usual workup yielded a pale yellow oil (10.8 g, quant.), 97% pure by GC analysis. ^{1}H NMR ($CDCl_3$): δ 1.66 (3 H, s, methyl-H); 1.73 (3 H, s, methyl-H); 5.37 (1 H, s, 2-H). MS (EI) m/e (relative abundance): 88 (M^+, 15); 73 (100); 70 (26); 69 (15); 55 (16); 45 (37); 43 (18); 42 (17); 41 (13).

[1,1-D_2]-3-Methyl-2-butenyl bromide (D_2-**54**)

Dimethylsulfide (19 mmol) was added dropwise to N-bromosuccinimide (NBS, 18 mmol) dissolved in 100 mL anhyd CH_2Cl_2 cooled to 0 °C. The reaction mixture was stirred at 0 °C for 30 min. The suspension was cooled to -30 °C, then D_2-**53** (17 mmol) dissolved in anhyd CH_2Cl_2 (4 mL) was added dropwise. The reaction mixture was stirred for 1 h at 0 °C, then

warmed to RT. The usual workup, followed by bulb-to-bulb distillation of the product (RT at 4 mm Hg, with careful cooling of the collecting bulb) yielded a colorless oil (12 mmol, 70% yield), 97% pure by GC analysis. ^{1}H NMR ($CDCl_3$): δ 1.73 (3 H, s, methyl-H); 1.78 (3 H, s, methyl-H); 5.52 (1 H, s, 2-H). MS (EI) m/e (relative abundance): 152 (13); 150 (M^+, 14); 81 (8); 79 (6); 71 (100); 55 (17); 43 (48); 42 (76); 41 (39).

[5,5-D_2]-7-Methyl-3-methylene-2-octen-1-yne (D_2-**55**)

Butyllithium (17.5 mmol of a 2.5 M soln in hexane) was added dropwise to a soln of 3-methyl-3-buten-1-yne (7.95 mmol) in anhyd THF (20 mL), cooled to -50 °C and stirred with an overhead mechanical stirrer. The soln turned a pale yellow. The soln was then cooled to -70 °C and potassium t-butoxide (17.5 mmol) dissolved in THF (10 mL) was carefully added dropwise. The reaction was extremely exothermic, and the reaction mixture turned a deep orange and developed a heavy precipitate. The temperature was maintained below -60 °C during the addition. After stirring for 30 min at -70 °C, the reaction mixture (which by this time had turned bright yellow) was warmed to +5 °C, stirred 10 min, then cooled to -20 °C. Anhyd LiBr (17.5 mmol) dissolved in THF (10 mL) was added dropwise. The orangy-red reaction mixture was stirred for 20 min at -20 °C, then cooled to -60 °C. The bromide **54** (7.95 mmol) dissolved in THF (5 mL) was added dropwise. The reaction mixture was stirred at -60 to -50 °C for 1 h, during which time the color disappeared. The reaction was quenched by addition to water (80 mL). After the usual workup the product (1.98 g) was 65% pure. The crude product was usually carried directly through to the next step. Some was purified (92% pure by GC analysis) by flash chromatography (pentane) for analyses. ^{1}H NMR ($CDCl_3$): δ 1.62 (3 H, s, methyl-H); 1.70 (3 H, s, methyl-H); 2.17 (2 H, s, 4-H); 2.89 (1 H, s, 1-H); 5.09 (1 H, br s, 6-H); 5.28 (1 H, s, 3-methylene-H); 5.54 (1 H, s, 3-methylene-H). MS (EI) m/e (relative abundance: 119 (M-15, 20); 105 (8); 91 (43); 77 (12); 69 (36); 51 (36); 41 (100). MS (CI): 135

(M+1, 100); 121 (56); 109 (22); 107 (44); 93 (36); 91 (22); 83 (20).

[5,5-D_2]-7-Methyl-3-methylene-1,6-octadiene (D_2-Myrcene) (D_2-**56**)

To a suspension of Zn powder (29 mmol) in absolute ethanol (2.6 mL) was added 1,2-dibromoethane (127 μL). The suspension was heated to reflux with vigorous stirring until ethylene no longer evolved. After cooling, more dibromoethane (127 μL) was added and the suspension was brought to reflux again. After 5 min, the heat was removed. A soln of anhyd LiBr (8.8 mmol) and CuBr·Me_2S (4.4 mmol) dissolved in THF (4 mL) was added, and the reaction mixture was brought to reflux again for another 15 min. The heat was removed, and the alkenyne **55** (1.98 g of the crude product, roughly 6.4 mmol, assuming an 80% yield) dissolved in THF (1 mL) was added. The reaction mixture was refluxed for 1 h. The reaction was quenched by pouring into saturated NH_4Cl (50 mL). The aqueous layer was extracted with pentane (7 x 20 mL). The combined pentane extracts were washed once with brine, and dried over anhyd $MgSO_4$. Removal of solvent by distillation through a 12" Vigreux column left a colorless oil (2.66 g) 64% pure by GC. The crude product was purified by flash chromatography on silica impregnated with 10% $AgNO_3$ (pentane/diethyl ether, 90/10). The final product (0.23 g, 21% from the bromide) was 97% pure by GC analysis and had the characteristic odour of myrcene. ^1H NMR ($CDCl_3$): δ 1.62 (3 H, s, methyl-H); 1.70 (3 H, s, methyl-H); 2.21 (2 H, s, 4-H); 4.99-5.08 (3 H, m, 1-H and 3-methylene-H's); 5.16 (1 H, s, 6-H); 5.25 (1 H, d, $J_{1,2}$=17.5; 1-H); 6.35-6.42 (1 H, dd, $J_{1,2}=J_{2,3\text{-methylene}}$=17.5, 2-H). MS (EI) m/e (relative abundance): 138 (M^+, 8); 123 (6); 96 (10); 95 (100); 94 (11); 93 (23); 92 (11); 79 (12); 78 (11); 71 (69); 43 (22); 42 (35); 41 (26).

Preparation of [5-D]-myrcene (Scheme 13)

3-Methyl-2-butenol (**53**)

3-Methyl-2-butenoic acid chloride **52** (84 mmol, prepared as described

in Scheme 12) was reduced with $LiAlH_4$ (52 mmol) in anhyd diethyl ether at 0 °C as described in Scheme 12. The usual workup, and distillation of the crude product, yielded a colorless oil (6.2 g, 86% yield), 99% pure by GC analysis, bp 68-70 °C (33 mm Hg). ^1H NMR ($CDCl_3$): δ 1.66 (3 H, s, methyl-H); 1.73 (3 H, s, methyl-H); 1.99 (1 H, br s, -OH); 4.08 (1 H, d, $J_{1,2}$=1.5, 1-H); 5.38 (1 H, m, $J_{1,2}$=1.5, 2-H). MS (EI) m/e (relative abundance): 86 (M^+, 30); 71 (100); 69 (5); 68 (8); 67 (15); 53 (21).

3-Methyl-2-butenal (**57**)

3-Methyl-2-butenol (51 mmol) dissolved in anhyd CH_2Cl_2 (20 mL) was added to 56 mmol of "activated" oxalyl chloride (prepared by the addition of 56 mmol of oxalyl chloride to 112 mmol DMSO at -60 °C, as described in *Chapter II*, Scheme 5). The soln was stirred with an overhead mechanical stirrer for 1 h at -50 °C, then triethylamine (38 mL) was added to quench the reaction. The usual workup followed by distillation yielded a colorless oil (4.3 g, 96% yield), >99% pure by GC analysis, bp 55-60 °C (40 mm Hg). ^1H NMR ($CDCl_3$): δ 1.96 (3 H, s, methyl-H); 2.15 (3 H, s, methyl-H); 5.87 (1 H, d, 2-H); 9.94 (1 H, d, 1-H). MS (EI) m/e (relative abundance): 84 (M^+, 100); 83 (46); 69 (9); 55 (64); 53 (16); 41 (48).

[1-D]-3-Methyl-2-butenol (**D-53**)

The aldehyde **57** (55 mmol) was dissolved in 95% ethanol (5 mL) and cooled to 0 °C. $NaBD_4$ (21 mmol) dissolved in 95% ethanol (5 mL) was added, and the soln was stirred at RT for 45 min. The usual workup and distillation under vacuum (78-80 °C at 40 mm Hg) yielded a colorless oil (2.89 g, 61% yield), 97% pure by GC analysis. NMR ($CDCl_3$): δ 1.65 (3 H, s, methyl-H); 1.709 (3 H, s, methyl-H); 4.08 (1 H, br s, 1-H); 5.37 (1 H, br s, 1-H). MS (EI) m/e (relative abundance): 87 (M^+, 20); 86 (17); 72 (100); 71 (78); 69 (31); 68 (35); 67 (14); 54 (19); 53 (24); 44 (34); 43 (40); 42 (27); 41 (50).

[1-D]-3-Methyl-2-butenyl bromide (D-54)

The bromide was prepared from the alcohol D-53 (30 mmol) with NBS as described in Scheme 12, in quantitative yield. ^{1}H NMR ($CDCl_3$): δ 1.73 (3 H, s, methyl-H); 1.78 (3 H, s, methyl-H); 4.00 (1 H, d, 1-H); 5.52 (1 H, d, 2-H).

D-Myrcene (D-56)

The bromide D-54 (8 mmol) was coupled with the dilithiated anion of 3-methyl-3-buten-1-yne, and the alkyne product reduced with activated Zn, as described for D_2-myrcene in Scheme 12. The final product (0.5 g, 45% yield after purification) was 99% pure by GC analysis. ^{1}H NMR ($CDCl_3$): δ 1.62 (3 H, s, methyl-H); 1.70 (3 H, s, methyl-H); 2.14-2.25 (3 H, m, 4-H and 5-H); 4.99-5.08 (3 H, m, 1-H and 3-methylene-H's); 5.13-5.18 (1 H, br d, $J_{5,6}$=6.5, 6-H); 5.25 (1 H, d, $J_{1,2}$=17.5, 1-H); 6.35-6.42 (1 H, dd, $J_{1,2}=J_{2,3\text{-methylene}}$=17.5, 2-H). MS (EI) m/e (relative abundance): 137 (M^+, 10); 122 (9); 95 (10); 94 (100); 93 (20); 92 (27); 91 (12); 81 (11); 80 (12); 79 (13); 78 (12); 77 (12); 70 (60); 53 (9). By SIM-MS, the myrcene was 1.6% unenriched; 6% enriched for 1 D, and 92% enriched for 2 D per molecule.

Preparation of D_4-ipsenone (Scheme 14)

7-Methyl-3-methylene-1-octen-5-one (Ipsenone) (58)

Chromic acid soln (2.5 M) was added dropwise to a soln of ipsenol (12.4 mmol) in acetone (50 mL) cooled to 0 °C until the yellow color persisted. The reaction mixture was stirred an additional 30 min at 0 °C, then warmed to RT. Isopropanol was added dropwise until the yellow color was gone, and the soln was green. The reaction mixture was filtered through Celite, then the Celite was washed with diethyl ether (3 x 20 mL). The filtrate and washes were combined, about 2/3 of the solvent was removed by rotary evaporation, and the remaining organic phase was

extracted with 1% HCl. The aqueous phase was washed with diethyl ether (3 x 15 mL). The combined organic layers were washed with brine (1 x 10 mL) and dried over anhyd $MgSO_4$. The crude yellow oil (87% pure by GC analysis) was flash chromatographed (pentane/diethyl ether, 90/10) to yield a colorless oil (1.10 g, 58% yield) 98% pure by GC analysis. ^1H NMR ($CDCl_3$): δ 0.88 (6 H, d, methyl-H); 2.07-2.18 (1 H, m, 2-H); 2.32 (2 H, d, 3-H); 3.26 (2 H, s, 5-H); 5.08-5.17 (3 H, m, 6-methylene H's and 8-H); 5.25 (1 H, s, 8-H); 6.36-6.45 (1 H, dd, 7-H). MS (EI) m/e (relative abundance): 152 (M^+, 32); 95 (10); 85 (100); 67 (18); 65 (12); 57 (92).

D_4-Ipsenone (D_4-**58**)

Ipsenone (6.58 mmol) dissolved in pentane (1 mL) was added to a soln of Na metal (1.3 mmol) dissolved in D_2O (25 mL). The reaction mixture was stirred vigorously at RT overnight, then worked up as described for the preparation of D_2-ipsdienone. The crude product was flash chromatographed (pentane/diethyl ether, 95/5) to yield 0.83 g (80% yield) product, 99% pure by GC analysis. ^1H NMR ($CDCl_3$): δ 0.88 (6 H, d, $J_{2,methyl-H}$=6.5, methyl-Hs); 2.05-2.16 (1 H, m, $J_{2,3}=J_{2,methyl-H)}$=6.5, 2-H); 2.32 (0.8 H, d, $J_{6,7}$=6.5, 3-H); 3.25 (0.6H, s, 5-H); 5.08-5.16 (3 H, m, 6-methylene H's and 8-H); 5.25 (1 H, s, 8-H); 6.36-6.46 (1 H, dd, $J_{7,8}=J_{6\text{-methylene},7}$=17.5, 7-H). MS (EI) m/e (relative abundance): 156 (M^+, 39); 155 (23); 154 (12); 153 (5); 152 (11); 98 (12); 97 (20); 87 (79); 86 (56); 85 (76); 70 (13); 69 (40); 68 (12); 67 (21); 66 (12); 59 (100); 58 (65); 57 (80). By GC/SIM-MS analysis, the product was 21% unenriched; 5% enriched for one deuterium atom/molecule; 14% for two; 22% for three; and 38% for four. The product was stored as an 0.5 g/mL soln in pentane, under Ar gas, at -20 °C.

C) RESULTS AND DISCUSSION

1. Confirmation of results of Fish *et al.*

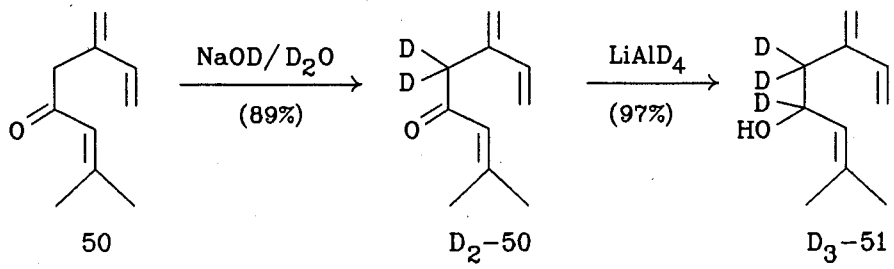
The first objective of this study was to attempt to duplicate the experiments of Fish *et al.* (1979, 1984).

As mentioned in the *Introduction* to this chapter, Fish *et al.* (1979) demonstrated that when *I. paraconfusus* was exposed to ipsdienol deuterated at the carbinol carbon, the ipsdienol recovered from the insects had lost some deuterium. The deuterated ipsdienol used in their study was deuterated only in the "reactive" position. In the present study insects were exposed to ipsdienol labelled with two deuteriums in relatively nonreactive positions, as well as with deuterium on the carbinol carbon (the reactive postion). This was done to ensure that the apparent loss of deuterium was not simply due to the dilution of labelled ipsdienol with endogenous unlabelled ipsdienol.

Fish *et al.* (1984) also demonstrated that *I. paraconfusus* exposed to ipsdienone produced (*R*)-ipsdienol (28% ee) and (*S*)-ipsenol (86% ee). This experiment was repeated in this study, by exposing the insects to D_2-ipsdienone and checking for the production of deuterium-enriched ipsdienol and ipsenol. The ee of the ipsdienol produced was determined by GC analysis of the acetyl-(*S*)-lactyl derivatives (Slessor *et al.*, 1985; Miller *et al.*, 1989). The derivatives of ipsenol were not separable by GC, and the chirality of ipsenol was not determined.

a) Syntheses of [5,5-D_2]-ipsdienone and [4,5,5-D_3]-ipsdienol

[5,5-D_2]-Ipsdienone was prepared in high yield through the base catalyzed exchange of the acidic protons of ipsdienone with deuteriums in D_2O (Scheme 11). The starting material, unlabelled ipsdienone, was obtained through the purification of crude Lippa oil by column chromatography. [4,5,5-D_3]-Ipsdienol was formed through $LiAlD_4$ reduction of D_2-ipsdienone.



50 D_2-50 D_3-51

Scheme 11: Synthetic route to [5,5-D_2]-ipsdienone (D_2-**50**) and [4,5,5-D_3]-ipsdienol (D_3-**51**).

b) Exposure of *I. paraconfusus* to D_3-ipsdienol

I. paraconfusus was exposed to vapors of D_3-ipsdienol for 48 hours, and the recovered ipsdienol and ipsenol were analyzed for deuterium content by GC/SIM-MS (CI). The results in Table 43 indicate that the ipsdienol recovered from *I. paraconfusus* was not as enriched in deuterium as the starting material. This is the same trend reported by Fish *et al.* (1979). The ipsenol recovered from *I. paraconfusus* showed a dramatic loss of deuterium: about three-quarters of the ipsenol was enriched for only two deuteriums, instead of three. Fish *et al.* (1979) also observed a much greater loss of deuterium in the ipsenol than in the ipsdienol. This observation will be discussed in more detail later in this Chapter.

c) Exposure of *I. paraconfusus* to D_2-ipdienone

I. paraconfusus exposed to D_2-ipsdienone produced ipsdienol and ipsenol enriched in deuterium (Table 44), as expected from the report by Fish *et al.* (1984). The ipsdienol produced by *I. paraconfusus* from ipsdienone was largely of the (*R*)-chirality (Table 45). The ee of the (*R*)-ipsdienol determined in this study (71 ± 6%) was larger than that reported earlier (28%) by Fish *et al.* (1984). The reason for the difference is not clear. However, if it is true that (*R*)-ipsdienol is the ultimate precursor of (*S*)-ipsenol, then a decreased rate of reduction of

Table 43: Deuterium content of ipsenol and ipsdienol isolated from *I. paraconfusus* exposed to D_3-ipsdienol[1] for 48 hours. [Relative abundance of peaks of interest by GC/SIM-MS (CI), $\bar{x}$ ± SE].

Sample[1]	Ipsenol		Ipsdienol	
	$(M+2)^+$	$(M+3)^+$	$(F+2)^+$	$(F+3)^+$
D_3-ipsdienol standard	n.d.	n.d.	15.6 ± 0.3	100.0
I. paraconfusus (3)	243 ± 35	100.0	17.6 ± 0.6	100.0

[1] Each biological sample was prepared from one beetle.

(*R*)-ipsdienol to (*S*)-ipsenol would result in a larger ee. However, if this is true then the quantity of ipsenol formed during exposure to ipsdienone [which is largely the (*S*)-isomer according to Fish *et al.* (1984)] should be added to the quantity of (*R*)-ipsdienol extracted from *I. paraconfusus* to give the total quantity of (*R*)-ipsdienol formed from ipsdienone. In this study the ee of (*R*)-ipsdienol formed from reduction of ipsdienone would then be roughly 86% [based on the estimate by Fish *et al.* (1984) that the ee of the (*S*)-ipsenol formed upon reduction of ipsdienone was 87%].

Table 44: Deuterium content of ipsenol and ipsdienone, produced by *I. paraconfusus* exposed to D_2-ipsdienone for 24 hours[1]. [Relative abundances of peaks of interest by GC/SIM-MS (CI), $\bar{x}$ ± SE].

Species	Ipsenol		Ipsdienol	
	$(F)^+$	$(F+2)^+$	F^+	$(F+2)^+$
I. paraconfusus	13 ± 1	100	4 ± 4	100

[1] Three samples (three beetles per sample) were analyzed.

Table 45: Quantity and chirality of ipsdienol and ipsenol produced by *I. paraconfusus* after exposure to D_2-ipsdienone for 24 hours[1] ($\bar{x}$ ± SE).

Species	Ipsdienol		Ipsenol
	quantity (ng/beetle)	% ee of (*R*)-ipsdienol	quantity (ng/beetle)
I. paraconfusus	11 ● 3 (5)	71 ± 6 (3)	192 ± 42 (5)

[1] Each sample was prepared from three beetles.

2. Do other bark beetles lose the carbinol-D of D_3-ipsdienol?

MPB and *I. pini* (P)* were exposed to vapors of D_3-ipsdienol and extracted after 48 hours. The extracts were analyzed as described for *I. paraconfusus*, and the results are presented in Table 46. The D_3-ipsdienol recovered from *I. pini* (P) and MPB did not appear to lose deuterium. This does not rule out the possibility that an ipsdienone-ipsdienol interconversion occurred in these beetles. This process may simply not have been detected in this experiment, due to the fact that the beetles were exposed to a relatively large quantity of D_3-ipsdienol (80 μg/beetle for 48 h) compared to the quantity of ipdienol that they normally produce (less than 1 μg/beetle/24 h). Even if the beetles had removed deuterium from some ipsdienol, this D_2-ipsdienol would be diluted by the relatively large pool of D_3-ipsdienol. This effect could have been minimized by decreasing the quantity of D_3-ipsdienol that the insects were exposed to, and by increasing the time of exposure. However an alternate approach (using deuterated myrcene, discussed at the end of this section) was used to circumvent this problem.

* The two populations of *I. pini* (from Princeton and Kimberly) will be designated as "*I. pini* (P)" and "*I. pini* (K)", respectively.

Table 46: Deuterium content of ipsenol and ipsdienol isolated from bark beetles after exposure to D_3-ipsdienol for 48 hours. [Relative abundance of peaks of interest by GC/SIM-MS (CI), ($\bar{x}$ ± SE)].

Sample[1]	Ipsenol		Ipsdienol	
	$(M+2)^+$	$(M+3)^+$	$(F+2)^+$	$(F+3)^+$
D_3-ipsdienol standard	n.d.	n.d.	15.6 ± 0.3	100.0
I. paraconfusus (3)	243 ± 35	100.0	17.6 ± 0.6	100.0
I. pini (P) (2)	n.d.	n.d.	15.4 ± 0.0	100.0
MPB (3)	n.d.	n.d.	15.7 ± 0.1	100.0

[1] Each biological sample was prepared from one beetle.

3. Can other bark beetles reduce ipsdienone to ipsdienol?

MPB and both populations of *I. pini* exposed to D_2-ipsdienone produced ipsdienol that was enriched in two deuteriums (Table 47). MPB produced almost racemic ipsdienol, while that produced by the two *I. pini* populations was almost pure (*R*)-ipsdienol. These species are not known to produce ipsenol, but a peak with the same GC-R.T. and SIM-MS pattern as the D_2-ipsenol produced by *I. paraconfusus* was observed. Unfortunately this observation was not explored due to time constraints.

Table 47: Deuterium content of ipsdienol, produced by bark beetles exposed to D_2-ipsdienone for 24 hours[1]. [Relative abundances of peaks of interest by GC/SIM-MS (CI) ($\bar{x}$ ± SE)].

Species	Ipsdienol	
	F^+	$(F+2)^+$
I. pini (K) (3)	7 ± 1	100
I. pini (P) (3)	5.5 ± 0.4	100
MPB	5 ± 4	100

[1] Each sample was prepared from three beetles.

Table 48: Quantity and chirality of ipsdienol and ipsenol produced by different bark beetles after exposure to D_2-ipsdienone for 24 hours[1] ($\bar{x}$ ± SE).

Species	Ipsdienol		Ipsenol
	quantity (ng/beetle)	% ee of (*R*)-ipsdienol	quantity (ng/beetle)
I. paraconfusus	11 ± 3 (5)	71 ± 6 (3)	192 ± 42
I. pini (K)	458 ± 38 (2)	97 ± 1.1 (3)	?[2]
I. pini (P)	130 ± 75 (2)	98 ± 1.4 (2)	?
MPB	82 ± 18 (7)	15 ± 5 (3)	?

[1] Each sample was usually prepared from three beetles; [2] a peak with the same fragmentation pattern and retention time as D_2-ipsenol was detected by GC/SIM-MS but was not quantified.

4. Alternate approaches to study the ipsdienol-ipsdienone interconversion

a) Use of deuterated myrcene

An alternate approach used to determine if ipsdienol was in equilibrium with ipsdienone was to expose the insects to deuterated myrcene (labelled at the pre-carbinol carbon), and then to determine the deuterium enrichment of the products. Loss of deuterium by the ipsdienol and ipsenol products could indicate the existence of an ipsdienol-ipsdienone interconversion *in vivo*. This is illustrated in Figure 57.

Synthesis of deuterated myrcene

The synthetic route to [5,5-D_2]-myrcene, D_2-**56**, involved coupling of [1,1-D_2]-3-methyl-2-butenyl bromide, D_2-**53**, with a synthetic equivalent of the isoprene anion: dilithiated isopropenylacetylene (Scheme 12). This is an adaptation of the method used by Klusener *et al.* (1987) to synthesize ipsenol and ipsdienol. This coupling reaction can be extremely

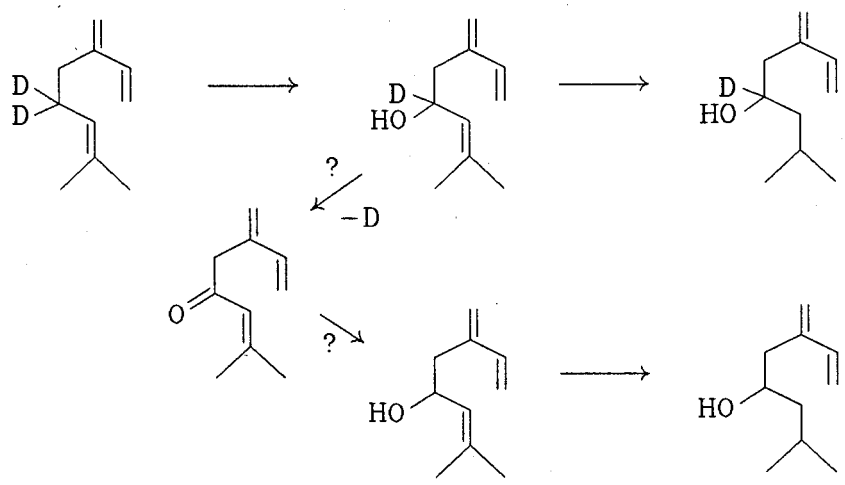
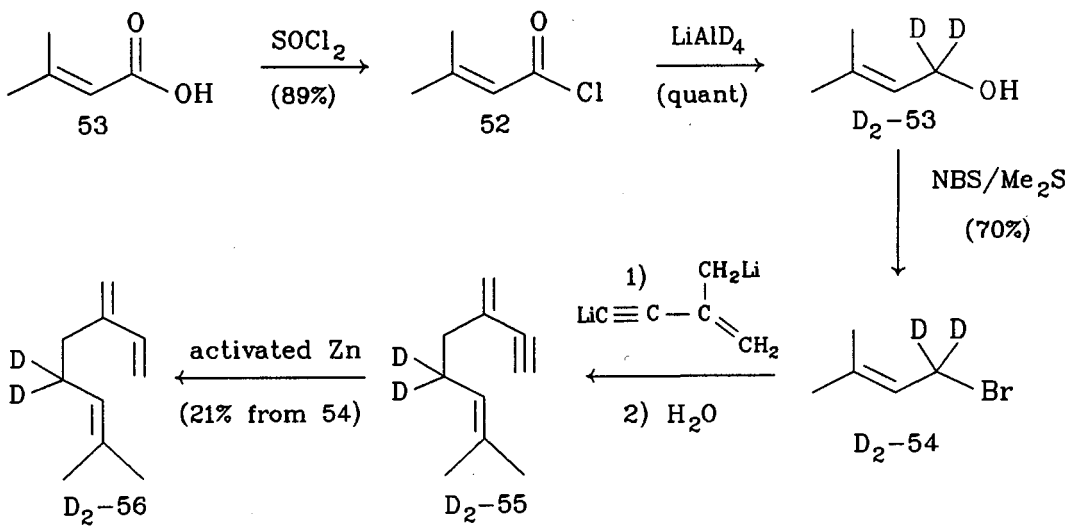


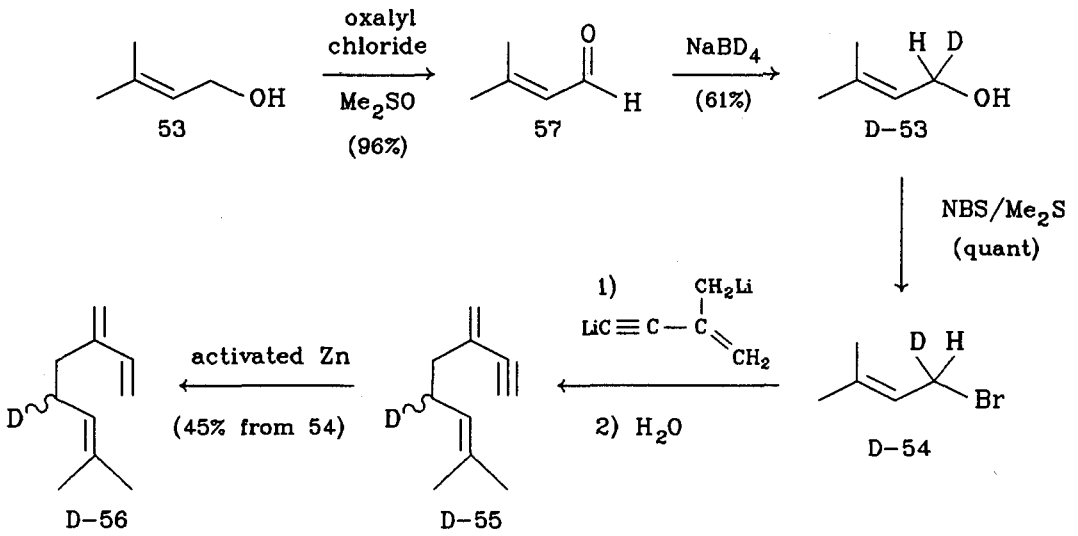
Figure 57: Expected loss of deuterium from deuterated myrcene *en route* to ipsdienol and ipsenol, if the ipsdienol-ipsdienone equilibrium occurs *in vivo*.

high-yielding: in cases where scrupulously dry conditions were attained, near quantitative yields were obtained. The deuterated precursor required for this coupling was easily prepared as outlined in Scheme 12.



Scheme 12: Synthetic route to [5,5-D_2]-myrcene (D_2-**56**).

[5-D]-Myrcene was prepared by a scheme analogous to that described for the preparation of D_2-myrcene, the major difference being that [1-D]-3-methyl-2-butenol, D-**53**, was used in place of D_2-**53**. This precursor was prepared in good yield through the $NaBD_4$-reduction of 3-methyl-2-butenal, **57** [prepared through the oxidation of 3-methyl-2-butenol by the method of Mancuso *et al.* (1978)]. This route is represented in Scheme 13.



Scheme 13: Synthetic route to [5-D]-myrcene (D-**56**).

Exposure of beetles to deuterated myrcene

As expected from the results of other studies (Hughes, 1974; Byers *et al.*, 1979; Hendry *et al.*, 1980; Hunt, 1986) extracts of all of the male bark beetles tested contained ipsdienol after exposure to vapors of deuterated myrcene for 24 hours (Table 49). *I. paraconfusus* was the only beetle to produce ipsenol.

The chiralities of the ipsdienol contained in the extracts were determined by GC analyses of the acetyl-(*S*)-lactyl derivatives. Most of the results were in agreement with previous reports. The major discrepancy occurred with *I. pini* (K). Although both this and the other

studies (Slessor *et al.*, 1985; and Miller *et al.*, 1989) indicate that this population produces (*R*)-ipsdienol, the other studies reported a much higher ee (88% and 78% ee, respectively, as compared to 24% determined in this study). This difference may be due to an isotope effect [which would decrease the turnover rate of an enzyme catalyzing the conversion of 5-D-(*S*)-ipsdienol to 5-D-(*R*)-ipsdienol, discussed in a later section.]

Table 49: Quantity and chirality of ipsdienol and ipsenol produced by male bark beetles after exposure to myrcene for 24 hours[1] ($\bar{x}$ ± SE).

Species	Ipsdienol		Ipsenol
	quantity (ng/beetle)	chirality, % ee	quantity (ng/beetle)
I. paraconfusus	34 ± 6 (7)	*S*, 88 ± 5 (9)	33 ± 6 (7)
I. pini (K)	725 ± 164 (10)	*R*, 24 ± 6 (7)	n.d.
I. pini (P)	533 ± 186 (10)	*S*, 25 ± 6 (12)	n.d.
MPB	803 ± 229 (12)	*S*, 93 ± 2 (9)	n.d.

[1] Each sample was prepared from one MPB or three *Ips* beetles.

As expected, the ipsdienol and ipsenol produced by the beetles exposed to D_2-myrcene were enriched in deuterium (Table 50). In all cases at least some of the ipsdienol produced was unlabelled. This was most obvious with *I. pini* (K): about one-fifth of the ipsdienol extracted from the beetles was not deuterium-labelled. The ipsenol present in extracts of *I. paraconfusus* was found to be dramatically depleted in deuterium: about three-quarters of the ipsenol did not contain deuterium (after correcting for the natural abundance of stable isotopes in the unenriched ipsenol), as compared to only 4% of the ipsdienol!

Table 50: Deuterium content of ipsenol and ipsdienol isolated from three scolytid species after exposure to D_2-myrcene[1]. [Relative abundance of peaks of interest by GC/SIM-MS (CI) ($\bar{x}$ ± SE)].

Sample	Ipsenol		Ipsdienol	
	$(M+1)^+$	$(M+2)^+$	F^+	$(F+1)^+$
I. paraconfusus (36 h) (3)	208 ± 5	100.0	4.4 ± 0.6	100.0
I. pini (K) (48 h) (3)	n.d.	n.d.	23 ± 2	100.0
I. pini (P) (36 h) (3)	n.d.	n.d.	5.9 ± 1	100.0
MPB (36 h) (3)	n.d.	n.d.	1.3 ± 0.02	100.0
(48 h) (2)	n.d.	n.d.	7.0 ± 0.1	100.0

[1] Each MPB sample was prepared from one beetle; each *Ips* sample was prepared from three beetles.

b) Exposure of beetles to D_2-myrcene and ^{18}O-labelled water or oxygen

The results of Fish *et al.* (1976, 1984) and this study indicate that *I. paraconfusus* converts exogenous ipsdienone to ipsdienol and ipsenol, and that during the conversion of D_2-myrcene to ipsdienol some of the deuterium at the carbinol carbon is lost. However, there is no evidence that these two facts are related. For this reason, experiments were performed in which *I. paraconfusus* was exposed to both D_2-myrcene and either $^{18}O_2$ or $^{18}OH_2$.

Ipsdienol is widely believed to be formed through the PSMO-mediated oxidation of myrcene (Vanderwel and Oehlschlager, 1987, and references cited therein). The source of oxygen for PSMO activity is molecular oxygen (Guengerich and Macdonald, 1984). When *I. paraconfusus* was exposed to both D_2-myrcene and $^{18}O_2$, the ipsenol that was formed was, as expected, enriched in ^{18}O (Table 51).

As the oxidation of myrcene involves molecular oxygen, beetles exposed to D_2-myrcene and $^{18}OH_2$ should not incorporate ^{18}O into ipsdienol

Table 51: Incorporation of deuterium and ^{18}O into ipsenol[1] produced by male *I. paraconfusus* exposed to unlabelled myrcene or to D_2-myrcene and $^{18}O_2$. [Relative abundance of peaks of interest by GC/SIM-MS (CI)].

Treatment[2]	Ipsenol			
	M^+	$(M+1)^+$	$(M+2)^+$	$(M+3)^+$
unlabelled	100	11	1.3	n.d.
+ $^{18}O_2$ + D_2-myrcene	100	67	92	13

[1] Unfortunately there was not enough ipsdienol in the sample for analysis. As mentioned in the *Experimental*, the $(M+1)^+$ peak in the GC/MS (CI) spectrum of ipsdienol is quite weak. Analyses were usually performed using the peak corresponding to the loss of water, but this was clearly not suitable in this experiment; [2] each sample was prepared from 3 beetles.

and ipsenol unless the ipsdienone/ipsdienol equilibrium does exist *in vivo*. As discussed in *Chapter III* of this thesis, ketone oxygens are vulnerable to exchange with water *in vivo*. Thus the oxygens of ipsdienone could exchange with the ^{18}O-oxygens of ^{18}O-water. Upon reduction, the ^{18}O would end up in ipsdienol and, subsequently, ipsenol (see Figure 58).

When *I. paraconfusus* was exposed to D_2-myrcene and $^{18}OH_2$, the products ipsenol and ipsdienol were enriched in either D or ^{18}O, but not both (Table 52). This was the expected pattern according to Figure 58. After correcting for the natural abundance of stable isotopes, the ipsdienol produced in this experiment was 48% unlabelled, 37% enriched in D alone and 14% enriched in ^{18}O alone. The ipsenol synthesized by the beetles under these conditions was 72% unlabelled, 8% enriched in D alone, 20% enriched in ^{18}O alone.

These data support the hypothesis by Fish *et al.* (1976, 1984) that an equilibrium between ipsdienol and ipsdienone functions *in vivo* in *I. paraconfusus*.

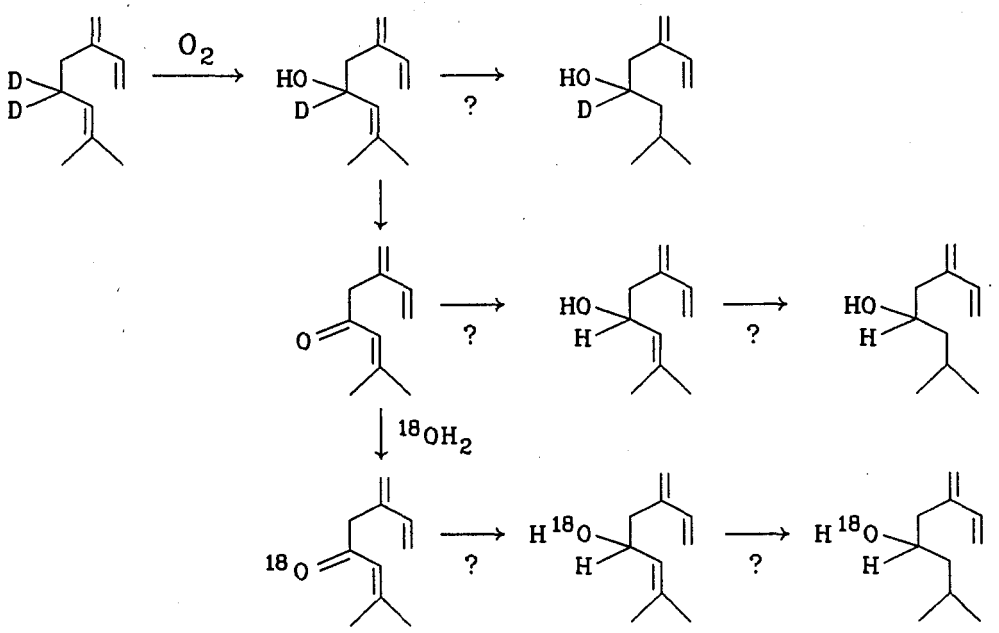


Figure 58: Expected pattern of incorporation of ^{18}O from $^{18}OH_2$ into ipsdienol and ipsenol through an ipsdienone intermediate.

Table 52: Incorporation of D and ^{18}O into ipsdienol and ipsenol produced by male *I. paraconfusus* exposed to unlabelled myrcene or to D_2-myrcene and $^{18}OH_2$. [Relative abundance of peaks of interest by GC/SIM-MS (CI)].

	Ipsdienol				Ipsenol			
Treatment[1]	M^+	$(M+1)^+$	$(M+2)^+$	$(M+3)^+$	M^+	$(M+1)^+$	$(M+2)^+$	$(M+3)^+$
unlabelled	100	11	n.d.	n.d.	100	11	1.3	n.d.
+ $^{18}OH_2$ **A**	100	87	41	4.9	100	28	10	0.7
B	100	92	35	5.1	100	14	53	6.1

[1] Each sample was prepared from 3 beetles. The letters **A** and **B** refer to different samples.

5. Is the ipsdienol-ipsdienone equilibrium involved in the biological resolution of ipsdienol?

To this point, this study has served to confirm the findings of Fish *et al.* (1976, 1980) that ipsdienone is reduced to ipsdienol, and that an equilibrium between ipsdienol and ipsdienone may exist *in vivo* in *I. paraconfusus*. This study also indicates that this process might occur in the other bark beetle species tested. The hypothesis of Fish *et al.* (1976, 1980), that this equilibrium is involved in the biochemical manipulation of the chirality of the ipsdienol produced by the beetles, will now be addressed.

According to Fish *et al.* (1979, 1984), in *I. paraconfusus* only (*R*)-ipsdienol is oxidized to ipsdienone, whereas both (*S*)- and (*R*)-ipsdienol are formed upon the reduction of ipsdienone. They suggested that the greater the extent of the ipsdienol-ipsdienone interconversion, the greater the proportion of *(S)*-ipsdienol that will develop. If this hypothesis is correct, then the more (*S*)-ipsdienol produced by beetles exposed to myrcene deuterated at the precarbinol carbon, the more deuterium should be lost by the ipsdienol.

This strategy was used to test the hypothesis of Fish *et al.* (1984). Insects were exposed to deuterated myrcene, then extracted as usual. The chirality of the ipsdienol in each extract was determined [through analysis of the acetyl-(*S*)-lactyl diastereomeric derivatives (Slessor *et al.*, 1985)] and compared to the proportions of (*S*)- and (*R*)-ipsdienol that had lost deuterium [determined by GC/SIM-MS (CI)]. (Since more experiments were performed with [5-D]-myrcene than with [5,5-D_2]-myrcene, the results of the former are presented here.)

The results are presented graphically in Figure 59. Each point corresponds to a single sample (prepared from one MPB or three *Ips* beetles). In Graph 1, the proportion of (*S*)-ipsdienol in the sample that did not contain deuterium (as a percentage of the total (*S*)-ipsdienol) was plotted against the percentage of (*S*)-ipsdienol in that sample. There

does not appear to be a significant correlation between increased loss of deuterium in *(S)*-ipsdienol and an increased proportion of *(S)*-ipsdienol. In the Graph 2 of Figure 59, the percentage of *(R)*-ipsdienol that did not contain deuterium was plotted against the percentage of *(S)*-ipsdienol in that sample. There is a negative correlation between the two: the more *(S)*-ipsdienol in the sample, the less the deuterium loss in the *(R)*-ipsdienol. If only the data for the *I. pini* populations are considered (Graph 3 of Figure 59), there is an even more significant correlation.

These results clearly do not support the hypothesis that an increase in the extent of the ipsdienol/ipsdienone interconversion effects a higher proportion of *(S)*-ipsdienol. There was no discernable relationship between D loss in *(S)*-ipsdienol and the percentage of *(S)*-ipsdienol. Moreover, the relationship between deuterium-loss in *(R)*-ipsdienol and the percentage of *(S)*-ipsdienol produced was opposite to that expected. This is also demonstrated in Table 53: *I. pini* (K), which produced the lowest proportion of *(S)*-ipsdienol (Table 49), exhibited the greatest loss of deuterium.

Other results in Table 53 were also unexpected. For example the *(R)*-ipsdienol produced by *I. pini* (P) exposed to [5-D]-myrcene was expected to be less enriched for D than the *(S)*-isomer, taking into account the facts that this population produced largely *(R)*-ipsdienol (ee 98%) from exogenous ipsdienone (Table 48), and produced much less *(R)*-ipsdienol than *(S)*-ipsdienol from exogenous myrcene (Table 49). However, according to Table 53 there was no statistically significant difference between the deuterium enrichments of the *(R)*- and *(S)*-ipsdienol produced by this beetle. MPB produced almost racemic ipsdienol from ipsdienone [*(R)*-ipsdienol, ee 15%, Table 48], and produced largely *(S)*-ipsdienol from myrcene (ee 94%, Table 49), and therefore was expected to produce a higher proportion of unlabelled *(R)*-ipsdienol than unlabelled *(S)*-ipsdienol after exposure to [5-D]-myrcene. In fact significantly more of the *(S)*-

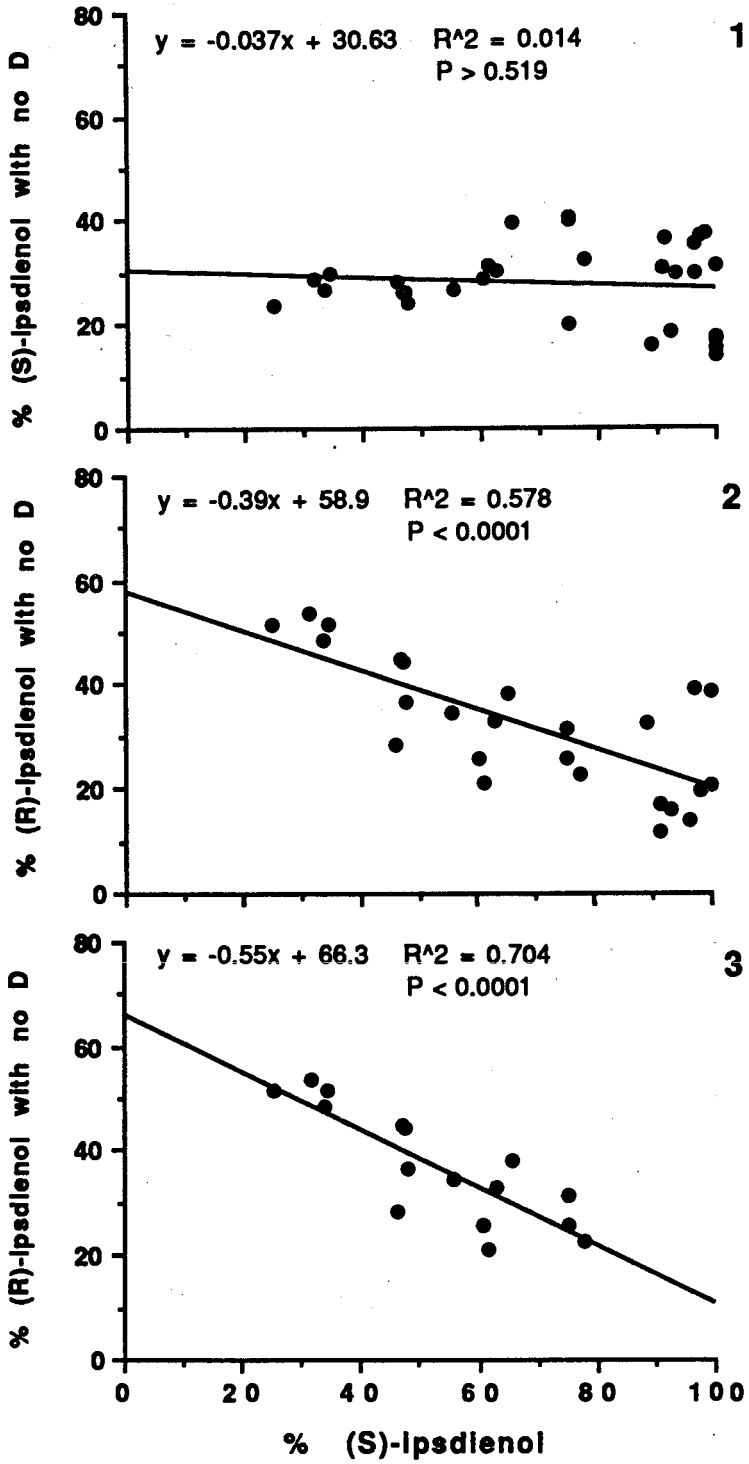


Figure 59: Quantity of ipsdienol not enriched in D produced by bark beetles after exposure to D-myrcene, compared to the quantity of (*S*)-ipsdienol produced, for both (*S*)- and (*R*)-ipsdienol. Graphs 1 and 2 include data from *I. paraconfusus*, *I. pini* (K), *I. pini* (P) and MPB. Graph 3 includes only data from the *I. pini* populations.

Table 53: GC/SIM-MS (CI) abundance of $(F+1)^+$ and $(M+1)^+$ peaks [relative to $(F+)^+$ and $(M+1)^+$ peaks] of ipsdienol and ipsenol, respectively, produced by different bark beetle species after exposure to D-myrcene for 36 hours[1]. ($\bar{x}$ ± SE).

Species	Relative abundance of $(F+1)^+$ peak of ipsdienol		Relative abundance of $(M+2)^+$ peak of ipsenol
	(S)-	*(R)*-	
I. paraconfusus	21 ± 1^{c} (8)	53 ± 5^{a} (3)	285 ± 44^{e} (8)
I. pini (K)	37 ± 1^{abc} (10)	89 ± 6^{d} (10)	n.d.
I. pini (P)	51 ± 4^{a} (9)	42 ± 4^{ab} (9)	n.d.
MPB	50 ± 2^{a} (10)	26 ± 6^{bc} (8)	n.d.

[1] Each sample was prepared from one MPB or three *Ips* beetles.

ipsdienol produced by this beetle was unenriched. These results are not consistent with the explanation that the deuterium loss is due solely to the ipsdienol-ipsdienone equilibrium as suggested by Fish *et al.* (1984).

6. Is there an alternate biosynthetic route to ipsenol?

Several observations reported by Fish *et al.* (1979, 1984) and presented in this chapter do not support the hypothesis that ipsdienol is the major ultimate precursor of ipsenol. These observations include:

1. the ipsenol produced by *I. paraconfusus* exposed to deuterated ipsdienol or deuterated myrcene always contained much less deuterium than ipsdienol, the supposed ultimate precursor (Fish *et al.*, 1979; Tables 43, 50, 52 and 53);

2. the ipsenol produced by *I. paraconfusus* exposed to both D_2-myrcene and $^{18}OH_2$ was more enriched in ^{18}O than the ipsdienol produced under these conditions (20% as compared to 14%);

3. extracts of *I. paraconfusus* exposed to ipsdienone (0.088 μL ipsdienone/beetle for 24 h) contained as much ipsenol as those of beetles exposed to ipsdienol (0.094 μL ipsdienol/beetle for 48 h);

4. *I. paraconfusus* exposed to both D_2-myrcene and $^{18}O_2$ (Table 52), produced little ipsenol enriched in both D and ^{18}O. The ipsenol produced in this experiment was 40% unlabelled, 24% enriched in D alone and 35% enriched in ^{18}O alone. The $(M+3)^+$ peak was largely due to the natural abundance of stable isotopes, and only about 1% of the ipsenol was enriched in both D and ^{18}O. It was expected that a significant proportion of ^{18}O,D-ipsdienol would be reduced to reduced to ^{18}O,D-ipsenol (Figure 60).

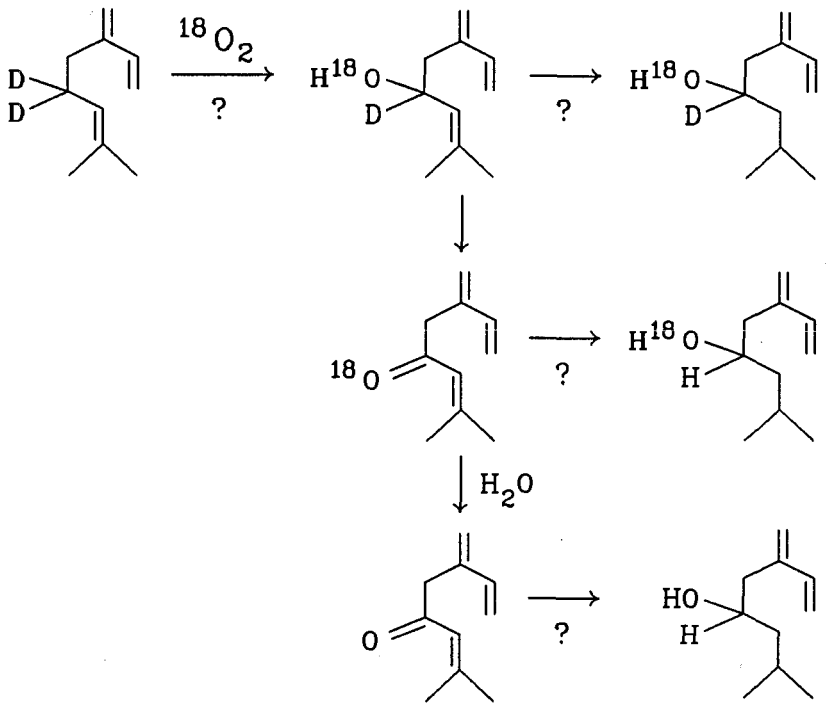


Figure 60: Expected pattern of incorporation of ^{18}O from $^{18}O_2$ into ipsenol through an ipsdienone intermediate.

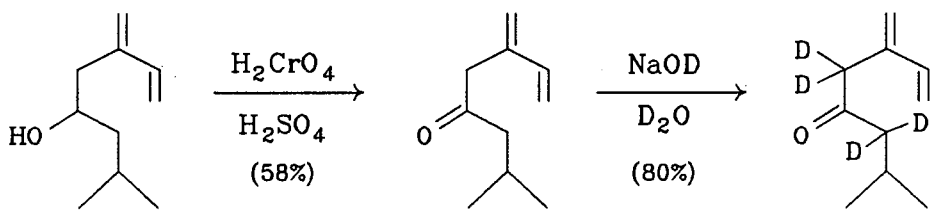
For these reasons, it was suspected that ipsenol could also be synthesized through an alternate path where ipsdienol is not the ultimate

precursor to ipsenol. One possibility is that ipsenol is formed directly through the reduction of ipsdienone. Evidence in favor of this hypothesis is that MPB and both populations of *I. pini* (which do not normally produce ipsdienol) produced small quantities of ipsenol when exposed to ipsdienone.

Another possibility is that ipsdienone could be reduced to ipsenone, which then might serve as the direct precursor to ipsenol. Ipsenone has been detected in the vapors of *I. paraconfusus* (Byers and Birgersson, 1990). The possibility that ipsenone is a precursor to ipsenol was tested by exposing beetles to deuterated ipsenone, and checking for the production of deuterium-enriched ipsenol.

a) Synthesis of [3,3,5,5-D_4]-ipsenone

[3,3,5,5-D_4]-Ipsenone was synthesized according to the route in Scheme 14. Ipsenol was oxidized to ipsenone by a Jones oxidation. Deuteriums were inserted through the base-catalyzed exchange of the acidic protons with the deuteriums of D_2O. Unfortunately, the reaction was terminated before the hydrogen exchange was complete, so that the "D_4-ipsenone" was in fact 21% unlabelled, 5% enriched for one deuterium, 14% enriched for two deuteriums, 22% enriched for three deuteriums, and 38% enriched for four deuteriums.



58 D_4–59

Scheme 14: Synthetic route to [3,3,5,5-D_4]-ipsenone (D_4-**59**).

b) Exposure of beetles to D_4-ipsenone

All beetles exposed to D_4-ipsenone produced ipsenol enriched in deuterium (Tables 54 and 55). The ipsenol produced by the beetles was not as enriched in deuterium as the starting material (Table 55), likely due to the exchange of the deuteriums (in acidic positions) with the hydrogens of water *in vivo*. *I. paraconfusus* did not produce significantly more ipsenol than the other beetles tested (Table 54). This was surprising, since *I. paraconfusus* was the only species known to produce ipsenol under normal conditions. It is not clear why Fish *et al.* (1984) did not detect ipsenol in extracts of *I. paraconfusus* that had been exposed to ipsenone.

Table 54: Quantity of ipsenol produced by beetles exposed to ipsenone[1].

Beetle species (population)	Quantity ipsenol in extract (ng/beetle, $\bar{x}$ ± SE)
I. paraconfusus	118 ± 16 (3)^{abc}
I. pini (K)	195 ± 13 (2)^{a}
I. pini (P)	90 ± 22 (4)^{b}
MPB	83 ± 4 (4)^{c}

[1] Each sample was prepared from three beetles.

Ipsdienone and ipsenone were converted to ipsenol equally well by *I. paraconfusus* (Figure 61). There was no statistically significant difference between the quantities of ispenol produced by *I. paraconfusus* exposed to ipsenone, ipsdienone, or ipsdienol. The quantity of ipsenol produced after exposure to myrcene was significantly less than that after exposure to ipsdienol or ipsdienone.

Table 55: Incorporation of deuterium into ipsenol produced by beetles exposed to D_4-ipsenone. [Relative abundances of peaks of interest by GC/SIM-MS (CI)].

Sample[1]	Relative abundance ($\bar{x}$ ± SE) F^+	$(F+1)^+$	$(F+2)^+$	$(F+3)^+$	$(F+4)^+$
D_4-ipsenone	51	16	36	58	100
I. paraconfusus (3)	100	46 ± 5	74 ± 7	74 ± 2	63 ± 2
I. pini (K) (3)	100	43 ± 1	63 ± 2	80 ± 2	56 ± 1
I. pini (P) (3)	100	49 ± 3	64 ± 3	90 ± 2	61 ± 1
MPB (3)	100	42 ± 4	55 ± 2	78 ± 3	59 ± 3

[1] Each sample was prepared from three beetles.

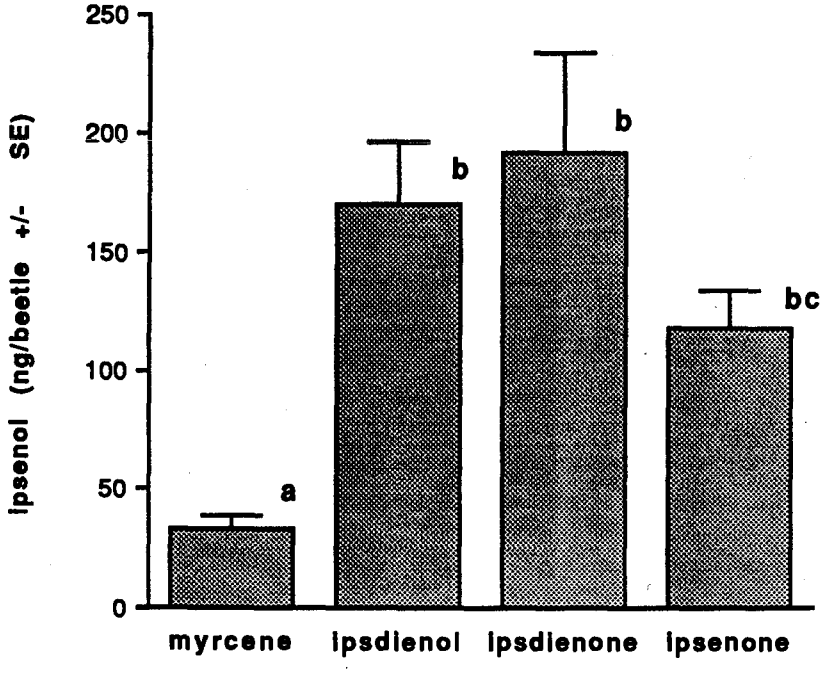


Figure 61: Quantities of ipsenol produced by male *I. paraconfusus* exposed to various precursors.

Thus, for reasons listed earlier, there is evidence for an alternate route to ipsenone where ipsdienol is not the ultimate precursor. I believe that this route is through the reduction of ipsenone, formed by the reduction of ipsdienone. Although *I. paraconfusus* converted ipsdienone and ipsenone to ipsenol equally well, MPB and both populations of *I. pini* produced only small quantities of ipsenol after exposure to ipsdienone, but produced about the same quantity of ipsenone as *I. paraconfusus* when exposed to ipsenone (Table 54).

The data presented herein do not provide conclusive evidence that ipsenol is formed through the reduction of ipsdienone or ipsenone. This will likely only be provided by *in vitro* experiments. It would have been very interesting to determine the chirality of the ipsenol produced from exogenous ipsdienone and ipsenone, but unfortunately time constraints prohibited such studies.

7. Possible mechanism for the biological resolution of ipsdienol in *I. pini*

Steve Seybold (personal communication*) has suggested that an epimerase might be involved in biological resolution of ipsdienol in some bark beetles. The trend observed in Figure 59, that the more (*R*)-ipsdienol produced, the more deuterium is lost by the (*R*)-ipsdienol, can be interpreted in this light. Some epimerases are believed to act through oxidation at the carbinol carbon followed by reduction to the other isomer (Martin *et al.*, 1985; Flentke and Frey, 1990; Wainwright *et al.*, 1990). The conversion of (*S*)-ipsdienol to (*R*)-ipsdienol by such an epimerase would be consistent with the data for *I. pini* (K), namely: (1) exogenous ipsdienone is reduced to (*R*)-ipsdienol; (2) the conversion of [5-D]-myrcene to ipsdienol proceeds with a greater loss of deuterium by the (*R*) isomer of ipsdienol; (3) the correlation between deuterium-loss in (*R*)-

---

* Department of Entomology, University of California, 201 Wellman Hall. Berkeley, CA. 94720.

ipsdienol and decreased production of (*S*)-ipsdienol demonstrated in Figure 59 was highly significant when the data for *I. pini* was considered; and (4) *I. pini* (K) produces more (*R*)-ipsdienol than (*S*)-ipsdienol. It would be interesting to determine if *I. pini* (K) produces (*R*)-ipsdienol after exposure to (*S*)-ipsdienol.

Although such a mechanism might operate in *I. pini* (K), it apparently does not operate in *I. paraconfusus*. Fish *et al.* (1979) reported that *I. paraconfusus* exposed to (*S*)-ipsdienol did not produce ipsenol (which is formed from (*R*)-ipsdienol).

8. Possible mechanism for the biological resolution of ipsdienol by *I. paraconfusus*

This study does not support the hypothesis of Fish *et al.* (1984) that an enantioselective oxidation of (*R*)-ipsdienol to ipsdienone, and a reduction of ipsdienone to both (*R*)- and (*S*)-ipsdienol, is involved in the biological resolution of ipsdienol by *I. paraconfusus*. Firstly, as discussed previously, an increase in the proportion of (*S*)-ipsdienol produced did not correlate with an increase in the interconversion (Figure 59, Table 53). Secondly, this study failed to corroborate the report by Fish *et al.* (1984) that the reduction of ipsdienone forms appreciable quantities of (*S*)-ipsdienol. In the present study, *I. paraconfusus* reduced ipsdienone to largely (*R*)-ipsdienol. As discussed previously, it seems likely *I. paraconfusus* produces ipsenol through two pathways: through the direct reduction of (*R*)-ipsdienol, and through the indirect reduction of (*R*)-ipsdienol through an ipsdienone intermediate (which may proceed through an ipsenone intermediate). When *I. paraconfusus* was exposed to D_2-myrcene, 96% of the ipsdienol was deuterated, as compared to 25% of the ipsenol (Table 50). This indicates that about 26% of the ipsenol was synthesized through direct reduction of ipsdienol. If the same proportion held true when *I. paraconfusus* was exposed to D_2-ipsdienone, then about 50 ng of ipsenol was synthesized through the

reduction of (*R*)-ipsdienol (26% of the 192 ± 42 ng ipsenol/beetle, Table 48). The ipsdienol remaining in the extract (11 ± 3 ng/beetle) was 85.5% *R*, indicating that there was about 9.4 ng (*R*)- and 1.6 ng (*S*)-ipsdienol. These calculations indicate that, overall, ipsdienone was reduced to (*R*)-ipsdienol with an ee of about 96%*.

A possible mechanism for the biological resolution of ipsdienol in *I. paraconfusus* could involve the selective removal of (*R*)-ipsdienol through conversion to (*S*)-ipsenol (either through direct reduction or through reduction via the ipsdienone intermediate, as shown in Figure 62). This process should effectively increase the ee of (*S*)-ipsdienol that is ultimately emitted by the beetle.

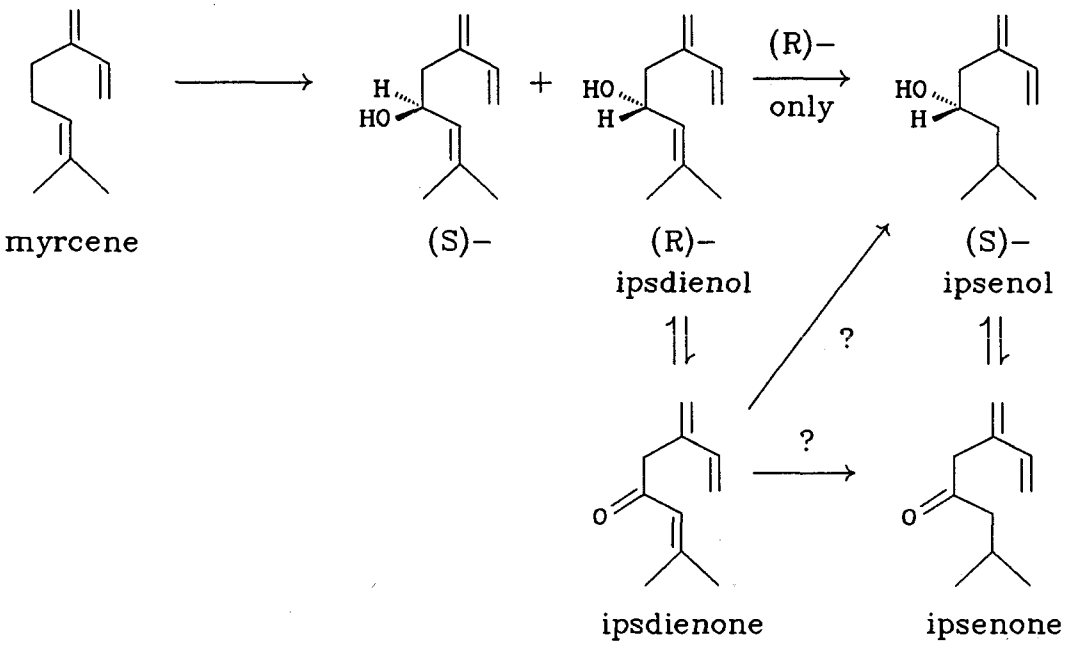


Figure 62: Possible mechanism for the biological resolution of ipsdienol in *I. paraconfusus*. (The conversion of ipsdienone to ipsenone is not represented by equilibrium arrows since no ipsdienol was detected in the extracts of *I. paraconfusus* exposed to ipsenone).

---

* These figures do not take into account the report by Fish *et al.* (1984) that the (*S*)-ipsenol formed from ipsdienone has an ee of only 87%. If this was true in this study, then the overall ee of the (*R*)-ipsdienol formed from ipsdienone in this study would be about 92%.

9. Possible mechanism for the biological resolution of ipsdienol by MPB

The data presented in this thesis do not indicate the mechanism of the biological resolution of ipsdienol in MPB. It seems unlikely that an ipsdienol-ipsdienone equilibrium is involved since the chirality of the ipsdienol produced upon the reduction of ipsdienone was almost racemic. It is possible that one of the isomers of ipsdienol is enantioselectively rearranged to as yet unknown products. It is also possible that the initial products of the oxidation of myrcene to ipsdienol are the products ultimately emitted by the beetle.

D) CONCLUSIONS

This study supports earlier reports by Fish *et al.* (1979, 1984) that an equilibrium between ipsdienol and ipsdienone is involved in ipsdienol and ipsenol biosynthesis in *I. paraconfusus*. As reported by Fish *et al.* (1979, 1984), ipsdienol and ipsenol recovered from beetles exposed to ipsdienol deuterated at the carbinol carbon were less enriched than the starting material. Exogenous ipsdienone was reduced to (*R*)-ipsdienol and ipsenol.

Evidence presented in this study does not support the hypotheses of Fish *et al.* (1984) that an enantioselective oxidation of (*R*)-ipsdienol to ipsdienone, and a reduction of ipsdienone to both (*R*)- and (*S*)-ipsdienol, is involved in the biological resolution of ipsdienol by *I. paraconfusus*.

Furthermore the results of this study indicate that, although some (*S*)-ipsenol may be formed through the direct reduction of (*R*)-ipsdienol, the major biosynthetic route to ipsenol in *I. paraconfusus* proceeds through reduction of ipsdienone (which may proceed through an ipsenone intermediate). Since (*R*)-ipsdienol is the precursor to ipsdienone, this route provides a mechanism for the biological resolution of ipsdienol by *I. paraconfusus*.

Like *I. paraconfusus*, MPB and both populations of *I. pini* studied reduced exogenous ipsdienone to ipsdienol. MPB produced (*R*)-ipsdienol with an ee of 15%, while *I. pini* (K) and (P) produced (*R*)-ipsdienol of 97% and 98% ee, respectively. These bark beetles did not effect a detectable loss in deuterium from the carbinol carbon of ipsdienol. However the ipsdienol produced by these beetles when exposed to myrcene deuterated at the pre-carbinol carbon did exhibit a loss of deuterium. The more (*R*)-ipsdienol produced by these beetles, the more deuterium was lost. It was suggested that an epimerase, catalyzing the conversion of (*S*)-ipsdienol to (*R*)-ipsdienol, might be involved in regulating the chirality of the ipsdienol ultimately emitted by *I. pini*.

CONCLUDING REMARKS

Pheromone biosynthesis in several species of bark and grain beetles was studied using both radio- and stable isotope-labelling techniques. Radiolabelled precursors were most useful to study the biosynthesis of pheromones from substrates incorporated at very low levels (such as through *de novo* biosynthesis from acetate and mevalonate). Stable isotope-labelled precursors were more useful to study the biosynthesis of pheromones from more immediate precursors. The incorporation of stable isotopes was determined by GC/SIM-MS, obviating requirements to extensively purify the pheromones prior to analyses.

The information collected in this study supports the idea that coleopteran pheromones are often produced through adaptations of "normal" biosynthetic pathways (Vanderwel and Oehlschlager, 1987). The macrolide aggregation pheromones produced by the cucujid grain beetles *C. ferrugineus* and *O. mercator* were formed through modifications of fatty acid and terpenoid metabolism (*Chapter II*). Ipsdienol, utilized as an aggregation pheromone by many bark beetles, is apparently formed through the modification of normal monoterpene-detoxification pathways (references summarized in Vanderwel and Oehlschlager, 1987; *Chapter IV*). The biosynthetic origins of the cyclic ketals *endo*- and *exo*-brevicomin remain to be determined.

Though the biosynthesis of coleopteran pheromones may have evolved from basic metabolic pathways, considerable sophistication has crept into the scheme. Bark beetles are apparently able to control the chirality of the pheromones that they emit, through several ingenious biosynthetic mechanisms. In MPBs, both enantiomers of 6,7-epoxynonan-2-one were converted to (+)-*exo*-brevicomin. In the *Ips* bark beetles, the chirality of ipsdienol ultimately emitted by the insects may be manipulated through the selective removal of one enantiomer of ipsdienol, or through the conversion of one enantiomer to the other.

Studies such this only begin to enhance our appreciation and

understanding of coleopteran biochemistry. Continued investigations should yield information that will not only satisfy academic curiosity, but should lead the way to environmentally-acceptable pest control strategies.

LITERATURE CITED

Ahmad, S. (1986). Enzymatic adaptations of herbivorous insects and mites to phytochemicals. *J. Chem. Ecol.* 12: 533-559.

Anderson, W. K. and Veysoglu, R. (1973). A simple procedure for the epoxidation of acid-sensitive olefinic compounds with *m*-chloroperbenzoic acid in an alkaline biphasic solvent system. *J. Org. Chem.* 38:2267-2268.

Armstrong, R. N. (1987). Enzyme-catalyzed detoxication reactions: mechanisms and stereochemistry. *CRC Crit. Rev. Biochem.* 22: 39-88.

Bakke, A., Froyen, P., and Skattebol, L. Field. (1977). Field response to a new pheromonal compound isolated from *Ips typographus*. *Naturwiss.* 64: 98-99.

Barth, Jr., R. H. (1965). Insect mating behavior: endocrine control of a chemical communication system. *Science*. 149: 882-883.

Bedard, W. D., Tilden, P. E., Lindahl, K. Q., Wood, D. L., and Rauch, P. A. (1980). Effects of verbenone and *trans*-verbenol on the response of *Dendroctonus brevicomis* to natural and synthetic attractant in the field. *J. Chem. Ecol.* 6: 997-1013.

Bellas, T. E., Brownlee, R. G. and Silverstein, R. M. (1969). Synthesis of brevicomin, principal sex attractant in the frass of the female Western Pine Beetle. *Tetrahedron* 25: 5149-5153.

Birch, M. C., Light, D. M., Wood, D. L., Browne, L. E., Silverstein, R. M., Bergot, B. J., Ohloff, G., West, J. R., and Young, J. C. (1980). Pheromone attraction and allomonal interruption of *Ips pini* in California by the two enantiomers of ipsdienol. *J. Chem. Ecol.* 6: 703-717.

Birgersson, G., Schlyter, F., Lofqvist, J., and Bergstrom, G. (1984). Quantitative variation of pheromone componenents in the spruce bark beetle *Ips typographus* from different attack phases. *J. Chem. Ecol.* 10: 1029-1055.

Bjostad, L. B., Linn, C. E., Du, J.-W., and Roelofs, W. L. (1984). Identification of new sex pheromone components in *Trichoplusia ni*, predicted from biosynthetic precursors. *J. Chem. Ecol.* 10: 1309-1323.

Bjostad, L. B., Wolf W. A. and Roelofs W. L. (1987) Pheromone biosynthesis in lepidopterans: desaturation and chain shortening. pp. 77-120 **in** *Pheromone Biochemistry* (G. D. Prestwich and G. J. Blomquist, eds.) Academic Press, Inc. Orlando, Florida, U.S.A.

Bligh, E. G., and Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Can. J. Biochem. and Physiol.* 37: 911-917.

Blomquist, G. J., Dwyer, L. A., Chu, A. J., Ryan, R. O. and de Renobales M. (1982) Biosynthesis of linoleic acid in a termite, cockroach and cricket. *Insect Biochem.* 12: 349-353.

Blomquist, G. J., Dillwith, J. W., and Adams, T. S. (1987). Biosynthesis and endocrine regulation of sex pheromone production in diptera. pp. 217-250 **in** *Pheromone Biochemistry* (G. D. Prestwich and G. J.

Blomquist, eds.) Academic Press, Inc. Orlando, Florida, U.S.A.

Borch, R. F., Bernstein, M. D., Durst, H. D. (1971) The cyanohydridoborate anion as a selective reducing agent. *J. A. C. S.* 93: 2897-2904.

Borden, J. H. (1985). Aggregation pheromones pp. 257-285 in *Comprehensive Insect Physiology Biochemistry and Pharmacology*, Volume 9 (G. A. Kerkut and L. I. Gilbert, eds.) Pergamon Press.

Borden, J. H., Nair, K. K., and Slater, C. E. (1969). Synthetic juvenile hormone: induction of sex pheromone production in *Ips confusus*. *Science*. 166: 1626-1627.

Borden, J. H., Dolinski, M. G., Chong, L., Verigin, V., Pierce, H. D., Jr. and Oehlschlager, A. C. (1979). Aggregation pheromone in the rusty grain beetle, *Cryptolestes ferrugineus* (Coleoptera: Cucujidae). *Can. Ent.* 111: 681-688.

Borden, J. H., Hunt, D. W. A., Miller, D. R., and Slessor, K. N. (1986). Orientation in forest Coleoptera: an uncertain outcome of responses by individual beetles to variable stimuli. in *Mechanisms in Insect Olfaction* (T. L. Payne and M. C. Birch, eds.) Oxford Univ. Press, Oxford.

Borden, J. H., Pierce, A. M., Pierce, H. D., Jr., Chong, L. J., Stock, A. J. and Oehlschlager, A. C. (1987a). Semiochemicals produced by western balsam bark beetle, *Dryocoetes confusus* Swaine (Coleoptera: Scolytidae). *J. Chem. Ecol.* 13:823-836.

Borden, J. H., Ryker, L. C., Chong, L. J., Pierce, H. D., Jr., Johnston, B. D., and Oehlschlager, A. C. (1987b). Response of the mountain pine beetle, *Dendroctonus ponderosae* Hopkins (Coleoptera: Scolytidae) to five semiochemicals in British Columbia lodgepole pine forests. *Can. J. For. Res.* 17: 118-128.

Bradshaw, J. W. S. (1985). Insect natural products--compounds derived from acetate, shikimate and amino acids in *Comprehensive Insect Physiology Biochemistry and Pharmacology* (G. A. Kerkut and L. I. Gilbert, eds.) 11: 655-703. Pergamon Press.

Brand, J. M., and S. J. Barras. (1977). The major volatile constituents of a basidiomycete associated with the southern pine beetle. *Lloydia*. 40: 398-400.

Brand, J. M., Bracke, J. W., Markovetz, A. J., Wood, D. L., Browne, L. E. (1975). Production of verbenol pheromone by a bacterium isolated from bark beetles. *Nature*. 254: 136-137.

Brand, J. M., Bracke, J. W., Britton, L. N., Markovetz, A. J., and Barras, S. J. (1976). Bark beetle pheromones: production of verbenone by a mycangial fungus of *Dendroctonus frontalis*. *J. Chem. Ecol.* 2: 195-199.

Brand, J. M., Schultz, J., Barras, S. J., Edson, L. J., Payne, T. L., and Hedden, R. L. (1977). Bark-beetle pheromones. Enhancement of *Dendroctonus frontalis* (Coleoptera: Scolytidae) aggregation pheromones by yeast metabolites in laboratory bioassays. *J. Chem. Ecol.* 3: 657-666.

Brand, J. M., Young, J. C., and Silverstein, R. M. (1979). Insect pheromones: a critical review of recent advances in their chemistry, biology and application. *Fortschritte d. Chem. Org. Naturst.* 37:

1-190.

Brandsma, L. (1971). *Preparative acetylenic chemistry*. Elsevier Publishing Company. Amsterdam.

Bright, D. E., Jr. (1976). The insects and arachnids of Canada and Alaska, 2. Coleoptera: Scolytidae. *Can Dep. Agric. Pub.* 1576.

Brown, C. A. and Ahuja, V. K. (1973). "P-2 Nickel" catalyst with ethylenediamine, a novel system for highly stereospecific reduction of alkynes to *cis*-olefins. *J.C.S. Chem. Comm.* 553-554.

Byers, J. A. (1983a). Bark beetle conversion of a plant compound to a sex specific inhibitor of pheromone attraction. *Science.* 220: 624-626.

Byers, J. A. (1983b). Influence of sex, maturity and host substances on pheromones in the guts of the bark beetles, *Ips paraconfusus* and *Dendroctonus brevicomis. J. Insect Physiol.* 29: 5-13.

Byers, J. A., and Birgersson, G. (1990). Pheromone production in a bark beetle independent of myrcene precursor in host pine species. *Naturwiss.* 77: 385-387.

Byers, J. A., and Wood, D. L. (1981). Antibiotic-induced inhibition of pheromone synthesis in a bark beetle. *Science.* 213: 763-764.

Byers, J. A., Wood, D. L., Browne, L. E., Fish, R. H., Piatek, B., and Hendry, L. B. (1979). Relationship between a host plant compound, myrcene and pheromone production in the bark beetle, *Ips paraconfusus. J. Insect Physiol.* 25: 477-482.

Campbell, I. M. (1974). Incorporation and dilution values--their calculation in mass spectrally assayed stable isotope labeling experiments. *Bioorg. Chem.* 3: 386-397.

Chararas, C. (1980). Physiologie des invertebres--Attraction primaire et secondaire chez trois especes de Scolytidae (*Ips*) et mecanisme de colonisation. *C. R. Acad. Sc. Paris, Series D.* 290: 375-378.

Chararas, C., Riviere, J., Ducauze, C., Rutledge, D., Delpui, G., and Cazelles, M. -T. (1980). Bioconversion d'un compose terpenique sous l'action d'une Bacterie du tube digestif de *Phloeosinus armatus* (Coleoptera, Scolytidae). *C. R. Acad. Sc. Paris, Series D.* 291: 299-302.

Chase, J., Jurenka, R., Schal, C., Halarnkar, P. P., and Blomquist, G. J. (1990). Biosynthesis of methyl branched hydrocarbons of the german cockroach *Blattella germanica* (L.) (Orthoptera, Blattellidae). *Insect Biochem.* 20: 149-156.

Clearwater, J. R. (1975). Pheromone metabolism in male *Pseudoletia separata* (Walk.) and *Mamestra configurata* (Walk.) (Lepidoptera: Noctuidae). *Comp. Biochem. Physiol.* 50B: 77-82.

Conn, J. E., Borden, J. H., Hunt, D. W. A., Holman, J., Whitney, H. S., Spanier, O. J., , H. D., Jr., and Oehlschlager, A. C. (1984). Pheromone production by axenically reared *Dendroctonus ponderosae* and *Ips paraconfusus* (Coleoptera: Scolytidae). *J. Chem. Ecol.* 10: 281-290.

Corey, E. J. and Suggs, J. W. (1975) Pyridinium chlorochromate. An efficient reagent for oxidation of primary and secondary

alcohols to carbonyl compounds. *Tett. Let.* 2647-2650.

Coyne, J. F., and Lott, L. H. (1976). Toxicity of substances in pine oleoresin to southern pine beetle. *J. Georgia. Entomol. Soc.* 11: 301-305.

Dillwith, J. W., Blomquist, G. J. and Nelson, D. R. (1981) Biosynthesis of the hydrocarbon components of the sex pheromone of the housefly, *Musca domestica* L. *Insect Biochem.* 11: 247-253.

Dowd, P. F., Smith, C. M., and Sparks, T. C. (1983). Detoxification of plant toxins by insects. *Insect Biochem.* 13: 453-468.

Evans, G. (1977). pp 17-21 in *The Life of Beetles.* George Allen & Unwin, Ltd. London, Great Britain.

Fish, R. H., Browne, L. E., Wood, D. L., and Hendry, L. B. (1979) Pheromone biosynthetic pathways: conversions of deuterium labelled ipsdienol with sexual and enantioselectivity in *Ips paraconfusus.* *Tet. Lett.* 17: 1465-1468.

Fish, R. H., Browne, L. E., and Bergot, B. J. (1984). Pheromone biosynthetic pathways: conversion of ipsdienone to (-)-ipsdienol, a mechanism for enantioselective reduction in the male bark beetle, *Ips paraconfusus. J. Chem. Ecol.* 10: 1057-1064.

Flentke, G. R., and Frey, P. A. (1990). Reaction of uridine diphosphate galactose 4-epimerase with a suicide inactivator. *Biochem.* 29: 2430-2436.

Folch, F., Lees, M., and Sloane Stanley, G. H. (1957). A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* 226: 497-509.

Francke, W., and Vité, J. P. (1983). Oxygenated terpenes in pheromone systems of bark beetles. *Z. angew. Ent.* 96: 146-156.

Francke, W., Heeman, V., Gerken, B., Renwick, J. A. A., and Vité, J. P. (1977). 2-Ethyl-1,6-dioxaspiro [4.4]nonane, principal aggregation pheromone of *Pityogenes chalcographus* (L.). *Naturwiss.* 64: 590-591.

Francke, W., Hindorf, G., and Reith, W. (1979). Alkyl-1,6-dioxaspiro[4.5]-decanes--a new class of pheromones. *Naturwiss.* 66: 618-619.

Galli, C., Illuminati, G., Mandolini, L. and Tamborra, P. 1977. Ring-closure reactions. 7. Kinetics and activation parameters of lactone formation in the range of 3- to 23-membered rings. *J. A. C. S.* 99: 2591-2597.

Gilman, A. G., Goodman, L. S., Rall, T. W. and Murad, F. (1985). *The Pharmacological Basis of Therapeutics* (7th Edition). Macmillan Publishing Company. New York, U.S.A.

Gonzalez-Pacanowska, D., B. Arison, C. M. Havel and J. A. Watson. (1988). Isopentenoid synthesis in isolated embryonic *Drosophila* cells: farnesol catabolism and *omega*-oxidation. *J. Biol. Chem.* 263: 1301-1306.

Gore, W. E., Pearce, G. T. and Silverstein, R. M. (1976). Mass spectrometric studies of the dioxabicyclo[3.2.1]octanes multistriatin, frontalin and brevicomin. *J. Org. Chem.* 41: 607-610.

Graham, K. (1967). Fungal-insect mutualism in trees and timber. *Ann. Rev. Entomol.* 12: 105-127.

Gries, G., Bowers, W. W., Gries, R., Noble, M., and Borden, J. H. (1990a). Pheromone production by the pine engraver *Ips pini* following flight and starvation. *J. Insect Physiol.* 36: 819-824.

Gries, G., Leufvén, A., Lafontaine, J. P., Pierce, Jr., H. D., Borden, J. H., Vanderwel, D., and Oehlschlager, A. C. (1990b). New metabolites of α-pinene produced by the mountain pine beetle, *Dendroctonus ponderosae* (Coleoptera: Scolytidae). *Insect Biochem.* (in press).

Gries, G., Smirle, M. J., Leufvén, A., Miller, D. R., Borden, J. H. and Whitney, H. S. (1990c). Conversion of phenylalanine to toluene and 2-phenylethanol by the pine engraver *Ips pini* (Say) (Coleoptera, Scolytidae). *Experentia* 329-331.

Gueldner, R. C., Sikorowski, P. P., Wyatt, J. M. (1977). Bacterial load and pheromone production in the boll weevil, *Anthonomus grandis. J. Inv. Path.* 29: 397-398.

Guengerich, F. P. and Macdonald, T. L. (1984). Chemical mechanisms of catalysis by cytochromes P-450: a unified view. *Acc. Chem. Res.* 17:9-16.

Hackstein, E., and Vité, J. P. (1978). Pheromon biosynthese und Reizkett in der Besiedlung von Fichten durch den Buchdrucker *Ips typographus. Mitt. dtsch. Ges. allg. angew. Ent.* 1: 185-188.

Hardee, D. D. (1970). Pheromone production by male boll weevils as affected by food and host factors. *Contr. Boyce Thompson Inst.* 24: 315-322.

Harring, C. M. (1978). Aggregation pheromones of the European fir engraver beetles *Pityokteines curvidens, P. spinidens* and *P. vorontzovi* and the role of juvenile hormone in pheromone biosynthesis. *Z. angew. Entomol.* 85: 281-317.

Hedin, P. A., Thompson, A. C., Gueldner, R. C., and Minyard, J. P. (1971). Malvaceae: constituents of the cotton bud. *Phytochem.* 10: 3316-3318.

Hedin, P. A., Rollins, C. S., Thompson, A. C., and Gueldner, R. C. (1975). Pheromone production of male boll weevils treated with chemosterilants. *J. Econ. Ent.* 68: 587-591.

Hendry, L. B., Piatek, B., Browne, L. E., Wood, D. L., Byers, J. A., Fish, R. H., and Hicks, R. A. (1980). *In vivo* conversion of a labelled host plant chemical to pheromones of the bark beetle *Ips paraconfusus. Nature.* 284: 485.

Hoyt, C. P., Osborne, G. O., and Mulcock, A. P. (1971). Production of an insect sex attractant by symbiotic bacteria. *Nature.* 230: 472-473.

Hughes, P. R. (1973a). *Dendroctonus*: production of pheromones and related compounds in response to host monoterpenes. *Z. angew. Entomol.* 73: 294-312.

Hughes, P. R. (1973b). Effect of α-pinene exposure on *trans*-verbenol synthesis in *Dendroctonus ponderosae. Naturwiss.* 60: 261-262.

Hughes, P. R. (1974). Myrcene: a precursor of pheromones in *Ips* beetles.

J. Insect Physiol. 20: 1271-1275.

Hughes, P. R. (1975). Pheromones of *Dendroctonus*: origin of α-pinene oxidation products present in emergent adults. *J. Insect Physiol.* 21: 687-691.

Hughes, P. R., and Renwick, J. A. A. (1977). Neural and hormonal control of pheromone biosynthesis in the bark beetle, *Ips paraconfusus*. *Physiol. Ent.* 2: 117-123.

Hunt, D. W. A. and Borden, J. H. (1989). Terpene alcohol pheromone production by *Dendroctonus ponderosae* and *Ips paraconfusus* (Coleoptera: Scolytidae) in the absence of readily culturable microorganisms). *J. Chem. Ecol.* 15: 1433-1463.

Hunt, D. W. A. and Smirle, M. J. (1988). Partial inhibition of pheromone production in *Dendroctonus ponderosae* (Coleoptera: Scolytidae) by polysubstrate monooxygenase inhibitors. *J. Chem. Ecol.* 14: 529-536.

Hunt, D. W. A., Borden, J. H., Pierce, H. D., Jr., Slessor, K. N., King, G. G. S., and Czyzewska, E. (1986). Sex-specific production of ipsdienol and myrcenol by *Dendroctonus ponderosae* (Coleoptera: Scolytidae) exposed to myrcene vapors. *J. Chem. Ecol.* (In press).

Klimetzek, D., and Francke, W. (1980). Relationship between the enantiomeric composition of α-pinene in host trees and the production of verbenols in *Ips* species. *Experentia*. 36: 1343-1345.

Klusener, P. A. A., Kulik, W. and Brandsma, L. (1987). Regiospecific functionalization of dimetalated isopropenylacetylene, a synthetic equivalent of the isoprene "anion". An efficient synthesis of the bark beetle pheromone (±)-ipsenol and (±)-ipsdienol. *J. Org. Chem.* 52: 5261-5266.

Johnston, J., Faulkner, J. D., Manderll, L. and Day, Jr., R. A. (1976). Electrochemical reduction of geranial, farnesal, and crotonaldehyde. *J. Org. Chem.* 15: 2611-2614.

Lanier, G. N., Classon, A. Stewart, T., Piston, J. J., and Silverstein, R. M. (1980). *Ips pini*: the basis for interpopulational differences in pheromone biology. *J. Chem. Ecol.* 6: 677-687.

Lanne, B. S., Ivarsson, P., Johnsson, P., Bergström, G., and Wassgren, A.-B. (1989). Biosynthesis of 2-methyl-3-buten-2-ol, a pheromone component of *Ips typographus* (Coleoptera: Scolytidae). *Insect Biochem.* 19: 163-167.

Leufven, A., Bergstrom, G., and Falsen, E. (1984). Interconversion of verbenols and verbenone by identified yeasts isolated from the spruce bark beetle *Ips typographus*. *J. Chem. Ecol.* 10: 1349-1361.

Libbey, L. M., Ryker, L. C., and Yandell, K. L. (1985). Laboratory and field studies of volatiles released by *Dendroctonus ponderosae*. *Z. angew. Entomol.* 100: 381-392.

Lyon, R. L. 1958. A useful secondary sex character in *Dendroctonus* bark beetles. *The Can. Entomol.* 582-584.

Mancuso, A. J., Huang, S.-L., and Swern, D. (1978). Oxidation of long-chain and related alcohols to carbonyls by dimethyl sulfoxide "activated" oxalyl chloride. *J. Org. Chem.* 43: 2480-2482.

Martin, D. W. Jr, Mayes, P. A., Rodwell, V. W., and Granner, D. K. (1985). pp. 110-112 and p. 191 **in** *Harper's Review of Biochemistry*. Lange Medical Publications. Los Altos, California.

Mecham, D. K. (1971). Lipids. pp. 393-451 **in** *Wheat: Chemistry and Technology* (Y. Pomeranz, ed.). American Association of Cereal Chemists, Incorporated. St. Paul, Minnesota, U.S.A.

Meijer, J., and DePierre, J. W. (1988). Cytosolic epoxide hydrolase. *Chem.-Biol. Interactions*. 64: 207-249.

Millar, J. G. J. O. (1983). Isolation and identification of aggregation pheromones for *Cryptolestes pusillus* (Schönherr) and *Cryptolestes turcicus* (Grouvelle) and the synthesis of macrolide pheromones for *Cryptolestes* and *Oryzaephilus* species (Coleoptera: cucujidae). Ph. D. Thesis, submitted to Department of Chemistry, Simon Fraser University, Burnaby, B.C., Canada.

Millar J. G., Pierce H. D., Jr., Pierce A. M., Oehlschlager A.C. and Borden J. H. (1985a) Aggregation pheromones of the grain beetle, *Cryptolestes turcicus* (Coleoptera: Cucujidae). *J. Chem. Ecol.* 11: 1071-1081.

Millar J. G., Pierce H. D., Jr., Pierce A. M., Oehlschlager A. C., Borden J. H. and Barak A. V. (1985b) Aggregation pheromones of the flat grain beetle, *Cryptolestes pusillus* (Coleoptera: Cucujidae). *J. Chem. Ecol.* 11: 1053-1070.

Miller, D. R., Borden, J. H., and Slessor, K. N. (1989). Inter- and intrapopulation variation of the pheromone, ipsdienol produced by male pine engravers, *Ips pini* (Say) (Coleoptera: Scolytidae). *J. Chem. Ecol.* 15: 233-247.

Miller, D. R., Gries, G., and Borden, J. H. (1990). *E*-Myrcenol: a new pheromone for the pine engraver, *Ips pini* (Say) (Coleoptera, Scolytidae). *Can. Ent.* 122: 401-406.

Mitlin, N., and Hedin, P. A. (1974). Biosynthesis of grandlure, the pheromone of the boll weevil, *Anthonomus grandis*, from acetate, mevalonate, and glucose. *J. Insect Physiol.* 20: 1825-1831.

Monsen, R. S. (1971). Functional group modifications: catalytic hydrogenation. *Advanced organic synthesis: methods and techniques*, pp. 39-44. Academic Press, New York.

Moore, R. N., Golumbic, C., and Fisher, G. S. (1956). Autoxidation of α-pinene. *J. A. C. S.* 78: 1173-1176.

Noyori, R., Tomino, I., Yamada, M., and Nishizawa, M. (1984) Synthetic applications of the enantioselective reduction by binaphthol-modified lithium aluminum hydride reagents. *J. A. C. S.* 106: 6717-6725.

Oehlschlager, A. C., Czyzewska, E., Aksela, R., and Pierce, Jr., H. D. (1986). Improved syntheses of hydroxy acid precursors of macrolide pheromones of cucujid grain beetles. *Can. J. Chem.* 64: 1407-1413.

Oehlschlager, A. C., King, G. G. S., Pierce, H. D., Jr., Pierce, A. M., Slessor, K. N., Millar, J. G. and Borden, J. H. (1987) Chirality of macrolide pheromones of grain beetles in the genera *Oryzaephilus* and *Cryptolestes* and its implications for species specificity. *J. Chem.*

Ecol. 13:1543-1554.

Oehlschlager, A. C., Pierce, A. M., Pierce, H. D., Jr. and Borden, J. H. (1988). Chemical communication in cucujid grain beetles. *J. Chem. Ecol.* 14: 2069-2096.

Pant, N. C., and Dang, K. (1972). Physiology and elimination of intracellular symbiotes in some stored product beetles. pp. 311-322 in *Insect and Mite Nutrition*. North-Holland, Amsterdam.

Pearce, G. T., Gore, W. E. and Silverstein, R. M. (1977) Carbon-13 spectra of some insect pheromones and related compounds of the 6,8-dioxabicyclo (3.2.1) octane system. *J. Mag. Res.* 27: 497-507.

Phillips, J. K., C. A. Walgenbach, J. A. Klein, W. E. Burkholder, N. R. Schmuff and H. M. Fales. (1985). (*R**,*S**)-5-hydroxy-4-methyl-3-heptanone: male-produced aggregation pheromone of *Sitophilus oryzae* (L.) and *S. zeamais* Motsch. *J. Chem. Ecol.* 11: 1263-1274.

Pierce, A. M., Borden, J. H., and Oehlschlager, A. C. (1981). Olfactory response to beetle-produced volatiles and host-food attractants by *Oryzaephilus surinamensus* and *O. mercator*. *Can. J. Zool.* 59: 1980-1990.

Pierce, A. M., Pierce, H. D., Jr., Millar, J. G., Borden, J. H., and Oehlschlager, A. C. (1984a). Aggregation pheromones in the genus *Oryzaephilus* (Coleoptera: Cucujidae). *Proc. Third Intern. Working Conf.* on *Stored-Prod. Entomol.*, Manhattan, Kansas. pp. 107-120.

Pierce, H. D., Jr., Pierce, A. M., Millar, J. G., Wong, J. W., Verigin, V. G., Oehlschlager, A. C., and Borden, J. H. (1984b) Methodology for isolation and analysis of aggregation pheromones in the genera *Cryptolestes* and *Oryzaephilus* (Coleoptera: Cucujidae). *Proc. Third Intern. Working Conf. on Stored-Prod. Entomol.*, Manhattan, Kansas. pp. 121-137.

Pierce, A. M., Pierce, H. D., Jr., Oehlschlager, A. C., and Borden, J. H. (1985). Macrolide aggregation pheromones in *Oryzaephilus surinamensis* and *Oryzaephilus mercator* (Coleoptera: Cucujidae). *J. Agric. Food Chem.* 33: 848-852.

Pierce, A. M., Pierce, H. D., Jr., Borden, J. H., and Oehlschlager, A. C. (1986). Enhanced production of aggregation pheromones in four stored-product coleopterans feeding on methoprene-treated oats. *Experentia*. 164-165.

Pierce, A. M., Pierce, H. D., Jr., Oehlschlager, A. C., Czyzewska, E., and Borden, J. H. (1987). Influence of pheromone chirality on response by *Oryzaephilus surinamensis* and *O. mercator* (Coleoptera: Cucujidae). *J. Chem. Ecol.* 13: 1525-1542.

Pierce, H. D., Jr., Conn, J. E., Borden, J. H., and Oehlschlager, A. C. (1987). Monoterpene metabolism in female mountain pine beetles, *Dendroctonus ponderosae* Hopkins, attacking ponderosa pines. *J. Chem. Ecol.* 13: 1455-1480.

Pitman, G. B., Vite, J. P., Kinzer, G. W., and Fentiman, A. F., Jr. (1969). Specificity of population-aggregating pheromones in *Dendroctonus*. *J. Insect Physiol.* 15: 363-366.

Plummer, E. L., Stewart, T. F., Byrne, K., Pearce, G. T., and Silverstein, R. M. (1976). Determination of enantiomeric composition of several

insect pheromone alcohols. *J. Chem. Ecol.* 2: 307-331.

Prestwich, G. D., Yamaoka, R., Phirwa, S., DePalma, A., Angelastro, M., and Gayen, A. K. (1984). Harnessing insect-specific enzymes to activate novel proinsecticides. pp. 127-143 in *Pesticide Synthesis through Rational Approaches* (American Chemical Society).

Prestwich, G. D., Yamaoka, R., and Carvalho, J. F. (1985). Metabolism of tritiated omega-fluorofatty acids and alcohols in the termite *Reticulitermes flavipes* (Kollar) (Isoptera, Rhinotermitidae). *Insect Biochem.* 15: 205-209.

Prestwich, G. D., Graham, S. McG., and Konig, W. A. (1989). Enantioselective opening of (+)- and (-)-disparlure by epoxide hydrase in gypsy moth antennae. *J. Chem. Soc. Chem. Commun.* 575-577.

Raffa, K. F., and Berryman, A. A. (1983). The role of host plant resistance in the colonization behavior and ecology of bark beetles (Coleoptera: Scolytidae). *Ecol. Monographs.* 53: 27-49.

Raffa, K. F., Berryman, A. A., Simasko, J., Teal, W., and Wong, B. L. (1985). Effects of Grand Fir monoterpenes on the fir engraver, *Scolytus ventralis* (Coleoptera: Scolytidae), and its symbiotic fungus. *Environ. Entomol.* 14: 552-556.

de Renobales M., Cripps C., Stanley-Samuelson D. W., Jurenka R. A. and Blomquist G. J. (1987) Biosynthesis of linoleic acid in insects. *TIBS* 12: 364-366.

Renwick, J. A. A., and Dickens, J. C. (1979). Control of pheromone production in the bark beetle, *Ips cembrae*. *Physiol. Ent.* 4: 377-381.

Renwick, J. A. A., Hughes, P. R., and Ty, T. D. (1973). Oxidation products of pinene in the bark beetle, *Dendroctonus frontalis*. *J. Insect Physiol.* 19: 1735-1740.

Renwick, J. A. A., Pitman, G. B., and Vite, J. P. (1976a). 2-Phenylethanol isolated from bark beetles. *Naturwiss.* 63: 198.

Renwick, J. A. A., Hughes, P. R., and Krull, I. S. (1976b). Selective production of *cis*- and *trans*-verbenol from (-)- and (+)-α-pinene by a bark beetle. *Science* 191: 199-200.

Renwick, J. A. A., Vité, J. P., and Billings, R. F. (1977). Aggregation pheromones in the ambrosia beetle, *Platypus flavicornis*. *Naturwiss.* 64: 226.

Rodin, J. O., Reece, C. A., Silverstein, R. M., Brown, V. H. and Degraw, J. I. (1971). Synthesis of brevicomin, principal sex attractant of Western Pine Beetle. *J. Chem. Eng. Data* 16: 380-381.

Roelofs, W., and Bjostad L. (1984) Biosynthesis of lepidopteran pheromones. *Bioorg. Chem.* 12: 279-298.

Rudinsky, J. A., Morgan, M. E., Libbey, L. M., and Putnam, T. B. (1977). Limonene released by the scolytid beetle *Dendroctonus pseudotsugae*. *Z. angew. Ent.* 82: 376-380.

Schmidt, S. and Monroe, R. E. (1976) Biosynthesis of the waxmoth sex attractants. *Insect Biochem.* 6: 377-380.

Schurig, V., R. Weber, G. J. Nicholson, A. C. Oehlschlager, H. Pierce, Jr., A. M. Pierce, J. H. Borden and L. C. Ryker. 1983. Enantiomer composition of natural *exo*- and *endo*-brevicomin by complexation chromatography/selected ion mass spectroscopy. *Naturwiss.* 70: 92-93.

Shrimpton, D. M. (1972). Variation in the extractives from lodgepole pine sapwood and heartwood. Northern Forest Research Centre Information Report NOR-X-18. Canadian Forestry Service, Department of the Environment. Edmonton, Alberta, Canada.

Shrimpton, D. M. (1973). Extractives associated with wound response of lodgepole pine attacked by the mountain pine beetle and associated microorganisms. *Can. J. Bot.* 51: 527-534.

Silverstein, R. M., Brownlee, R. G., Bellas, T. E., Wood, D. L., Browne, L. E. (1968). Brevicomin: principal sex attractant in the frass of the female western pine beetle. *Science* 159: 889-891.

Slessor, K. N., King, G. G. S., Miller, D. R., Winston, M. L., and T. L. Cutforth. (1985). Determination of chirality of alcohol or latent alcohol semiochemicals in individual insects. *J. Chem. Ecol.* 11: 1659-1667.

Smith, R. H. (1961). The fumigant toxicity of three pine resins to *Dendroctonus brevicomis* and *D. jeffreyi*. *J. Ec. Ent.* 54: 365-369.

Smith, R. H. (1965a). A physiological difference among beetles of *Dendroctonus ponderosae (= D. monticolae)* and *D. ponderosae (= D. jeffreyi)*. *Ann. Ent. Soc. Am.* 58: 440-442.

Smith, R. H. (1965b). Effect of monoterpene vapors on the western pine beetles. *J. Ec. Ent.* 58: 509-510.

Still, W. C., Kahn, M. and Mitra, A. (1978). Rapid chromatographic technique for preparative separations with moderate resolution. *J. Org. Chem.* 43: 2923-2925.

Stoakley, J. T., Bakke, A., Renwick, J. A. A., and Vité, J. P. (1978). The aggregation pheromone system of the larch bark beetle *Ips cembrae*. *Z. angew. Ent.* 86: 174-177.

Thompson, A. C., and Mitlin, N. (1979). Biosynthesis of the sex pheromone of the male boll weevil from monoterpene precursors. *Insect Biochem.* 9: 293-294.

Tumlinson, J. H., Gueldner, R. C., Hardee, D. D., Thompson, A. C., Hedin, P. A., and Minyard, J. P. (1970). The boll weevil sex attractant. in *Chemicals Controlling Insect Behavior* (M. Beroza, ed.) pp.41-59. Academic Press, New York.

Vanderwel D. and Oehlschlager A. C. (1987) Biosynthesis of pheromones and endocrine regulation of pheromone production in Coleoptera. pp. 175-215 in *Pheromone Biochemistry* (G. D. Prestwich and G. J. Blomquist, eds.) Academic Press, Inc. Orlando, Florida, U.S.A.

Vederas, J. C. (1987). The use of stable isotopes in biosynthetic studies. *Natural Product Reports.* 277-337.

Vité, J. P., Bakke, A. and Renwick, J. A. A. (1972). Pheromones in *Ips* (Coleoptera: Scolytidae): occurrence and production. *Can. Ent.* 104: 1967-1975.

Wainwright, S., Falck, J. R., Yadagiri, P., and Powell, W. S. (1990). Stereochemistry of leukotriene B_4 metabolites formed by the reductase pathway in Porcine polymorphonuclear leukocytes: inversion of stereochemistry of the 12-hydroxyl group. *Biochem.* 29: 1180-1185.

Walker, E. R. H. (1976) The functional group selectivity of complex hydride reducing agents. *Chem. Soc. Rev.* 5: 23-50.

Wasserman, H. H. and Barber, E. H. (1969). Carbonyl epoxide rearrangements. Synthesis of brevicomin and related [3.2.1] bicyclic systems. *J. A. C. S.* 91:3674-3675.

Weatherston, J., and Percy, J. E. (1976). The biosynthesis of phenethyl alcohol in the male bertha armyworm *Mamestra configurata*. *Insect Biochem.* 6: 413-417.

White, R. A., Franklin, R. T., and Agosin, M. (1979). Conversion of α-pinene to α-pinene oxide by rat liver and the bark beetle *Dendroctonus terebrans* microsomal fractions. *Pest. Biochem. Physiol.* 10: 233-242.

White, R. A., Jr., Agosin, M., Franklin, R. T., and Webb, J. W. (1980). Bark beetle pheromones: evidence for physiological synthesis mechanisms and their ecological implications. *Z. angew. Ent.* 90: 254-274.

Whitney, H.S., and Spanier, O.J. (1982). An improved method for rearing axenic mountain pine beetles, *Dendroctonus ponderosae* (Coleoptera: Scolytidae). *Can. Entomol.* 114: 1095-1100.

Wolf, W. A. and Roelofs, W. L. (1983) A chain-shortening reaction in orange tortrix moth sex pheromone biosynthesis. *Insect Biochem.* 13: 375-379.

Wong, J. W., Vergin, V., Oehlschlager, A. C., Borden, J. H., Pierce, Jr., H. D., Pierce, A. M., and Chong, L. (1983). Isolation and identification of two macrolide pheromones from the frass of *Cryptolestes ferrugineus* (Coleoptera: Cucujidae). *J. Chem. Ecol.* 9: 451-474.

Wood, S. L. (1982). pp. 692 and 703 **in** *Great Basin Naturalist Memoirs: the bark and ambrosia beetles of North and Central America (Coleoptera: Scolytidae), a taxonomic monograph.* Brigham Young University.

Yarger, R. G., Silverstein, R. M., and Burkholder, W. E. (1975). Sex pheromone of the female dermestid beetle *Trogoderma glabrum* (Herbst). *J. Chem. Ecol.* 1: 323-334.