Caste-Specific Biosynthesis of Mandibular Acids in Honey Bees (*Apis mellifera* L.)

by

Erika Plettner

B. Sc. (Honors), Simon Fraser University, 1990

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR IN PHILOSOPHY
in the
Department of Chemistry

SIMON FRASER UNIVERSITY

September 1995

Approval

Name: Erika Plettner

Degree: Doctor of Philosophy

Title of Thesis: Caste-Specific Biosynthesis of Mandibular Acids in Honey Bees (Apis mellifera L.)

Examining Committee:

Chairman: Dr. S. Holdcroft

Dr. K. N. Slessor, Professor, Senior Supervisor

Dr. M. L. Winston, Professor, Supervisory Committee

Dr. F. W. B. Einstein, Professor, Supervisory Committee

Dr. N. Haunerland, Assistant Professor, Internal Examiner

Dr. R. M. Crewe, Professor, External Examiner
Department of Zoology, University of the Witwatersrand

Date Approved: Sept 5, 1995

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:

Caste-Specific Biosynthesis of Mandibular Acids in Honey Bees (Apis mellifera L.)

Author: [signature]
(signature)

Erika Plettner
(name)

September 12, 1995
(date)

Abstract

Female honey bees (*Apis mellifera* L.) produce a caste-specific blend of functionalized 10-carbon fatty acids in their mandibular glands. Queens produce acids functionalized at the penultimate (ω-1) position, such as 9-hydroxy- and 9-keto-(E)2-decenoic acids (9-HDA and ODA). Both are components of the queen mandibular primer pheromone, a powerful attractant of worker bees. Workers produce 10-hydroxy-(E)2-decenoic acid and other acids fuctionalized at the terminal (ω) position, which are thought to be preservatives of brood food.

To study the biosynthesis of mandibular acids, specifically deuterated substrates were applied to the mandibular gland, and their conversion was followed by gas chromatography-mass spectrometry. Studies with fatty acids of different chain length indicated that octadecanoic acid is the entry point to the pathway. Experiments with labelled octadecanoic acid in the presence and absence of 2-fluorooctadecanoic acid, a β-oxidation inhibitor, indicated that 17- and 18-hydroxyoctadecanoic acids are the first functionalized intermediates in the pathway. Using labelled mandibular acids, the keto- and diacids were found to be derived from the corresponding hydroxy acids, and the (E)2-unsaturated hydroxy acids from the corresponding saturated acids.

The biosynthesis of mandibular acids is accomplished in a three-step bifurcated pathway. The ω and ω-1 branches are established at the first step: hydroxylation of octadecanoic acid at the 18^{th} and 17^{th} position. The resulting 18-carbon hydroxy acids are

chain shortened to the principal 10-carbon hydroxylated components. Oxidation of the ω and ω-1 hydroxy group, to give diacids and keto acids, completes the process. Both castes hydroxylate octadecanoic acid at the ω and ω-1 position. Queens chain shorten 18-hydroxyoctadecanoic acid to the 8-carbon length and the 17-hydroxy isomer to the 10-carbon length. Workers chain shorten 18-hydroxyoctadecanoic acid to the 10-carbon length, and they chain shorten the 17-hydroxy isomer to 9-HDA to a small extent. Hydroxy group oxidation also differs between the castes: workers are unable to oxidize 9-HDA to ODA, but they oxidize the 10-carbon ω-hydroxy acids to diacids. Queens oxidize 9-HDA and ω-hydroxy acids. Therefore, the last two steps in the pathway determine the caste-specificity of the mandibular gland secretions of queens and workers.

Acknowledgments

I would like to thank my mentors, Dr. Keith Slessor and Dr. Mark Winston, for the guidance, encouragement and generous support they have given me. I would also like to thank my parents for giving me the opportunity and the support to study science.

Dr. Skip King taught me how to operate and maintain the GC and GC-MS, and he was always willing to share his knowledge about synthesis. Mr. Greg Owen helped me with GC-MS and Ms. Marcy Tracey ran all the NMR spectra for this project. Mr. Phil Laflamme, Mr. Steve Mitchell and Ms. Heather Higo looked after the apiary and reared queens used for this work. Without their help, this project would not have been possible.

All the people in Dr. Slessor's, Dr. Winston's and Dr. Gries' laboratories have made my experience as a graduate student rewarding. In particular, I would like to thank Ms. Pingping Zhang, Mr. Ralph Wells, Mr. Greg Sutherland, Ms. Kirsten Bray, Mr. Peter Chua, Ms. Tanya Pankiw and Ms. Nicole Laurencelle for their help and ideas. I would also like to thank Ms. Elizabeth Brion for secretarial assistance.

This work was supported by Simon Fraser University, an NSERC postgraduate scholarship to E. P. and NSERC Operating and Strategic Grants to K. N. S. and M. L. W.

Table of Contents

Chapter II: Materials and Methods

List of Tables

List of Figures

List of Schemes

List of Abbreviations

abbreviation	meaning
BSTFA	bis trimethylsilyl trifluoroacetamide
C10:0	decanoic acid
C10:1	(E)2-decenoic acid
C10:1 Δ^9	9-decenoic acid
C12:0	dodecanoic acid
C14:0	tetradecanoic acid
C16:0	hexadecanoic acid
C18:0	octadecanoic acid
C18:1 Δ^{17}	17-octadecenoic acid
C10:0 DA	decanedioic acid
C10:1 DA	(E)2-decenedioic acid
CI	chemical ionization
CoA	coenzyme A
DAE	diaminoethane
3,10-diOH C10	3,10-dihydroxydecanoic acid
DMF	dimethylformamide
DMSO	dimethylsulphoxide
EI	electron impact
FAS	fatty acid synthase
2-F C18:0	2-fluorooctadecanoic acid
FFA	free fatty acid
FID	flame ionization detector
FR	intensity of an isotope peak of the M-15 fragment ion relative to the total intensity of the M-15 ion and the isotope peak
FR_o	FR value of an unlabelled standard
GC	gas chromatography
h	hour

abbreviation	meaning
9-HDA	(R,S) 9-hydroxy-(E)2-decenoic acid
10-HDA	10-hydroxy-(E)2-decenoic acid
10-HDAA	10-hydroxydecanoic acid
HMPA	hexamethylphosphoramide
7-HOAA	(R,S) 7-hydroxyoctanoic acid
8-HOAA	8-hydroxyoctanoic acid
IR	infrared spectroscopy/ - spectrum
JH III	juvenile hormone III
K. I.	Kovats Index
MC	mandibular complex
min	minute
MS	mass spectrometry
NMR	nuclear magnetic resonance
ODA	9-keto-(E)2-decenoic acid
11-OH C12:0	11-hydroxydodecanoic acid
11-OH C12:1	11-hydroxy-(E)2-dodecenoic acid
12-OH C12:0	12-hydroxydodecanoic acid
12-OH C12:1	12-hydroxy-(E)2-dodecenoic acid
13-OH C14:0	13-hydroxytetradecanoic acid
13-OH C14:1	13-hydroxy-(E)2-tetradecenoic acid
14-OH C14:0	14-hydroxytetradecanoic acid
16-OH C16:0	16-hydroxyhexadecanoic acid
17-OH C18:0	17-hydroxyoctadecanoic acid
18-OH C18:0	18-hydroxyoctadecanoic acid
PA	phosphatidic acid
PC, PE	phosphatidylcholine, -ethanolamine
PCC, PDC	pyridinium chlorochromate, - dichromate
rep	replicate
Qeq	queen equivalent

abbreviation	meaning
QMC	queen mandibular complex
S. E.	standard error
TAG	triacylglycerol
THF	tetrahydrofuran
THP	tetrahydropyranyl group
TLC	thin-layer chromatography
TMPA	trimethylphosphonoacetate
TMS	trimethylsilyl group
ω	terminal position in an alkyl chain
ω-1	penultimate position in an alkyl chain
WMC	worker mandibular complex

Introduction

A colony of honey bees exhibits an intricate social structure, at the heart of which are division of labor and an effective communication system. Labor is divided between the queen, who devotes her time to egg-laying, and the workers, who carry out numerous chores from tending brood to collecting food. Many signals coordinate the behavior of the queen and the workers, among them chemical cues. The best known among the honey bee signal chemicals are blends of isomeric fatty acids that are produced in both worker and queen mandibular glands. Although the structural differences between the components of the queen and worker mandibular complexes are small, these compounds have very different functions in the colony. The queen uses her mandibular complex to signal her presence; worker compounds are thought to act as a preservative and nutrient in brood food. The biosynthesis of the mandibular complexes has not been studied and is of interest because differentiation between queen and worker mandibular complexes is critical in the differentiation between queen and worker behaviors that are the key to colony integration. Insight into this physiological difference between queens and workers also may aid in the development of more efficient beekeeping techniques that make use of these powerful chemical signals.

The elucidation of the biosynthetic pathway of the queen and worker mandibular complexes is described in this thesis. Chapter I is a review of the literature on caste differentiation in the honey bee, the functions of the queen and worker mandibular complexes and the biosynthesis of fatty acid-derived semiochemicals. The synthesis of labelled precursors and the methods for following them are described in Chapter II; the experiments which led to the elucidation of the pathway are presented in Chapter III. Chapter IV is a discussion of caste-specificity in mandibular complex biosynthesis.

Chapter I: Literature Review

I.1. Caste determination and significance of the caste-specific mandibular acid blends in female honey bees

1.1 The honey bee castes and their determination during development

The existence of distinct queen and worker castes within a honey bee colony has fascinated people since ancient times. However, until the 16th century, little was known about the role of these castes in the colony. The queen, known then as "master bee", was first recognized as female in 1586 by L. M. de Torres who observed a queen laying eggs. Soon thereafter, the workers also were recognized as female. Detailed morphological comparisons of queens and workers led to the conclusion that only the queen has the ability to mate and, therefore, to reproduce (Crane 1946, Free 1982). The appearance of glass-walled hives allowed more detailed observations which led to the realization that a colony of honey bees consists of one queen, several thousand workers and a variable number of drones, and that the workers perform many non-reproductive tasks in the colony such as brood care, defense and foraging (Free, 1982). Eventually, the term "caste" was introduced to denote individuals of the same sex that vary in form and function. The queen and the workers are considered to be distinct castes because they differ morphologically and physiologically even though they have the same genetic complement (Wilson 1971, p. 136).

Once it was known that queens and workers are female, beekeepers and scientists became intrigued by how their caste is determined. In 1889, Perez observed that both female castes arise from a fertilized egg, but follow different developmental pathways, depending on the quality and quantity of food a young larva is given (Beetsma 1979). Queen larvae are fed a high proportion of royal jelly, a milky secretion from the mandibular glands of nurse bees, while worker larvae are fed mostly secretions from the hypopharyngeal glands and pollen. When the workers need to rear a new queen, either because of queen loss or in preparation for swarming, they elongate a cell containing a

young female larva and begin feeding her royal jelly (Winston 1987). A young female larva can develop into a queen or a worker up to the third day of the larval instars, depending on her feeding regime. Four day old worker larvae grafted into queen cells develop into workers, and larvae grafted during the third-fourth day develop into intercastes, individuals with mixed queen and worker characteristics (Beetsma 1979). Queen and worker cells differ in shape and orientation, and nurse bees feed larvae according to the cell type they are in (Beetsma 1985). Cell type is thus translated into food quality and quantity, both of which are important factors in caste determination in female honey bees.

Numerous experiments have been done to explore the chain of events leading from differences in food quality and quantity to caste differentiation. This work has culminated in two hypotheses: a) royal jelly contains a hormone-like substance that induces queen characters in larvae (trophic factor), or b) the nutritional value of the diet given to a larva translates into a hormonal stimulus that induces the caste-specific characters (Beetsma 1979, Rachinsky 1990). Some attempts have been made to isolate and characterize the elusive "trophic factor" in royal jelly, but the results are unclear, perhaps because the substance is unstable (Rembold *et al.* 1974). On the other hand, Asencot and Lensky (1976) were able to demonstrate that feeding young larvae worker food supplemented with glucose and fructose results in some of them developing into queens and intercastes. The higher the sugar content of the brood food, the greater the proportion of queens among the larvae reared. Royal jelly has a higher sugar content than worker food, and the sugar is thought to act as a phagostimulant, causing queen larvae to ingest more food than worker larvae (Beetsma 1979, Winston 1987). This higher feeding rate results in neural stimuli that are translated into higher juvenile hormone III (JH III) titers in the hemolymph of queen larvae (Rachinsky 1990). The hormonal and neural stimuli in queen larvae lead to a higher metabolic rate, faster growth and, therefore shorter development times for queens as compared to workers (Beetsma 1979, Winston 1987) . This difference in development ultimately results in morphological and physiological differences between queens and workers.

1.2 Mandibular components in *A. mellifera* queens and workers

Many morphological differences between queens and workers relate to their respective tasks in the colony and have been studied in detail. For instance, only queens possess fully developed ovaries and a spermatheca and only workers have pollen baskets on their hindlegs (Winston 1987). Unlike morphological differences, the physiological differences between queens and workers are only beginning to be explored. One such difference is the composition of the mandibular complex (MC), a blend of several compounds produced in the mandibular glands of female honey bees. Both queens and workers produce a group of functionalized 10-carbon fatty acids (among other compounds) in their mandibular glands. The fatty acids characteristic of queens have the functional group at the second-to-last (ω-1) position in the chain, while the acids typical of workers are functionalized at the last (ω) position. The major component of queen mandibular complex (QMC) is 9-keto-(E)2-decenoic acid (ODA), and the second most abundant is 9-hydroxy-(E)2-decenoic acid (9-HDA) (Slessor *et al.* 1988, 1990). The most abundant component of worker mandibular complex (WMC) is 10-hydroxy-(E)2-decenoic acid (10-HDA) (Callow *et al.* 1959), followed by 10-hydroxydecanoic acid (10-HDAA). The 8-carbon compound, 8-hydroxyoctanoic acid (8-HOAA), is a minor component, and so are the diacids corresponding to the ω-hydroxy acids (Weaver *et al.* 1968, Pain *et al.* 1962). The functions of these caste-specific blends also differ. QMC is a pheromone, which attracts nearby workers, giving rise to a retinue of workers around the queen (Free 1987, Slessor *et al.* 1988), among other effects. The worker MC components have been less well studied, but some of them could be involved in food preservation (Blum *et al.* 1959, Lukoschus and Keularts 1968) and nutrition (Kinoshita and Shuel 1975).

For many years, the composition of queen and worker MC had been thought to be mutually exclusive, until it was found that mated, laying queens always have some 10-HDA and 10-HDAA (Crewe *et al.*, 1982). Conversely, queenright workers always have trace amounts of 9-HDA in their mandibular glands (Plettner *et al.* 1995). Workers retain the same ratio of mandibular acids throughout their lives, and the titer of material found in the

gland increases as workers age (Figure I.1, Arnold and Roger 1979, Boch and Shearer 1967). Newly emerged queens have a blend with a high proportion of 10-HDA that changes to a blend with mostly ODA and 9-HDA during the first two weeks after emergence (Figure I.2).

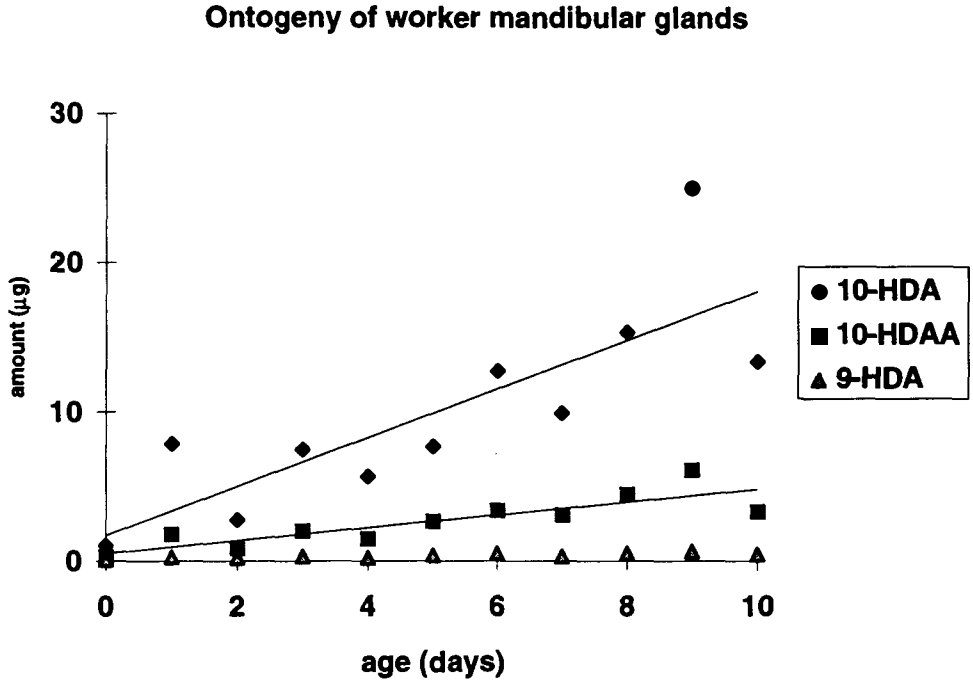


Figure I.1. Ontogeny of 10-hydroxy-(E)2-decenoic acid (10-HDA), 10-hydroxydecanoic acid (10-HDAA) and 9-hydroxy-(E)2-decenoic acid (9-HDA) in worker mandibular glands. Each point represents the average of 3 determinations. To estimate the average increase in titer, lines were obtained by linear regression. For 10-HDA, $y = 1.6x + 1.7$ ($R^2 = 0.67$) , for 10-HDAA, $y = 0.4x + 0.5$ ($R^2 = 0.73$) (from Plettner *et al.* 1995).

Even though queenright workers have 9-HDA in their MC, they seldom contain ODA. However, under queenless conditions, some workers have ODA (Ruttner *et al.* 1976). Further work has led to the realization that combinations of morphological and physiological queen and worker characters are possible in some individuals under special circumstances. For instance, when a colony becomes queenless and the workers are unable to rear a new queen, a fraction of the workers will develop their ovaries and begin laying

unfertilized eggs. Occasionally, one of these laying workers will attract a retinue of workers around her, just like a mated, laying queen would (Sakagami 1958). Such workers are referred to as "false queens", and their mandibular glands contain ODA and 9-HDA, along with a small proportion of the characteristic worker acids (Plettner *et al.* 1993). Laying workers that do not attract a retinue have a worker MC. An intercaste with developed ovaries, a small spermatheca, queen-like quantities and proportions of ODA and 9-HDA in her mandibular glands, and a worker-like external appearance has been reported (Plettner *et al.* 1993). There appears to be a continuum from worker to false queen to intercaste to virgin queen to mated queen with respect to mandibular gland states. Along this gradation, the proportion of ω-1-functionalized queen acids increases relative to the ω-functionalized worker acids. In European honey bee subspecies, the intermediate states are rare, but in some African subspecies they are quite common. In particular, *A. mellifera capensis* workers easily become false queens under queenless conditions (Crewe and Velthius 1980, Cooke 1987, Allsopp and Crewe 1993).

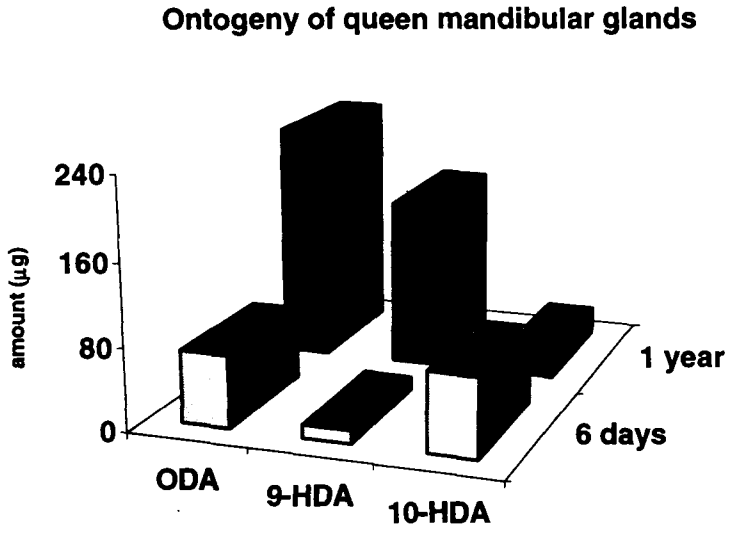


Figure I. 2. Ontogeny of 9-keto-(E)2-decenoic acid (ODA), 9-HDA and 10-HDA in queens. For 1 year old queens N = 29, for 6 day old queens N = 10 (Plettner *et al.*, unpublished observation).

1.3 Role of the mandibular gland secretion in colony integration

All social insects have developed the ability to coordinate the effort of many individuals towards collective survival. Even though the social insects have adapted to a wide variety of conditions, all the advanced insect societies share three characteristics. First, there are at least two overlapping generations cohabiting in a colony. This is an important difference to solitary insects, such as many wasp species, in which a mother dies before her offspring emerge. Overlap of generations is a prerequisite for the second characteristic: extended brood care and provisioning. Females of solitary wasp species lay their eggs on a paralyzed prey item before they die, the resulting brood having to survive on these provisions. In contrast, social insects continuously provision and tend their brood until shortly before pupation. The third characteristic of highly social insects is division of labor into reproductive and non-reproductive tasks carried out by morphologically distinct castes. The queen, the only fertile female in the colony, lays eggs, while the workers tend the brood, forage for food and build, clean and defend the nest. This sharp division of reproductive and other tasks among distinct castes sets the eusocial insects (such as the honey bees) apart from other insects that exhibit less complex social behavior (Wilson 1971).

Another feature of highly advanced insect societies is reciprocal communication among nestmates, which is needed to achieve coordinated colony-level responses to internal and external stimuli. Reciprocal communication is essential for the establishment of a hierarchy among the individuals, for recruitment of foragers to food sources and for alarm (Wilson 1971, Hölldobler and Wilson 1990). Among the various means used for communication in insect societies, chemical signals are prominent. Semiochemicals are signal chemicals used for communication between members of the same or different species, the former being known as pheromones. Compounds that elicit a specific short-term behavior are releasers and compounds that have a long-term physiological effect are primers. The queen MC can be classified as both, since it is very attractive to the workers and elicits retinue formation around the queen and swarm attraction (releasers), but also has

been found to inhibit JH III biosynthesis in workers and to inhibit queen rearing by workers (primers). No releaser or primer functions have been found for worker MC, but some components seem to be involved in food preservation and larval nutrition.

Queen MC is a signal by which the queen makes her presence felt throughout the nest: it delays swarming, inhibits queen rearing, stimulates pollen collection and brood rearing in small colonies and affects division of labor among the workers. The workers in the retinue around the queen remove QMC from her body surface and spread it throughout the nest mostly by grooming and exchanging food with other workers (Naumann *et al.* 1991). When a colony becomes overcrowded, QMC can no longer circulate sufficiently among the workers and queen rearing in preparation for swarming begins (Winston *et al.* 1991). When a colony loses its queen, the workers sense the loss of QMC within half an hour and begin emergency queen rearing within one day (Winston *et al.* 1989, 1990). Swarming and emergency queen rearing can be delayed by the introduction of synthetic QMC into slightly overcrowded or queenless colonies, respectively, which confirms the role of QMC in the inhibition of queen rearing (Winston *et al.* 1989, 1990, 1991). Furthermore, synthetic QMC added to small, newly established colonies in the spring can stimulate pollen foraging and brood rearing (Higo *et al.* 1992).

The major component of QMC, ODA, inhibits the biosynthesis of JH III in workers, which allows the queen to influence JH III-regulated aspects of worker behavior and physiology, such as worker age polyethism (Kaatz *et al.* 1992). As workers age, they progress through various tasks starting with work in the center of the nest, then proceeding to the periphery and finally flying out and foraging (Winston 1987). This ordered change in behavior is mediated by an increasing JH III titer in workers as they age (Robinson *et al.* 1991). A rapid rise of the JH III titer in newly emerged bees causes them to skip the tasks in the nest and to begin foraging precociously (Robinson 1987). QMC may be one signal that slows the rapid programmed rise in JH III in workers, thereby ensuring that workers progress through all the tasks (Kaatz *et al.* 1992, Pankiw personal communication). In spite of the large number of effects QMC has, it is not the only chemical signal that controls

and integrates worker behavior and physiology. In particular, aspects of worker physiology that are not under the control of JH III do not appear to be influenced by QMC. One such aspect is worker ovary development, which is not affected by QMC, but appears to be controlled by a primer pheromone emanating from the brood (Willis *et al.* 1990). All the observations about QMC point to its central, albeit not unique, role in colony integration: this semiochemical allows the queen to coordinate some activities of the workers and to prevent the rearing of rival reproductives.

Honey bee workers gather, process and distribute the food supply for the entire colony, and the mandibular glands (among others) are involved in food processing (Winston 1987). Therefore, one would expect the worker MC to play a role in this activity. The provisioning of brood and storage of food require antibacterial and antifungal compounds for preservation. Such compounds have been found among several social insects, in particular those that live in moist environments and/or store food. For instance, *Crematogaster deformis* ants produce a mixture of phenols, which exhibit antibacterial activity, in their metapleural glands (Attygalle *et al.* 1989). Fire ants (*Solenopsis invicta*) spray their brood with a small quantity of venom that contains a mixture of piperidine alkaloids with antibiotic properties (Obin and Van der Meer 1985). In honey bees, the worker MC component 10-HDA is found in royal jelly (Barker *et al.* 1959), along with the other ω-hydroxy acids and their corresponding diacids (Weaver *et al.* 1968). Blum and coworkers (1959) found 10-HDA to exhibit antifungal and antibacterial activity in laboratory tests, so this compound may be involved in the preservation of royal jelly. The 10-HDA also inhibits the germination of pollen, which is important if pollen is to be stored (Lukoschus and Keularts 1968). Moreover, 10-HDA may be involved in larval nutrition. Kinoshita and Shuel (1975) found that larvae fed with delipidated brood food pupated precociously and exhibited high mortality. The readdition of 10-HDA to the delipidated food partially reversed the effect of lipid removal; the readdition of the sterol fraction did not lead to as strong a reversal. The authors suggest that 10-HDA may inhibit JH III production in the larvae and, in the full royal jelly blend, help to disperse the sterol

components. Thus, the best understood functions of 10-HDA are related to food preservation and larval nutrition.

I.1.4 Existing and potential commercial applications of the mandibular pheromone and the importance of biosynthetic studies for their development.

The effects of QMC on workers have led to the development of several commercially important applications of synthetic QMC. For instance, packaged worker bees can be shipped with a dose of synthetic QMC instead of a live queen. The QMC prevents the queenless workers from becoming restless and makes shipping easier. Since QMC is very attractive to the workers, it can be used to lure workers to blooming crops and thus enhance pollination efficiency. Furthermore, QMC can be used to suppress queen rearing and, hence, swarming (review of practical applications: Winston and Slessor 1992). All the known effects of QMC are dose dependent, and so are the practical applications developed so far: an overdose may cause an unforseen side effect, but the administered dose must be high enough to achieve the desired effect. To find suitable doses and release rates for the various practical applications of QMC, one needs to know the natural rates of production and dispersion of the QMC components in the colony. The rate of QMC dispersion in a colony has been studied (Naumann *et al.* 1991), but the rate of production was only determined indirectly as part of the dispersion studies. Determination of the production rate of QMC components requires the elucidation of the biosynthetic pathway. The breakdown of ODA in workers has been studied by Johnston *et al.* (1965), but the biosynthesis of the acid components of QMC has not been studied.

I.2. Biosynthesis of fatty acid-derived semiochemicals

2.1 Fatty acid and fatty acid-derived semiochemicals

Few species use free fatty acids (FFA) as semiochemicals, possibly because of the low volatility of these compounds. Some examples include the carpet beetles which have unsaturated fatty acids as sex pheromones (Silverstein *et al.* 1967, Kuwahara and Nakamura 1985). The death's head hawkmoth is able to enter and live in honey bee colonies, without being detected by the bees, because its cuticular FFA profile mimics that of the bees (Moritz *et al.* 1991). (R)3-Hydroxybutanoic acid is the contact sex pheromone of *L. triangularis*, a forest spider (Schulz and Toft 1993). However, the honey bee queen mandibular pheromone is the most extensively studied semiochemical with FFA components.

Semiochemicals that are derived from fatty acids are numerous and can be classified into fatty alcohols, esters, aldehydes, lactones, hydrocarbons, oxiranes and ketones. Several moths, such as *B. mori* and *T. pityocampa*, have fatty alcohols as the main component of their sex pheromone (Ando *et al.* 1988, Fabrias *et al.* 1989). Many other moths, among them *A. velutinana* and *T. ni*, have acetate esters of fatty alcohols (Wolf and Roelofs 1989); *A. variana* and *C. fumiferana* are two species from a long list that have fatty aldehydes as their sex pheromone (Gries *et al.* 1994, Weatherston *et al.* 1971). Lactones of 12- and 14-carbon hydroxy acids are components of the aggregation pheromone of several grain beetles (Vanderwel and Oehlschlager 1989). Examples of hydrocarbon semiochemicals are the housefly sex pheromone, (Z)9-tricosene, and the Arctiid moth sex pheromone, 2-methylheptadecane (Blomquist *et al.* 1993, Charlton and Roelofs 1991). The sex pheromone of the German cockroach, 3,11-dimethylnonacosan-2-one is derived from the corresponding alkane (Blomquist *et al.* 1993).

The biosynthesis of many fatty acid-derived semiochemicals has been studied. All of these biosynthetic routes consist of three processes: 1) synthesis of the precursor fatty acid, 2) functionalization and chain shortening or elongation and 3) carboxyl group modification. Combinations of these processes account for all the known biosynthetic patterns of fatty

acid-derived semiochemicals. Almost all the species studied are able to synthesize the precursor fatty acid *de novo* from acetate. However, most of them also are able to incorporate the preformed precursor fatty acid directly. The second process, functionalization of the fatty acid, consists of the introduction of a second functionality such as a C=C double bond in many moth pheromones. In many cases, the chain length of the precursor fatty acid does not correspond to that of the final product, because the precursor fatty acid is chain shortened or elongated before or after functionalization.

The last process in the biosynthesis of fatty acid-derived semiochemicals is the modification of the carboxyl group. Fatty alcohols are formed by reduction of the fatty acyl coenzyme A (CoA) ester, first to the aldehyde, then to the alcohol. The acetates are formed from the alcohols by acetylation (Jurenka and Roelofs 1993). The aldehyde pheromone of *C. fumiferana* is formed from the corresponding acetate ester by hydrolysis and oxidation on the gland surface during release (Morse and Meighen 1987). Lactones are formed by cyclization of the precursor hydroxy acyl CoA esters (Vanderwel *et al.* 1992). Hydrocarbons are formed by reduction of the precursor fatty acyl CoA ester to an aldehyde that is decarbonylated to give the hydrocarbon (Blomquist *et al.* 1993). The first two processes, precursor fatty acid synthesis and combinations of functionalization and chain shortening or elongation, are applicable to honey bee mandibular acid biosynthesis and are discussed further.

2.2 Fatty acid biosynthesis and degradation

De novo biosynthesis of fatty acids

Most of the species that have been studied are able to incorporate labelled acetate into their pheromone components, as well as into free and lipid-bound fatty acids. This indicates that the fatty acid precursor can be synthesized in the pheromone gland. However, preformed labelled fatty acids also are incorporated into the pheromone, indicating that the source of the fatty acid is not important to the pheromone biosynthetic

pathway. One exception is *P. gossypiella*, which is unable to incorporate hexa- or octadecanoic acid into its pheromone, but can incorporate (Z)9-octadecenoic acid. The gland is unable to synthesize this precursor which may come from elsewhere in the insect (Foster and Roelofs 1988).

Fatty acid synthase (FAS) is the enzyme complex responsible for fatty acid biosynthesis from acetate. The sequence of reactions starts with the carboxylation of acetyl-CoA to give malonyl-CoA. Malonyl-CoA is condensed with acetyl-CoA (or the growing fatty acyl-CoA in subsequent rounds) to give 3-ketobutyryl-CoA. The 3-keto acyl-CoA is reduced to give 3-hydroxyacyl-CoA, and this is followed by dehydration to give an (E)2-enoyl-CoA. Reduction yields the fatty acyl-CoA two carbons longer (Stanley-Samuelson *et al.* 1988). Most FAS require acetyl-CoA as starter, but some accept other CoA esters such as 3-methylbutyryl CoA in the biosynthesis of 2-methyl-branched fatty acids (Charlton and Roelofs 1991) or 18-carbon acyl-CoA esters in the biosynthesis of very long chain fatty acids (Vaz *et al.* 1988).

In insects, as in vertebrates, these reactions occur in close succession, without release of the intermediates. Furthermore, several rounds of condensation occur before the growing fatty acyl-CoA is released and hydrolyzed by a thioesterase domain of the FAS (Stanley-Samuelson *et al.* 1988). In most insect FAS studied, release occurs when the chain has reached a 16 or 18 carbon length. For instance, Morse and Meighen (1987) studied the chain-length specificity of a FAS found in the pheromone gland of *C. fumiferana* by determining the ratio of labelled acetyl-CoA to malonyl-CoA incorporated into the fatty acid. Since in their *in vitro* system only one acetyl-CoA was incorporated per fatty acyl chain, the ratio of malonyl- to acetyl-CoA incorporated gave an indication of the chain length of the FAS product. The ratios found corresponded to incorporation into octa- and hexadecanoic acid. More direct methods, such as gas chromatography of the FAS products, have been used to study the chain length specificity of insect FAS. For instance, FAS from *T. ni* and *M. domestica* both produce hexa- and octadecanoic acids (Stanley-Samuelson *et al.* 1988). The FAS from the pea aphid synthesizes mainly tetradecanoic acid. Ryan *et al.* (1982) demonstrated that this is due to a separate thioesterase that specifically

hydrolyzes tetradecanoyl-CoA before it is elongated. In the absence of the thioesterase, the aphid FAS produces hexa- and octadecanoic acids. Thus, in most insects, the FAS synthesizes mainly hexa- and octadecanoic acids.

Chain shortening of fatty acids

Fatty acids are chain shortened by β-oxidation, a sequence of reactions that leads to the removal of acetyl-CoA from the acyl-CoA substrate. The process starts with desaturation to give an (E)2-enoyl-CoA. This is followed by hydration to a 3-hydroxyacyl-CoA and oxidation to a 3-ketoacyl-CoA. This intermediate is attacked by coenzyme A to give acetyl-CoA and the shortened acyl-CoA. Both, the chain length specificity and the extent of chain shortening vary between β-oxidation systems: some preferentially accept 18-carbon acyl CoA esters for the first round, others start with acyl-CoA esters that are 10 to 14 carbons long (Christensen *et al.* 1989). Furthermore, the extent of β-oxidation varies, depending on the subcellular location of the enzyme complex. In vertebrates, the mitochondrial system appears to degrade fatty acids completely to acetate, while the peroxisomal system performs limited chain shortening (Lazarow and de Duve 1976, van den Bosch *et al.* 1992). In insects, both limited and complete β-oxidation are observed, but the subcellular location of these activities has not been determined.

Limited β-oxidation is common in pheromone biosynthetic pathways. For instance, in *A. velutinana* and *S. littoralis*, hexadecanoic acid undergoes one round of chain shortening before the Δ11 double bond or the diene system, respectively, are introduced (Wolf and Roelofs 1989, Martínez *et al.* 1990). Limited chain shortening can also take place on functionalized fatty acids. For example, the grain beetles chain-shorten (Z)9-octadecenoic acid to (Z)3-dodecenoic acid, an intermediate in the biosynthesis of (Z)3-dodecen-11-olide (Vanderwel *et al.* 1992). Females of *T. ni* biosynthesize their sex pheromone, (Z)7-dodecenyl acetate, by chain shortening of (Z)11-hexadecenoic acid to the 12-carbon length (Wolf and Roelofs 1989, Jurenka and Roelofs 1993).

Distinction of limited β-oxidation from complete degradation and resynthesis

Conversion of a fatty acid to shorter fatty acids (and derived compounds) can occur through two routes: limited β-oxidation to the required chain length or complete degradation to acetate and resynthesis of the shorter fatty acid. Which of the two routes is the major one depends on the type of β-oxidation system as well as the chain length specificity of the FAS. Often these enzyme systems have not been studied in sufficient detail to delineate the route of fatty acid incorporation directly, so indirect methods have to be used. Incorporation patterns of radiolabelled acetate and fatty acids and stable isotope labelled fatty acids are useful in distinguishing the two possibilities. For instance, in a study of (Z)7-dodecenyl acetate biosynthesis in *T. ni*, the position of radiolabel in the pheromone formed from 16 ^{3}H-(Z)11-hexadecenoic acid was used to distinguish limited β-oxidation from complete degradation and resynthesis of the precursor. The pheromone isolated from treated glands was ozonolyzed, and the fragments were separated by gas chromatography. Only the fragment corresponding to the methyl end of the pheromone was labelled, which indicated that the (Z)11-hexadecenoic acid was incorporated mainly by limited chain shortening (Bjostad *et al.* 1987).

In another example, females of *A. velutinana* incorporated 1-^{14}C tetradecanoic acid into (Z)11-tetradecenoic acid and the pheromone, but not into hexa- and octadecanoic acids, which meant that very little tetradecanoic acid was degraded to acetate and resynthesized. Furthermore, 16,16,16-D_3 hexadecanoic acid was incorporated into the shorter fatty acids and the pheromone. If the deuterium labelled hexadecanoic acid had been completely degraded and resynthesized to tetradecanoic acid, the mass label would not have been detectable, because the deuterated acetate would be diluted in the endogenous acetate pool and deuterium from D_3 acetate would be lost during resynthesis. The detectable incorporation of mass labelled fatty acid into the pheromone confirmed that degradation and resynthesis was not a major route of fatty acid incorporation into the pheromone of *A. velutinana* (Bjostad *et al.* 1987).

The lipid pool as a source and sink of fatty acyl intermediates in pheromone biosynthesis

The majority of the fatty acid found in pheromone glands is not free, but esterified to glycerol in several forms of glycerolipid. These include neutral lipids such as triacylglycerol (TAG) and phospholipids such as phosphatidylcholine and -ethanolamine. In the previously discussed *de novo* studies, labelled acetate was incorporated into free and lipid-bound fatty acids, indicating that the lipid is synthesized in the pheromone gland. The fatty acid profile of pheromone gland lipids typically includes hexa- and octadecanoic acids; (Z)9-octadecenoic and polyunsaturated acids. In addition, some unusual fatty acids are bound in the TAG of some species. For example, TAG from the pheromone gland of *B. mori* contains (Z)11-hexadecenoic acid and (E)10,(Z)12-hexadecadienoic acid (Bjostad *et al.* 1987). The latter has the same diene system as the pheromone and both are known to be precursors of the pheromone (Ando *et al.* 1988). The lipid may, therefore, serve as a pheromone precursor storage site in this species.

The role of glycerolipid-bound fatty acids was studied in detail in *A. velutinana* which has large quantities of (E) and (Z) 11-tetradecenoic acid bound in the TAG. In a time-course of 1-^{14}C acetate incorporation into the TAG-bound Δ11 acids and the pheromone, label was incorporated equally fast into both. If the TAG-bound Δ11 acids had been pheromone precursors, incorporation of label into the pheromone should have lagged behind incorporation into the Δ11 acids. Further studies with TAG containing labelled (E) and (Z) 11-tetradecenoic acid confirmed that the TAG-bound Δ11 acids are not intermediates in pheromone biosynthesis in this species. The TAG-bound Δ11 acids are mostly E, but the Δ11 desaturase produces more of the Z isomer which is preferentially incorporated into the pheromone. If the E isomer were allowed to accumulate in the free acid pool, the Z:E ratio in the pheromone would change with time. (E)11-Tetradecenoic acid is thought to be preferentially bound into the TAG to prevent its accumulation and thereby maintain a constant Z:E ratio in the pheromone (Bjostad *et al.* 1987).

2.3 Functionalization of fatty acids

Functionalization is the introduction of a structural feature into an alkyl chain. In the biosyntheses of fatty acid-derived semiochemicals, initial functionalization frequently occurs before modification of the fatty acid carboxyl group, and can take place before or after chain shortening or elongation. Initial functionalization reactions include desaturation and hydroxylation. Subsequent reactions can introduce further functional groups or modify the one originally introduced. Examples include further desaturations or hydroxylations, epoxidation of double bonds and oxidation of alcohol to carbonyl functionalities. Desaturation, hydroxylation and hydroxy group oxidation are applicable to honey bee mandibular acid biosynthesis and will be discussed further.

Desaturation

Desaturation of fatty acyl CoA's is a common motif in the biosynthesis of many moth pheromones. It can occur at different positions, the 9^{th} (Δ9), 11^{th}(Δ11) and 13^{th} (Δ13) being frequently encountered. For example, a Δ9 desaturase converts (Z)11-tetradecenoic acid to (Z)9,(Z)11-tetradecadienoic acid in *S. littoralis* (Martinez *et al.* 1990); Δ11 desaturases are found in *T. ni* and *A. velutinana* (Bjostad *et al.* 1987); Δ13 desaturase activity has been recently discovered in *T. pityocampa* as part of the biosynthetic pathway of (Z)13-hexadecen-11-ynyl acetate (Arsequell *et al.* 1990).

Wolf and Roelofs (1986) studied the Δ11 desaturase in *T. ni* in detail. The activity was only found in the pheromone gland and, like other desaturases, was associated with the microsomal fraction. The substrates were the CoA esters of hexa- and octadecanoic acid, and NADH was required as a cofactor. Only the Z isomer of the Δ11 alkenoic acids was produced. The desaturase activity peaked at the same age at which pheromone production is known to be highest, thus confirming the unique role of this enzyme in pheromone production.

Hydroxylation

Terminal and internal hydroxylation of fatty acids is part of many metabolic processes, among them fatty acid catabolism in mammals, cutin biosynthesis in plants and semiochemical biosynthesis in insects. A few examples of fatty acid hydroxylations are presented in Table I.1. Fatty acid hydroxylation was first studied in mammalian liver (Lu *et al.* 1969, Christensen *et al.* 1991). The major cuticular component of plants, cutin, contains long-chain hydroxy fatty acids which are synthesized by hydroxylation of the precursor (Soliday and Kolattukudy 1977, 1978). Yeast and bacteria also hydroxylate long-chain fatty acids (Heinz *et al.* 1969, Ho and Fulco 1976, Boddupalli *et al.* 1990). In insects, fatty acid hydroxylation has been studied in the housefly (Clarke *et al.* 1989, Ronis *et al.* 1989). Semiochemical biosynthesis in gain beetles involves hydroxylation of (Z)3-dodecenoic acid in the penultimate position prior to lactonization (Vanderwel *et al.* 1992).

Early studies of Lu *et al.* (1969) revealed that fatty acid hydroxylation in rat liver was catalyzed by a microsomal hemoprotein and required O_2 and NADPH. This enzyme was classified as a cytochrome P450 because it exhibited a characteristic absorption band at 450 nm and inhibition by CO. Rat liver enzyme preparations were observed to hydroxylate dodecanoic acid at the 11^{th} (ω-1) and 12^{th} (ω) position. These two activities were suspected to be on different enzymes based on differences in isotope effects (Hamberg and Bjorkhem 1971) and inducibility. Gibson *et al.* (1982) separated the two activities into different chromatographic fractions, thereby confirming that the ω-1 and ω hydroxylase activities were located on different enzymes. Similar results were obtained for analogous enzymes isolated from houseflies (Ronis *et al.* 1988).

The hydroxylation of an alkyl chain could occur in two ways: direct hydroxylation or hydration of a C=C double bond. The former reaction involves only the center to be hydroxylated, but the latter involves an additional neighbouring center. Therefore, substrates with ^2H or ^3H at the hydroxylation position and the neighbouring positions have enabled researchers to distinguish the two possibilities: a one-center reaction will proceed with loss of label at the hydroxylation position, but retention at all neighbouring positions.

Another difference between the two routes is the source of the hydroxyl O: in the one-center reaction it comes from O_2 and in double bond hydration, from water. The incorporation of labelled ^{18}O from $^{18}O_2$ or $H_2{}^{18}O$ has also been useful in distinguishing the two hydroxylation routes. Cytochrome P450-mediated reactions studied in this way have been found to be one-center reactions with the O coming from O_2 (Heinz *et al.* 1969, Hamberg and Bjorkhem 1971). However, there are a few examples of hydroxylations that are not mediated by cytochrome P450 and involve the hydration of a double bond. For example, several species of *Pseudomonas* hydrate (Z)9-octadecenoic acid to 10-hydroxyoctadecanoic acid (Schroepfer 1966, Gotuda 1991).

Table I.1. Examples of fatty acid hydroxylation.

organism (tissue)	hydroxylation position	preferred substrate(s)	references
rat (liver)	ω	C10:0, C12:0	Lu *et al.* 1969 Ichihara *et al.* 1969
	ω	"	Christensen *et al.* 1991
	ω/ω-1	"	Hamberg and Bjorkhem 1971
	ω/ω-1	"	Gibson *et al.* 1982
housefly (abdomen)	ω/ω-1	C 12:0	Clarke *et al.* 1989 Ronis *et al.* 1988
grain beetles	ω-1	3(Z) C12:1	Vanderwel *et al.* 1992
Vicia faba (leaves)	ω	C16:0	Soliday and Kolattukudy (1977)
	midchain	16-OH C16:0	" (1978)
yeast, *Torulopsis*	ω-1	C18:0	Heinz *et al.* (1969)
bacteria, *Bacillus megaterium*	ω-1,-2,-3	C16:0	Ho and Fulco 1976 Boddupalli *et al.* 1990

Hydroxy group oxidation

In many organisms, primary and secondary alcohols are oxidized to aldehydes and ketones, respectively, and aldehydes are further oxidized to acids. Some examples of oxidations involved in pheromone or hydroxy acid metabolism are presented in Table I.2.

Table I. 2. Examples of enzymatic alcohol and aldehyde oxidations.

organism (tissue)	type of activity	substrate	product	reference
C. fumiferana (pheromone gland)	alcohol oxidase	(Z)11 tetradecen-1-ol	aldehyde	Morse and Meighen 1984
rat (liver)	alcohol dehydrogenase	17-OH C18:0	17-keto C18:0	Bjorkhem and Hamberg 1971
horse (liver)	"	17-OH C18:0 18-OH C18:0	17-keto C18:0 C18:0 diacid	Bjorkhem *et al.* 1973.
potato (tuber)	"	16-OH C16:0	16-oxo C16:0	Agrawal and Kolattukudy 1978b
"	aldehyde dehydrogenase	16-oxo C16:0	C16:0 diacid	" 1978a
C. fumiferana (cuticular epithelium)	"	(Z)11-tetradecenal	acid	Morse and Meighen, 1984
H. virescens (cuticular epithelium)	"	misc. C 8- C 16 aldehydes	acids	Tasayco and Prestwich, 1990
" (antennae)	aldehyde oxidase	(Z)9-tetradecenal	acid	"

Two types of enzyme that use different electron acceptors are responsible for the oxidation of alcohols and aldehydes: oxidases (which require O_2) and dehydrogenases (which require NAD^+ or $NADP^+$). For example, an alcohol oxidase found in the pheromone glands of females of *C. fumiferana* oxidizes (Z)11-tetradecen-1-ol to the aldehyde (Morse and Meighen 1984). The antennae of males of *H. virescens* contain an aldehyde oxidase which oxidizes the sex pheromone, (Z)9-tetradecenal, to the corresponding acid to prevent desensitization of the antennae (Tasayco and Prestwich 1990). Dehydrogenases from rat and horse liver oxidize long-chain ω-1- and ω-hydroxy acids to the corresponding keto- and diacids (Bjorkhem and Hamberg 1971, Bjorkhem *et al.* 1973). Potatoes contain

dehydrogenases that oxidize 16-hydroxyhexadecanoic acid to the corresponding oxo acid and further to the diacid (Agrawal and Kolattukudy 1978 a,b).

2.4 Specificity in pheromone blends

Many pheromones are blends with a major and several minor components in a precise ratio. These compounds are often synergistic in their effect, which makes the composition of a pheromone important for optimal activity. For example, QMC elicits highest retinue responses when all the components are present in the correct ratio (Slessor *et al.* 1988, Kaminsky *et al.* 1990). The specificity of a semiochemical blend arises because each step in the biosynthetic pathway has an inherent degree of substrate and product flexibility, thus allowing for a distribution of substrates to be used and a number of products to be formed. A single pathway can therefore give rise to a blend with one major component, resulting from the major substrate and product preference at each step, and some minor components. For example, pheromone biosynthesis in *T. ni* starts with chain shortening of octadecanoic acid, mostly by one round, but minor products from two and three rounds are observed. (Z) Δ11 desaturation occurs on 12- to 18-carbon substrates, 16 being the preferred length. The products of the desaturase are further chain shortened by one or, more frequently, two rounds of β-oxidation. Because carboxyl group modification is non-specific in this species (Jurenka and Roelofs 1989, 1993), the preceding desaturation and chain shortening determine the specificity of the pheromone blend which features (Z)7-dodecenyl acetate as the major component and Z(5)-dodecenyl acetate, their 14-carbon homologs and 11-dodecenyl acetate as minor components.

The composition of a semiochemical often changes during an insect's ontogeny. Females of *M. domestica* produce a sex pheromone comprised of mostly (Z)9-tricosene, some *cis* 9,10-epoxytricosane and (Z)14 tricosen-10-one. Newly emerged females and males produce mainly (Z)9-heptacosene, and as females mature the proportion of this alkene decreases and the proportion of (Z)9-tricosene, the epoxide and the ketone increase. The 23- and 27-carbon alkenes are produced from (Z)9-octadecenoic acid by 3 and 5

rounds of chain elongation, respectively, followed by reduction and decarbonylation. (Z)9-Tricosene is further oxidized to the ketone or the epoxide. Only females produce (Z)9-tricosene, the sex-specific step being the release of (Z)15-tetracosenoyl CoA from further elongation. This release is stimulated by 20-hydroxyecdysone which also controls ovary development, thereby linking sex pheromone production to ovary maturation (Blomquist *et al.* 1993).

2.5 Objectives of this work

The first objective of this work was to find the fatty acid precursor(s) to the mandibular acids and to determine whether the bees are able to synthesize the mandibular acids *de novo* from acetate. The lipid-bound fatty acids from mandibular glands also were analyzed to determine whether biosynthesis proceeds *via* a lipid-bound intermediate.

The next objective was to screen these compounds for interconversion, because the major and minor components in the mandibular blends are structurally related. For example, the keto- and diacids could be derived from the corresponding hydroxy acids by oxidation. The saturated and (E)2-unsaturated hydroxy acids could be derived from each other. Finally, the possibility that ω- and ω-1-hydroxy acids interconvert also was investigated.

The final targets were to determine the order of steps in the biosynthetic pathway and the route of ω- and ω-1-hydroxylation. This knowledge gave some insight into how the caste-specific blends in queens and workers arise and at which points in the pathway the control over caste-specificity resides.

Chapter II: Materials and Methods

II. 1. Sources of deuterated compounds

1.1 Purchased/donated chemicals

Terminal D_3 octadecanoic acid (D_3 C18:0) was purchased from MSD Isotopes, D_3 hexadecanoic acid (D_3 C16:0) was a gift from Dr. R. Cushley[1] and 7,7,8,8-D_4 decanoic acid (D_4 C10:0) was synthesized by Dr. S. King[1]. The D_3 C18:0 was 96 % pure (by GC) and contained 0.8 % unlabelled, 0.3 % D_1, 0.4 % D_2, 94 % D_3 and 4.9 % D_4 (calculated from M-15 ion in the mass spectrum (EI) of the TMS derivative). The D_3 C16:0 was 98 % pure (by GC) and contained 1.6 % D_2, 93 % D_3 and 5.6 % D_4. The D_4 C10:0 was 97 % pure by GC and contained 0.1 % unlabelled, 4.6 % D_2, 19 % D_3, 71 % D_4 and 5.5 % D_5. Mr. A. Yim[1] provided 1-^{13}C acetate, and 2-fluorooctadecanoic acid (2-F C18:0) was a gift from Dr. J. E. Oliver[2] (Oliver *et al.* 1994).

1.2 Chromatographic methods and determination of deuterium content

The purity of products was determined by gas chromatography (GC) on a Hewlett-Packard (HP) 5890 instrument equipped with a 30 m DB-1 fused-silica column (0.25 mm I. D., 0.25 μm film thickness) a flame ionization detector (FID) and a HP 3390A integrator. The gas chromatograph was run in the splitless mode and was programmed 100°C (1 min), 10°/min to 185 (4 min), 3°/min to 200 (0 min), 25°/min to 260 (20 min), flow 40 mL/min, head pressure 125 kPa. Fatty acids were converted to the trimethylsilyl (TMS) derivatives by reaction with *bis*-trimethylsilyl trifluoroacetamide (BSTFA) (Sigma) according to Slessor *et al.* 1990. The silylation mixture was diluted with hexane, and an aliquot was injected on the GC. GC-mass spectrometry (MS) was performed on a Varian Saturn Ion trap

[1] Dept. of Chemistry, S. F. U.
[2] USDA, ARS Beltsville Agriculture Research Center, Beltsville, MD.

instrument coupled to a Varian 3400 GC with a 30 m DB-5 fused-silica column (0.25 mm I. D., 0.25 μm film thickness) programmed 100°C (1 min), 10°/min to 180 (7 min), 25°/min to 240 (18.2 min), flow 20 mL/min, head pressure 105 kPa. Mass spectra were recorded with the automatic gain control (AGC) on, at 70 eV in the electron impact (EI) mode, with an ionization current of 10 μA. Most spectra were scanned from 70 - 350 amu. Chemical ionization (CI) spectra were obtained on a HP 5985B GC-MS instrument with isobutane as ionization gas.

NMR spectra were recorded on a 400 MHz Brucker instrument. Splitting patterns are described as singlet (s), doublet (d), triplet (t), multiplet (m) where the splitting was not resolved and "b" when peaks were broadened. Splitting constants are given in Hertz (Hz). IR spectra were recorded on a Perkin Elmer instrument. Intensity of IR absorptions are described as strong (s), medium (m), weak (w) and broad (b). Melting points were determined on a Fisher-Johns melting point apparatus and were not corrected.

Whenever possible, deuterium content was determined from ^{1}H NMR spectra by monitoring the disappearance of the appropriate signal. The signal due to residual ^{1}H at the site of interest was integrated relative to an isolated reference signal from the same compound and compared to an unlabelled standard. For example, when octanal was deuterated (Scheme 2), the signal at 2.41 ppm decreased from 2 in the unlabelled material to 0.14 H, the reference signal for integration being the aldehyde H at 9.76 ppm. The proportion of deuterium at the α position was (2-0.14)/2 = 0.93. Deuterium content was also estimated from the MS. Again, it was necessary to have an unlabelled standard in order to correct for natural isotope abundance according to Biemann (1962).

1.3 Synthesis of deuterated compounds

Five methods were used for the introduction of deuterium: 1) reduction of carbonyl groups with $NaBD_4$, 2) exchange of protons α to an aldehyde carbonyl with D_2O and pyridine, 3) deprotonation of a terminal alkyne followed by quenching with D_2O, 4) partial

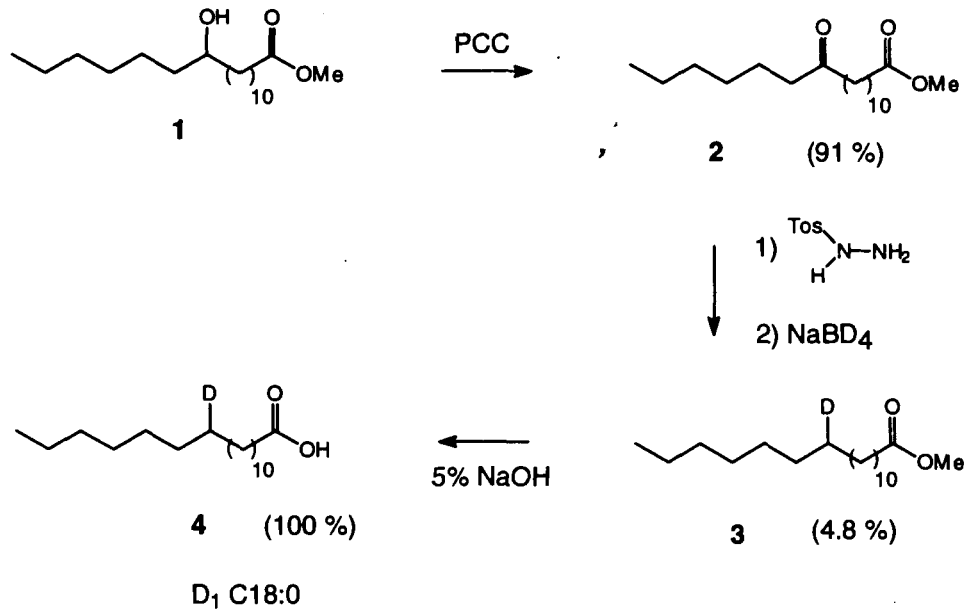
or complete catalytic reduction of alkynes with D_2 and 5) solvomercuration of terminal alkenes followed by demercuration with $NaBD_4$. The extent of deuteration was monitored by GC-MS and ^1H NMR. In some cases, ^2H NMR was used to confirm the deuteration pattern.

Solvents used for synthesis were reagent grade; solvents used for analytical work were HPLC grade. Tetrahydrofuran (THF) was distilled over $LiAlH_4$ prior to use; dimethylformamide (DMF) and hexamethylphosphoramide (HMPA) were distilled over CaH_2 and stored over molecular sieves (3 A). Pyridine (BDH) was stored over NaOH pellets. Dimethyl sulphoxide (DMSO) was distilled before use. In general, reactions were worked up by extraction of an aqueous mixture with diethyl ether (3X). The extract was washed with brine and dried over anhydrous Na_2SO_4. Reactions that required anhydrous, air-free conditions were performed in flame-dried glassware under Ar.

Reactions were monitored by thin-layer chromatography (TLC) which was performed on 0.25 mm thick films of silica gel (Merck, Kieselgel 60G) on glass plates. Acidic TLC plates were prepared with aqueous 2% H_3PO_4 instead of water. Acidic silica gel for the purification of free fatty acids was prepared by suspending one part of silica (Merck Kieselgel 60, 230-400 mesh) in two parts of aqueous 2% H_3PO_4, leaving the silica to settle, decanting the supernatant and drying the slurry in an oven at 100 °C. Acidic Florisil^R (60-100 mesh) was prepared the same way as acidic silica gel.

12-D_1 octadecanoic acid (D_1 C18:0)

This compound was synthesized from methyl 12-hydroxyoctadecanoate by the route outlined in Scheme 1.



Scheme 1. Synthesis of 12-D_1 octadecanoic acid (D_1 C18:0)

Oxidation of methyl 12-hydroxyoctadecanoate (**1**) to methyl 12-ketooctadecanoate (**2**)

The methyl ester **1** (Sigma, 1.00 g, 3.18 mmol) was added in small portions to a well-stirred suspension of pyridinium chlorochromate (PCC, Aldrich, 1.03 g, 4.77 mmol) in CH_2Cl_2 (50 mL) at room temperature (procedure of Corey and Schmidt, 1979). The initially orange suspension went brown soon after the addition and was left stirring at room temperature. After 2 h, ether (100 mL) was added, and the resulting solution filtered through a column of Florisil^R (40 g) with some silica gel (5 g) on top. The black residue that remained in the reaction vessel was rinsed with ether (100 mL), and this solution was also filtered through the column. After evaporation of the solvent, 0.90 g of **2**, a white solid, m. p. 43-44 °C, was obtained in 91 % yield. The material was 100 % pure (GC). IR (KBr): 2920 (s); 2849 (s); 1736 (s); 1704 (s); 1460 (m); 1209 (s) cm^{-1}. ^1H NMR (in $CDCl_3$): δ 0.87 (3 H, t, $J_{17,18}$= 7 Hz, 18-H); 1.26 (27 H, m, 4-9-, 15-17-H); 1.59 (10 H, m, 2, 10 and 14-H); 2.30 (2.3 H, t, $J_{2,3}$= 7 Hz, 2-H); 2.37 (4 H, tb $J_{10,11}$= $J_{13,14}$= 7 Hz, 11, 13-H); 3.66 (3 H, s, CH_3). MS (EI): m/e (relative abundance) 314 (4.6); 313 (18.1); 312 ($M^{+\cdot}$,4.9); 281 (28.4); 242 (McLafferty, 30.3); 227 (36.9); 185 (30.6); 167 (33.6); 153

(85.6); 152 (88.9); 135 (63.8); 128 (McLafferty, 43.5); 113 (83.8); 112 (100); 85 (93.7); 83 (97.8); 81 (79.3); 71 (86.0). The ions due to McLafferty rearrangement confirm that the keto group is in the 12 position (Biemann 1962).

Reduction of **2** to methyl octadecanoate (**3**) and hydrolysis of **3**

p-Tolylsulphonhydrazine (Sigma , 132 mg, 0.43 mmol) and **2** were mixed in methanol and the solution was heated to 80°C for 2 h, after which the mixture was cooled to 50°C and $NaBD_4$ (Aldrich, 13.4 mg, 0.32 mmol) was added. Two h after the addition of $NaBD_4$, the apparatus was cooled to room temperature and the reaction quenched with cold water. The reaction mixture was neutralized with 10% HCl and extracted with ethyl acetate (3 X 50 mL). The extract was washed with brine and dried over Na_2SO_4. The crude product had 10% of the desired product **3**, along with 52% of unreacted **2**, as well as 44% of methyl 12-hydroxy-octadecanoate which probably formed in a competing $NaBD_4$ reduction of the keto group. Chromatography on a column of silica gel (30 g) with hexane:ether (10:1) afforded 6 mg of **3** in 4.8 % yield. The GC retention time of **3** matched that of a commercial methyl octadecanoate standard.

Methyl octadecanoate **3** from two rounds of reduction (8.5 mg, ca. 0.03 mmol) was hydrolyzed with 5% NaOH to give 8.5 mg of **4**. Purification on a column of acidic silica gel (1 g, in a Pasteur pipette) with hexane afforded a white solid, m. p. 66-70 °C (commercial standard, Anachemia, 68-70°C), that was 93 % pure by GC (TMS derivative). MS (EI) of the TMS derivative, m/e (relative abundance): 358 (5.1); 357 ($M^{+\cdot}$, 19); 356 (3.4); 343 (28); 342 (M-15, 100); 341 (20) 129 (8); 117 (45); 75 (42); 73 (42). MS (CI) of the TMS derivative, m/e: 361 (1.5); 360 (6.8); 359 (29); 358 (M+1, 100); 357 (22). ^2H NMR (in $CHCl_3$): δ 1.30 (sb). The material was 83 % D_1, as judged by MS (CI), which is consistent with the value obtained from the M-15 fragment in the EI spectrum (84 % D_1).
For comparison, the MS (EI) of the TMS derivative of an unlabeled standard was obtained: m/e (relative abundance) 357 (4.6); 356 ($M^{+\cdot}$, 13); 355 (0.5); 343 (8.2); 342 (22); 341 (M-15, 82); 340 (2.1); 129 (38); 117 (84); 75 (45); 73 (56).

4,4-D_2 (E)2-decenoic acid (D_2 C10:1)

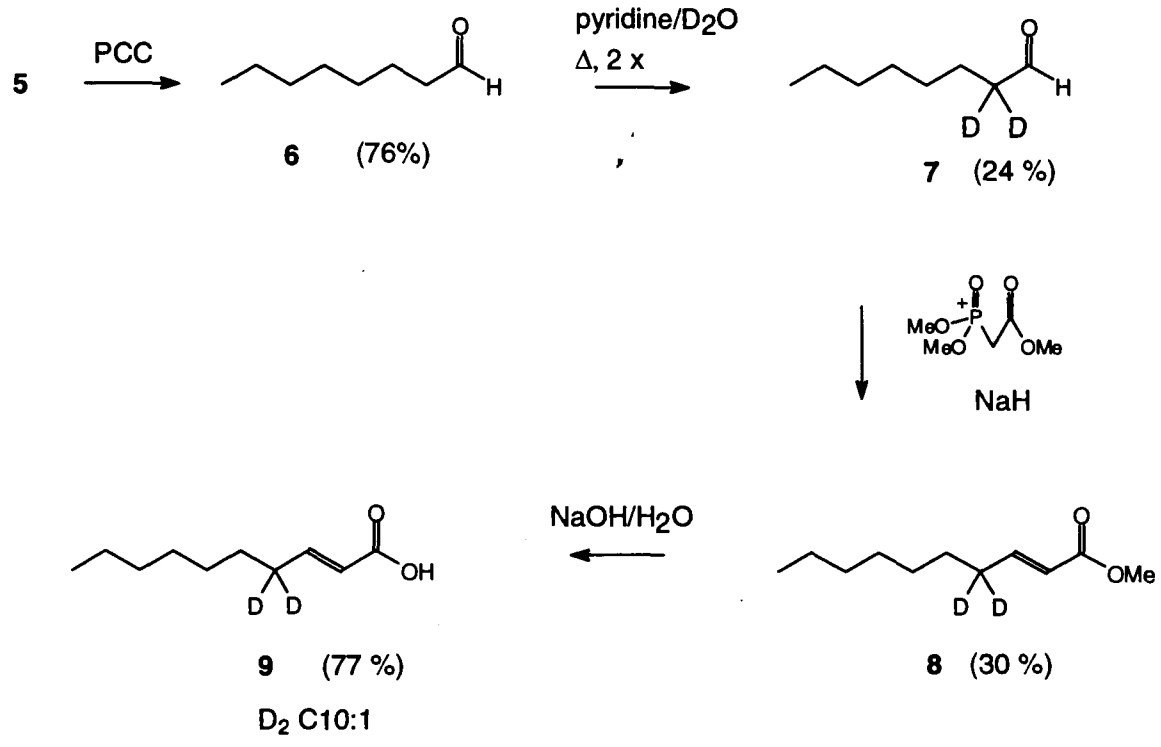
This compound was synthesized by condensation of octanal and trimethylphosphonoacetate. Deuterium was introduced by exchange of the protons α to the carbonyl group in octanal (Scheme 2).

Oxidation of 1-octanol (**5**)

1-octanol (**5**, Aldrich, 2.09 g, 16 mmol) was oxidized with PCC (6.92 g, 32 mmol) by the same procedure as described in Scheme 1, which gave 1.56 g of octanal (**6**), a colorless oil that was 100% pure by GC. IR: 2928 (s); 2857 (s); 1711 (s); 1465 (m); 1274 (w) cm^{-1}. ^1H NMR (in $CDCl_3$): δ 0.90 (8 H, t $J_{7,8}$ = 8 Hz, 8-H); 1.25 (22 H, m, 4-7-H); 1.61 (5 H, m, 3-H); 2.41 (2 H, t $J_{2,3}$ = 10 Hz, 2-H); 9.76 (1 H, t $J_{1,2}$ < 5 Hz, 1-H). MS (EI): m/e (relative intensity) 129 (0.3); 128 ($M^{+\cdot}$, 0.7); 110 (1.3); 109 (1.2); 44 (11); 40 (100).

Deuteration of octanal (**6**)

Two rounds of deuteration were carried out, starting with 0.584 g (4.5 mmol) of **6** and 10 mL of D_2O/pyridine (1:1, v/v). In both rounds, the mixture was heated to 100°C for 3 h, then cooled to room temperature. Once cool, cold tap water was added to the mixture and the product extracted with pentane (2 X 50 mL). The combined pentane extract was washed with water (2 X 5 mL), then with brine and dried over Na_2SO_4. Purification on a column of silica gel (300 g) with hexane:ether (5:1) afforded 0.142 g of a colorless oil **7** (100 % pure by GC) in 24 % yield. ^1H NMR (in $CDCl_3$): δ 0.90 (3.7 H, t $J_{7,8}$ = 8 Hz, 8-H); 1.25 (12 H, m, 4-7-H); 1.61 (3 H, m, 3-H); 2.41 (0.14, m, residual 2-H); 9.76 (1 H, s, 1-H). MS(EI): m/e (relative intensity) 130 ($M^{+\cdot}$, 0.3); 112 (0.5); 111 (1.2); 46 (19); 41 (100). As calculated from the ^1H NMR, 93 % of the α protons had exchanged with D.



Scheme 2. Route to 4,4-D_2 (E)2-decenoic acid (D_2 C10:1).

4,4-D_2 (E)2-decenoic acid (**9**) from 2,2-D_2 octanal (**7**)

Labelled octanal was condensed with trimethylphosphonoacetate (TMPA) according to a procedure described by Kandil (1985). NaH (60% dispersion in mineral oil, 0.09 g, 2.3 mmol) was placed in a 2-neck round-bottom flask fitted with a dropping funnel. The hydride was rinsed twice with pentane, suspended in freshly distilled THF (20 mL) and cooled to 0°C (ice bath). TMPA (Aldrich, 0.22 g, 1.2 mmol) was added dropwise in THF (3 mL) with stirring. Five min after the addition, the ice bath was removed and the mixture stirred at room temperature for 1 h, after which **7** (0.13 g, 1.0 mmol) was added in THF (20 mL). The reaction was quenched 3 h after the addition of the aldehyde with water (20 mL) and enough 10% HCl to neutralize the mixture which was extracted with ether (3 X 70 mL). The combined extract was washed with brine and dried over Na_2SO_4. The crude product was 84 % pure by GC and contained one major impurity (13 %) which was identified as the Z isomer. Chromatography on a column of silica gel (300 g) with

hexane:ether 5:1 gave 56 mg of **8**, a colorless oil, in 30 % yield. The product contained 5% of the Z isomer by GC. ^{1}H NMR (in $CDCl_3$): δ 0.87 (4 H, t $J_{9,10}$= 6Hz, 10-H), 1.26 (10.3 H, m, 5-9-H); 2.20 (0.11 H, m, residual 4-H); 3.71 (3.5 H, s, CH_3); 5.80 (1 H, d $J_{2,3}$= 16 Hz, 2-H); 6.97 (1 H, d $J_{2,3}$=16 Hz, 3-H). MS (EI) : m/e (relative abundance) 189 (1); 188 ($M^{+\cdot}$, 6); 187 (39); 155 (32); 154 (35); 125(26); 115 (28); 97 (38); 87 (100); 83 (53); 82 (42); 81 (53). The material was 93% enriched for 2 D in the allylic position, which is consistent with the value obtained from the aldehyde intermediate.

An unlabelled standard of methyl (E)2-decenoate was synthesized by the same procedure, starting with 0.55 g (4.3 mmol) of **6**, in 42 % yield. IR: 2928 (s); 2856 (m); 1728 (s); 1658 (s); 1271 (s); 1175 (s) cm^{-1} . ^{1}H NMR (in $CDCl_3$): δ 0.87 (4 H, t $J_{9,10}$= 6 Hz, 10-H), 1.26 (11.3 H, m, 5-9-H); 2.20 (2.3 H, d $J_{3,4}$= 8 Hz, t $J_{4,5}$= 4 Hz, 4-H); 3.71 (3.3 H, s, CH_3); 5.80 (1 H, d $J_{2,3}$= 16 Hz, 2-H); 6.97 (1 H, d $J_{2,3}$= 16 Hz, t $J_{3,4}$= 8 Hz, 3-H). MS (EI): m/e (relative abundance) 187 (2.0); 186 ($M^{+\cdot}$,3.9); 185 (27); 153 (24); 152 (25); 123(25); 113 (35); 96(31); 87(100); 81(66).

The Z isomer eluted from the column before the E isomer, and the unlabelled material was isolated for identification. IR: 2926 (s); 2856 (m); 1728 (s); 1646 (m); 1170 (s). The ^{1}H NMR resonances for the vinylic protons were δ 5.75 (1 H, d $J_{2,3}$ = 10 Hz, 2-H); 6.21 (1 H, d $J_{2,3}$ = 10 Hz, t $J_{3,4}$= 6 Hz, 3-H).

A methanol solution of **8** (6.5 mg, 0.03 mmol) was added to 5% aqueous NaOH and stirred at room temperature overnight. Acidification and extraction with ether gave 4.6 mg of pure **9**, a colorless oil, in 77 % yield. MS (EI) of the TMS derivative: m/e (relative abundance) 245 (0.6); 244 ($M^{+\cdot}$, 2.5); 229 (M-15, 46); 139 (0.6); 131 (27); 129 (28); 117 (52); 83 (10) 75 (66); 73 (100).

An unlabelled standard was synthesized. MS (EI) of the TMS derivative: m/e (relative abundance) 243 (2.3); 242 ($M^{+\cdot}$, 0.7); 227 (M-15, 100); 152 (3.4); 137 (4.7); 129 (44); 117 (5.2); 81 (10); 75 (49); 73 (29).

17,18,18-D_3 17-octadecenoic acid (D_3 C18:1 Δ^{17}) and 17,17,18,18,18-D_5 octadecanoic acid (D_5 C18:0)

These compounds were synthesized *via* 17-octadecyn-1-ol, as outlined in Scheme 3. Deuterium was introduced by exchange of the acetylenic hydrogen and by catalytic deuteration. I thank Ms. P. Zhang[3] for helping with the synthesis of D_5 C18:0 and D_3 C18:1 Δ^{17}.

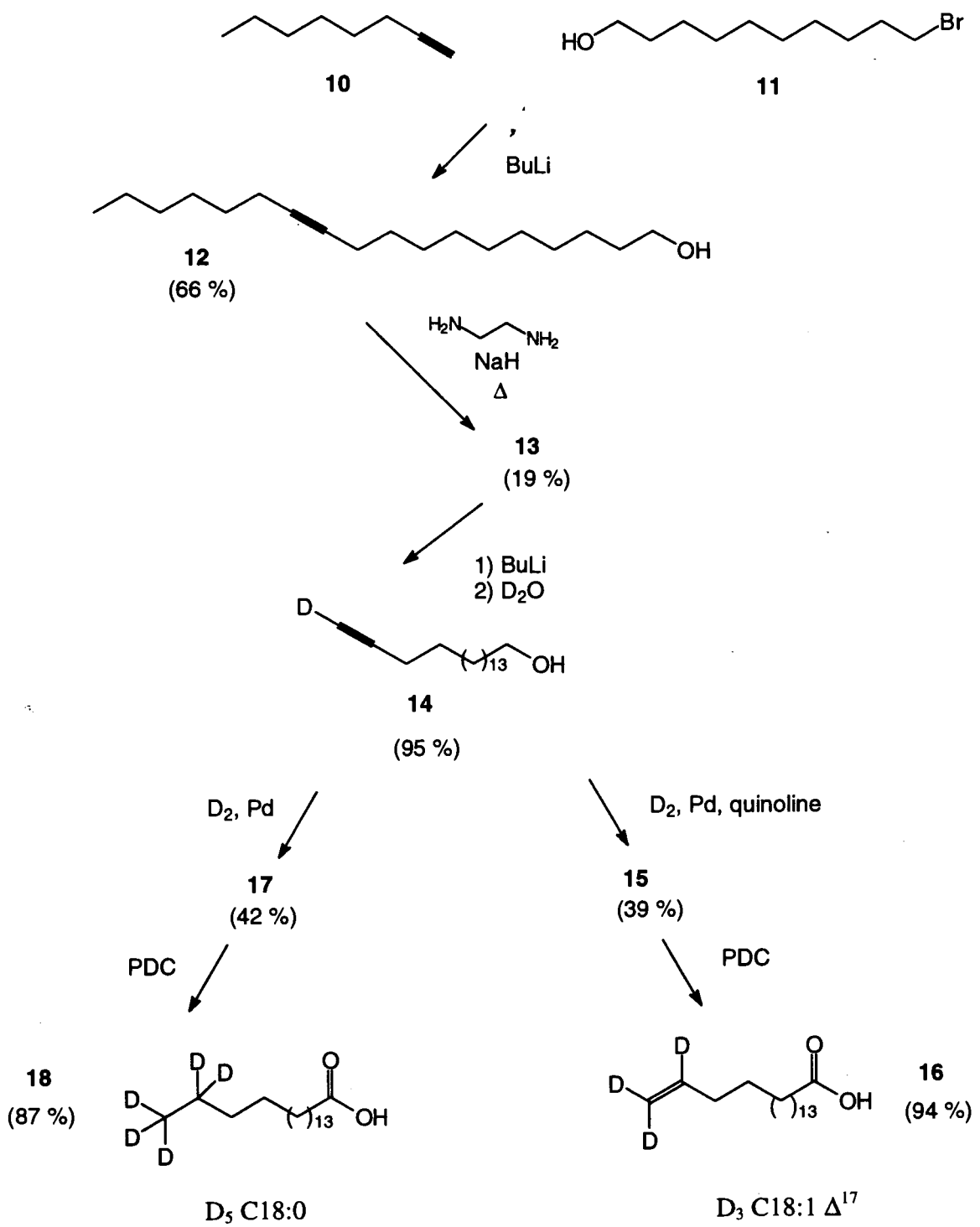
10-bromo-1-decanol (**11**)

1,10-Decanediol (Aldrich, 5.03 g, 29 mmol) was reacted for 24 h with 40 % HBr (20 mL) under continuous extraction with heptane. The crude product was recovered from the heptane phase and was purified by chromatography on silica gel with hexane:ether (1:1) to give 4.08 g of pure product (97 % by GC) in 60 % yield. IR: 3333 (sb); 2922 (s); 2850 (s); 1464 (s); 1372 (m); 1257 (s); 1057 (s); 722 (m); 646 (m) cm^{-1} . MS (EI): m/e (relative abundance) 218 ($M^{+\cdot}$, 1.1); 191 (8.4); 189 (8.9); 178 (2.6); 176 (3.2); 163 (18.4); 161 (20); 150 (22); 149 (61); 148 (74); 137 (39); 135 (40); 123 (3.2); 121 (4.2); 111 (13): 109 (23); 107 (13); 97 (61); 96 (16); 95 (27); 83 (100); 82 (33); 81 (45); 80 (6.3) 79 (12).

11-octadecyn-1-ol (**12**)

1-Octyne (**10**) and **11** were condensed according to the procedure of Hendry *et al.* 1975. A solution of **10** (CPL Inc., 1.16 g, 10.5 mmol) in THF (20 mL) was mixed with 2.5 M butyl lithium (BuLi) in hexane (Sigma, 4.2 mL) at 0°C. The resulting yellow

[3] Dept. of Chemistry, S. F. U.



Scheme 3. Synthesis of 17,17,18,18,18-D_5 octadecanoic and 17,18,18-D_3 17-octadecenoic acids.

suspension was stirred for 1.5 h at 0°C, after which a solution of **11** (1.00 g, 4.2 mmol) in HMPA (15 mL) was added slowly. The temperature was kept at < 5 °C during the addition. The mixture, which turned brown, was allowed to warm to room temperature and was stirred overnight. After aqueous workup, 1.24 g of crude product was obtained. Chromatography on a column of silica gel (300 g) with hexane:ether (1:1) afforded 0.738 g of pure product (94% by GC) in 66% yield. IR: 3348 (sb); 2926 (s); 2856 (s); 1942 (w); 1745 (w); 1466 (m); 1378 (m); 1196 (w) cm^{-1}. MS (EI) m/e (relative abundance) 206 (M-60, 0.5); 192 (1); 177 (1); 163 (2); 149 (3.2); 135 (9.5); 124 (18); 109 (25); 95 (68); 93 (24); 91 (13); 81 (100); 79 (44); 77 (13); 71 (7.6); 70 (7.4). MS(EI) of the TMS derivative: m/e (relative abundance) 337 (0.2); 325 (0.3); 324 (2.9); 323 (M-15, 9.8); 295 (2.4); 281 (1.1); 267 (1.8); 247 (4.8); 183 (5.6); 163 (6.7); 149 (12); 135 (17); 121 (20); 109 (26); 103 (13); 101 (8.7); 95 (47); 93 (21); 91 (11); 82 (29); 81 (64); 79 (28); 75 (100); 73 (45). ^{1}H NMR (in $CDCl_3$): δ 0.90 (4.5 H, t $J_{17,18}$ = 7 Hz, 18-H); 1.30 (26 H, m, 3-8 and 15-17-H); 1.53 (9 H, m, 9, 14, 2-H); 2.13 (4 H, tb $J_{9,10} = J_{13,14}$ = 8 Hz, 10-,13-H); 3.63 (2 H, t $J_{1,2}$ = 6 Hz, 1-H).

Isomerization of **12** to 17-octadecyn-1-ol (**13**)

Base-mediated isomerization of **12** was accomplished using the procedure of Abrams (1982). NaH (60 % dispersion in mineral oil, 700 mg, 17.5 mmol) was rinsed with pentane and mixed with diaminoethane (DAE, Fisher, 8 mL). Once the vigorous evolution of H_2 subsided, the mixture was heated to 80 °C. After 2 h, a solution of **12** (730 mg, 2.74 mmol) in DAE (8 mL) was added to the reagent at 80 °C. After addition, the mixture was allowed to cool to room temperature and was stirred overnight. The reaction was quenched with ice water, and the aqueous mixture acidified with 10 % HCl. Workup furnished 704 mg of crude **13** which was purified by chromatography with ether:hexane (1:1). The resulting product (137 mg, 19 % yield) was 93 % pure by GC. IR: 3285 (sharp peak due to alkyne H-C superimposed on the broad OH band); 2918 (s); 2849 (m); 1736 (w); 1462 (m); 1364 (w); 1123 (w); 1060 (m). MS(EI) of the TMS derivative: m/e (relative abundance) 338 ($M^{+\cdot}$, 0.5) 325 (1.6); 324 (6.3); 323 (M-15, 26); 295 (2.1) 267 (2.0); 247 (4.2); 149

(4.2); 103 (7.9); 95 (10); 91 (3.5); 81 (15); 75 (100). ^{1}H NMR: δ 1.26 (22 H, m, 3-14-H); 1.55 (9 H, m, 3- and 15-H); 1.94 (1 H, t $J_{16,18}$= 3 Hz, 18-H); 2.18 (2 H, t $J_{15,16}$= 8 Hz, d $J_{16,18}$= 3 Hz, 16-H); 2.38 (0.5 H, t J= 9 Hz, impurity); 3.64 (3.3 H, t $J_{1,2}$= 8 Hz, 1-H + impurity).

Exchange of the acetylenic H of **13** with D

Two mL of 2.5 M BuLi (5 mmol) in hexane were added to a solution of **13** (400 mg, 1.5 mmol) in THF (5 mL). The mixture was stirred for 2 h, after which D_2O (2 mL) was added. The mixture was extracted with hexane. After two rounds of deuteration by the above procedure, material that was 83 % pure by GC (380 mg) was recovered. IR: 3314 (sb); 2919 (s); 2850 (m); 2584 (w); 2248 (w); 1654(wb); 1462 (m); 1378 (w); 1057 (m) cm^{-1}. MS (EI) of the TMS derivative: m/e (relative abundance) 339 (M^+, 0.4); 326 (1.5); 325 (5.2); 324 (M-15, 12); 323 (6.9); 149 (1.6); 103 (7.3); 95 (7.7); 91 (3.0); 82 (12); 75 (100). ^{1}H NMR (in $CDCl_3$): δ 1.28 (28 H, m, 3-14-H); 1.57 (5.7, m, 2- and 15-H); 1.96 (0.2, sb, residual 18-H); 2.00 (0.9, m, impurity); 2.18 (1.1, t $J_{15,16}$= 8 Hz, 16 H); 3.64 (2 H, t $J_{1,2}$= 8 Hz, 1-H). According to the ^{1}H NMR, the material was 80 % D_1.

Catalytic deuteration of **14** to 17,18,18-D_3 17-octadecen-1-ol (**15**) and 17,17,18,18,18-D_5 octadecan-1-ol (**17**)

The alkynol **14** (100 mg, 0.37 mmol) was dissolved in hexane (50 mL). Pd (5 %) on $CaCO_3$ (Aldrich , 10 mg) and quinoline (Fisher, 0.5 mL) were added to the solution in a filtering flask that was fitted with a pipette bulb and a septum. The flask was pressurized with D_2 and the mixture stirred for 2 h at room temperature. The crude product that was obtained after filtration and washing of the filtrate with 10 % H_2SO_4, contained unreacted alkynol:alkenol 3:2 (106 mg total). Purification on a column of silica gel (40 g) with ether:hexane (1:1) gave 39 mg of **15** (84 % by GC) in 39 % yield. IR 3346 (sb); 2922 (s); 2854 (s); 2244(w); 1582 (w); 1466 (s); 1378 (m); 1056 (sb) cm^{-1}. MS (EI): m/e (relative abundance) 272 (4.0); 271 (M^+, 4.4); 270 (2.2); 269 (1.5); 252 (1.5); 168 (1.4); 167 (2.0);

153 (2.8); 152 (4.4); 151 (4.0); 139 (7.0); 138 (9.8); 137 (11); 125 (16); 124 (20); 123 (21); 111 (32); 110 (27); 109 (29); 97 (62); 96 (61); 95 (62); 83 (65); 82 (77); 81 (100). MS (EI) of the TMS derivative: m/e (relative abundance) 343 ($M^{+\cdot}$, 1.3); 342 (1.9); 341 (0.8); 333 (4.5); 332 (M-15 of over-reduced material, 7.3); 331 (2.5); 330 (1.1); 329 (3.4); 328 (M-15, 11); 327 (19); 326 (4.7); 325 (0.9); 299 (3.3); 298 (3.8); 297 (1.5); 296 (0.7); 185 (2.1); 149 (1.6); 143 (3.2); 129 (4.8); 111 (3.4); 103 (10); 95 (4.9); 91 (5.3); 89 (6.1); 81 (8.1); 75 (100). ^{1}H NMR (in $CDCl_3$): δ 1.27 (29 H, m, 3-14-H); 1.56 (9 H, m, 2- and 15-H); 2.02 (2 H, t $J_{15,16}$= 8.5 Hz, 16-H); 3.64 (2 H, t $J_{1,2}$= 8 Hz, 1-H).

The alkynol **14** (100 mg, 0.37 mmol) was deuterated by the same procedure as described above, except that quinoline was omitted. After filtration, 148 mg of crude product were recovered. Purification by chromatography as described above afforded 43 mg of **17** (88 % by GC) in 42 % yield. IR: 3376 (sb); 2925 (s); 2853 (s); 2251 (w); 2214 (w); 1464 (m); 1388 (s); 1056 (m) cm^{-1}. MS (EI) of the TMS derivative: m/e (relative abundance) 351 (0.5); 350 (1.6); 349 (4.1); 348 (8.0); 347 ($M^{+\cdot}$, 6.8); 346 (5.9); 345 (3.9); 344 (2.3); 343 (1.5); 342 (2.3); 336 (6.6); 335 (9.7); 334 (16); 333 (26); 332 (M-15 for D_5, 44); 331 (23); 330 (11); 329 (3.9); 328 (2.6); 327 (M-15 for unlabelled, 6.8); 221 (7.4); 149 (27); 129 (11); 115 (12); 111 (19); 103 (33); 97 (33); 91 (26); 83 (30); 81 (13); 75 (100). ^{1}H NMR (in $CDCl_3$): δ 1.26 (27.5 H, m, 3-16); 1.58 (4.9 H, m, 2-H); 3.64 (2 H, t $J_{1,2}$= 8 Hz, 1-H). From the MS it was apparent that a distribution of deuterated forms of the octadecanol had formed. The distribution was centered around the D_5 material as estimated from the relative intensity of the corresponding M-15 ions: D_9 (4.1 %); D_8 (5.7 %); D_7 (9.8 %); D_6 (14 %); D_5 (32 %); D_4 (17 %); D_3 (8.2 %); D_2 (2.9 %); D_1 (1.0 %); unlabelled (5.4 %).

Oxidation of **15** and **17** to the corresponding acids, **16** and **18**

The D_3 alkenol **15** (39 mg, 0.14 mmol) was oxidized, according to the procedure of Corey and Schmidt (1979), with pyridinium dichromate (PDC, Aldrich, 194 mg, 0.52 mmol) in DMF (4 mL). The solution was stirred at room temperature overnight. Water (5

mL) and 10 % HCl (5 mL) were added to the solution, and the mixture was extracted with ether (3 X). The ether extract was washed with 5 % HCl until it was colorless. The extract was dried over Na_2SO_4. The crude product was purified on a 20 g column of acidic silica gel with ether:hexane (1:1), which afforded 38 mg of product (76 % pure by GC) in 94 % yield. The impurities were identified as lower homologs by MS, and their proportion was estimated by GC: 17-C (16 %), 16-C (3.8 %), 15-C (3.3 %) and 14-C (1.3 %). MS (EI) of the TMS derivative of **16**: m/e (relative abundance) 359 (2.6); 358 (7.1); 357 (M^+,3.6); 356 (1.4); 346 (3.5); 344 (5.4); 343 (19); 342 (M-15, 60); 341 (13); 247 (3.4); 241 (2.6); 199 (10); 185 (11); 183 (6.6); 171 (6.4); 169 (3.5); 165 (2.8); 159 (3.5); 145 (17); 129 (63); 117 (99); 95 (21); 93 (8.5); 91 (4.0); 83 (18); 81 (25); 75 (90); 73 (100). The MS revealed that a distribution of under- and overdeuterated forms, centered around the D_3 material, had formed during the reduction: D_6 (3.1 %), D_5 (4.5 %), D_4 (8.0 %), D_3 (66 %), D_2 (14 %), D_1 3.9 %. ^{1}H NMR (in $CDCl_3$): δ 1.27 (20 H, m, 4-14); 1.63 (4 H, m, 3- and 15-H); 2.20 (2 H, t $J_{15,16}$= 7 Hz, 16-H); 2.34 (2 H, t $J_{2,3}$= 8.5 Hz, 2-H). ^{2}H NMR ($CHCl_3$): δ 0.73 (0.15 D, sb, 18-D of over-reduced material); 1.29 (0.16 D, sb, -CD_2- of over-reduced material); 5.02 and 5.07 (2.1 D, sb not resolved, 18-D); 5.91 (1 D, sb, 17-D).

The D_5-octadecanol **17** (43 mg, 0.15 mmol) was oxidized as described above for **15**. After column purification, 39 mg of **18** (65 % by GC) were obtained in 87 % yield. The impurities were the following lower homologs 17-C (15 %), 16-C (14 %), 15-C (4.3 %) and 14-C (1.9 %). MS(EI) of the TMS derivative: m/e (relative abundance) 365 (0.6); 364 (1.6); 363 (4.5); 362 (8.7); 361 (13); 360 (7.7); 359 (3.0); 358 (0.7); 357 (0.6); 356 (1.1); 350 (2.5); 349 (4.5); 348 (9.5); 347 (18); 346 (36); 345 (15); 344 (4.2); 343 (1.4); 342 (1.2); 341 (3.0); 129 (46); 117 (100); 75 (61); 73 (74). MS(CI) of the TMS derivative: m/e (relative abundance) 366 (6.8); 365 (12); 364 (25); 363 (54); 362 (M+1 for D_5, 100); 361 (51); 360 (19); 359 (12); 358 (19); 357 (M+1 for unlabelled, 14). Both mass spectra confirm that the following distribution of over- and underdeuterated forms of **18** were present: D_9 (1.9 %), D_8 (3.2 %), D_7 (6.8 %), D_6 (14 %), D_5 (35 %), D_4 (18 %), D_3 (6.6 %), D_2 (3.3 %), D_1 (6.2 %) and unlabelled (5.4 %). This is consistent with the values obtained from **17**. ^{1}H NMR (in $CDCl_3$): δ 1.27 (18 H, m, 3-16-H); 1.62 (2.7 H, m, 3-H); 2.35 (2 H,

t $J_{2,3}$= 8.5 Hz, 2-H). ^{2}H NMR ($CHCl_3$): δ 0.87 (3-D, sb, 18-D); 1.28 (4-D, sb, 17-D and additional D).

9,10-D_2 and 9,10,10-D_3 9-decenoic acids (D_2 and D_3 C10:1 Δ^9)

These acids were synthesized *via* 9-decyn-1-ol (Scheme 4).

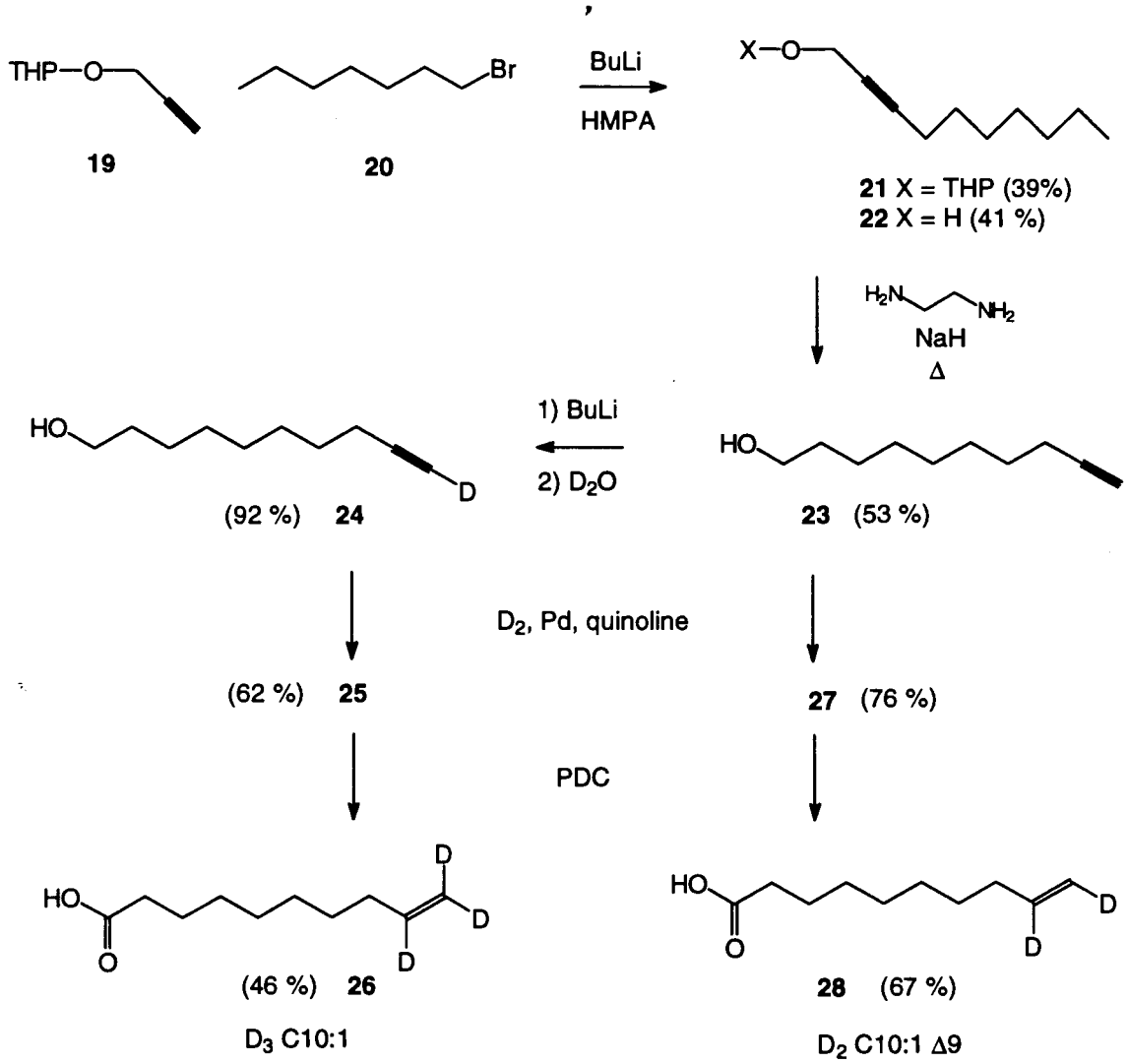
THP derivative of 2-decyn-1-ol (**21**)

The THP-derivative of propynol **19** (3.37 g, 24 mmol) and 1-bromoheptane **20** (Aldrich, 4.3 g, 24 mmol) were condensed by the same method used to synthesize **12** in Scheme 3. After purification on a column of silica gel with hexane:ether (9:1), 2.23 g of pure (94 % by GC) product was obtained in 39 % yield. IR: 2930 (s); 2875 (m); 2237 (w); 1466 (m); 1362 (m); 1118 (s); 1025 (s) cm^{-1}. MS(EI): m/e (relative abundance) 238 ($M^{+\cdot}$, 3.3); 195 (2.2); 180 (2.7); 167 (5.5); 153 (4.9); 137 (5.5); 125 (6.6); 111 (35); 101 (57); 95 (93); 85 (100); 81 (86); 79 (50). ^{1}H NMR: δ 0.89 (5 H, t $J_{9,10}$= 8 Hz, 10-H); 1.56 (22 H, m, 2'-4'(THP) and 6-10-H); 2.20 (2 H, t $J_{4,5}$= 8 Hz, t $J_{1,4}$= 3 Hz, 4-H); 3.53 (1-H, m, 5'-H (THP)); 3.84 (1 H, 5'-H (THP)); 4.15 (1 H, d $J_{1,1}$= 14 Hz, t $J_{1,4}$= 3 Hz, 1-H); 4.33 (1 H, d $J_{1,1}$= 14 Hz, t $J_{1,4}$= 3 Hz, 1-H); 4.81 (1 H, t $J_{1',2'}$= 5 Hz, 1'-H (THP)).

Removal of the THP group and isomerization of **22** to the terminal alkynol **23**

A solution of **21** (2.23 g; 9.3 mmol) in water:methanol:10% HCl (1:1:1, total 30 mL) was refluxed at 60°C for 2 h. After workup 0.916 g of crude product was obtained. This material was purified by column chromatography with ether:hexane (1:1), which afforded 0.586 g of **22** (100 % pure by GC) in 41 % yield. IR: 3329 (sb); 2930 (s); 2880 (s); 2288 (w); 2226 (w); 1475 (s); 1344 (m); 1137 (m); 1030 (s) cm^{-1}. MS(EI) of the TMS derivative: m/e (relative abundance) 213 (1.0); 212 (3.3); 211 (M-15, 15); 210 (2.2); 193 (3.3); 181 (16); 155 (9); 143 (12); 135 (11); 127 (13); 121 (7.7); 109 (6.6); 101 (7.7); 93 (14); 79 (16); 75 (100); 73 (56). ^{1}H NMR: δ 0.87 (3.4 H, t $J_{9,10}$= 8 Hz, 10-H); 1.31 (9.7 H,

m, 6-9-H); 1.50 (3.6 H, m, 5-H); 2.20 (2.3 H, t $J_{4,5}$= 8 Hz, t $J_{1,4}$= 3 Hz, 4-H); 4.24 (2 H, t $J_{1,4}$= 3 Hz, 1-H).



Scheme 4. Synthesis of 9,10,10-D_3 and 9,10-D_2 9-decenoic acids.

The isomerization of **22** (0.586 g, 3.8 mmol) was accomplished the same way as the isomerization of **12** (Scheme 3). After workup and purification by chromatography with ether:hexane (1:1), 0.310 g of pure **23** (100 % by GC) was obtained in 53 % yield. IR: 3309 (sharp peak due to alkyne C-H superimposed on broad OH band); 2931 (s); 2856 (m);

2361 (w); 2117 (w); 1463 (m); 1363 (w); 1050 (s) cm^{-1}. MS(EI) of the TMS derivative: m/e (relative abundance) 226 ($M^{+\cdot}$, 0.4); 225 (0.8); 213 (1.6); 212 (2.3); 211 (M-15, 9.3); 210 (3.1); 193 (4.6); 181 (16); 155 (10); 143 (13); 135 (15); 127 (17); 109 (7.7); 95 (9.3); 93 (10); 75 (100); 73 (60). ^{1}H NMR (in $CDCl_3$): δ 1.34 (10 H, m, 3-6-H); 1.54 (4.6 H, m, 2- and 7-H); 1.93 (1 H, t $J_{8,10}$= 3 Hz, 10-H); 2.18 (2 H, t $J_{7,8}$= 7 Hz, d $J_{8,10}$= 3 Hz, 8-H); 3.64 (2 H, tb $J_{1,2}$= 8 Hz, 1-H).

Exchange of the acetylenic H of **23** with D

The alkynol **23** (0.310 g, 2.0 mmol) was treated with BuLi and D_2O as described before for compound **13** (Scheme 3). After workup, 0.286 g of pure (100 % by GC) **24** was recovered in 92 % yield. MS(EI) of the TMS derivative: m/e (relative abundance) 214 (0.7); 213 (1.2); 212 (M-15, 3.8); 211 (1.1); 194 (1.6); 183 (2.2); 182 (1.1); 154 (1.6); 143 (1.6); 136 (15); 130 (3.8); 129 (6.5); 108 (6.6); 101 (11); 94 (9.2); 75 (100); 73 (32). ^{1}H NMR 1.33 (5.4 H, m, 3-6-H); 1.55 (2.6 H, m, 2- and 7-H); 1.93 (0.08 H, t $J_{8,10}$= 3 Hz, residual 10-H); 2.17 (2 H, t $J_{7,8}$= 7 Hz, 8-H); 3.64 (2 H, t $J_{1,2}$= 8 Hz, 1-H). This material was 92 % enriched for 1 D, as calculated from the ^{1}H NMR.

Deuteration of **23** to 9,10-D_2 9-decen-1-ol (**27**) and of **24** to 9,10,10-D_3 decen-1-ol (**25**)

The unlabelled alkynol **23** (288 mg, 1.87 mmol) was partially deuterated following the same procedure as in Scheme 3. Column purification of the crude product afforded 224 mg of pure **27** (96 % by GC) in 76 % yield. MS(EI) of the TMS derivative: m/e (relative abundance) 231 (0.9); 230 ($M^{+\cdot}$, 1.6); 229 (1.8); 228 (0.9); 217 (1.1); 216 (4.9); 215 (M-15, 16); 214 (6.6); 213 (1.6); 197 (3.8); 186 (2.7); 149 (6.6); 139 (2.7); 129 (3.3); 115 (3.8); 103 (9.9); 89 (6.0); 83 (8.2); 82 (9.3); 81 (7.7); 75 (100); 73 (26). ^{1}H NMR: δ 1.30 (11.6 H, m, 3-6-H); 1.60 (7 H, m, 2- and 7-H); 2.03 (2 H, tb $J_{7,8}$= 7 Hz, 8-H); 3.64 (2 H, tb $J_{1,2}$= 9 Hz, 1-H) 4.90 (0.2, m, residual 10-H); 4.96 (0.6 H, m, 10'-H); 5.80 (0.2, m, residual 9-H). The ^{1}H NMR revealed that this material was 80 % enriched with D in the 9- and 10-position and that there was some D in the 10' position.

The labelled alkynol **24** (255 mg, 1.66 mmol) was partially deuterated as described above. The crude product was purified by chromatography on silica gel with ether:hexane (1:1), which gave 162 mg of pure alkenol **25** (62 % yield). MS(EI) of the TMS derivative: m/e (relative abundance) 232 (0.8); 231 ($M^{+\cdot}$, 1.1); 218 (1.2); 217 (4.4); 216 (M-15, 16); 215 (3.3); 214 (1.1); 198 (4.3); 186 (2.3); 149 (1.3); 140 (3.3); 129 (2.7); 115 (3.2); 103 (8.8); 75 (100); 73 (26). ^{1}H NMR (in $CDCl_3$): δ 1.31 (14.8 H, m, 3-6-H); 1.57 (4.3 H, m, 2- and 7-H); 2.04 (2 H, tb $J_{7,8}$= 8 Hz, 8-H); 3.63 (2.4 H, tb $J_{1,2}$= 8 Hz, 1-H); the residual vinylic resonances were very small (not integrated).

Some unlabelled alkynol was partially hydrogenated to give an unlabelled standard of 9-decen-1-ol. MS(EI) of the TMS derivative: m/e (relative abundance) 229 (1.1) 228 ($M^{+\cdot}$, 3.2); 227 (1.3); 215 (1.2); 214 (1.4); 213 (M-15, 18); 212 (2.2); 195 (1.1); 183 (1.6); 143 (9.3); 137 (4.9); 129 (16); 115 (6.6); 109 (10); 101 (13); 95 (27); 81 (37); 75 (100); 73 (47). ^{1}H NMR (in $CDCl_3$): δ 1.32 (16 H, m, 3-6-H); 1.60 (13 H, m 2- and 7-H); 2.03 (2.4 H, tb $J_{7,8}$= 7 Hz, d $J_{9,8}$= 7 Hz, 8-H); 3.64 (2.4 H, t $J_{1,2}$= 7 Hz, 1-H); 4.92 (1 H, d $J_{10',10}$= 3 Hz, d $J_{9,10}$= 12 Hz, 10-H); 4.99 (1 H, d $J_{10,10'}$= 3 Hz, db $J_{9,10'}$= 18 Hz, 10'-H); 5.81 (1 H, d $J_{10',9}$= 18 Hz, d $J_{10,9}$= 12 Hz, t $J_{8,9}$= 7 Hz, 9-H).

Oxidation of the alkenols **25** and **27** to the corresponding acids

The alkenols **25** (210 mg, 1.33 mmol) and **27** (200 mg, 1.28 mmol) were oxidized with PDC as described for the analogous oxidations in Scheme 3. The products were purified on a column of acidic silica gel (50 g) with hexane:ether (2:1) to give **26** (105 mg, 46 % yield) and **28** (122 mg, 67 % yield), respectively. For compound **26**, MS(EI) of the TMS derivative: m/e (relative abundance) 248 (1.9); 247 (2.7); 246 (7.9); 245 ($M^{+\cdot}$, 2.0); 232 (2.2); 231 (7.5); 230 (M-15, 36); 229 (6.5); 228 (0.3); 185 (5.0); 155 (12); 129 (43); 117 (68); 96 (11); 75 (100); 73 (81). ^{2}H NMR (in $CHCl_3$): δ 0.93 (0.5 D, sb, -10-D of over-reduced material); 1.34 (0.7 D, sb, -CD_2- of over-reduced material); 5.10 (2.1 D, sb, 10-D); 5.93 (1 D, sb, 9-D). For compound **28**, MS (EI) of the TMS derivative: m/e (relative abundance) 246 (2.1); 245 (4.7); 244 ($M^{+\cdot}$, 2.8); 231 (2.0); 230 (6.5); 229 (20); 228 (13); 227 (3.1); 185 (4.1); 154 (5.6); 147 (2.1); 131 (14); 129 (38); 117 (61); 97 (3.6);

96 (8.8); 75 (100); 73 (82). ^{2}H NMR (in $CHCl_3$): δ 0.90 (0.13 D, sb, 10-D of over-reduced material); 1.32 (0.14 D, sb, -CD_2- of over-reduced material); 5.03 (1.2 D, sb, 10-D); 5.90 (1 D, sb, 9-D). The M-15 fragment ion in the mass spectra of the TMS derivatives of compounds **26** and **28** was used to estimate the distribution of over- and underdeuterated forms of these compounds. Compound **26** had 2.5 % D_4, 90 % D_3 and 7.9 % D_2, and compound **28** had 7.0 % D_3, 55 % D_2, 33 % D_1 and 6.0 % unlabelled.

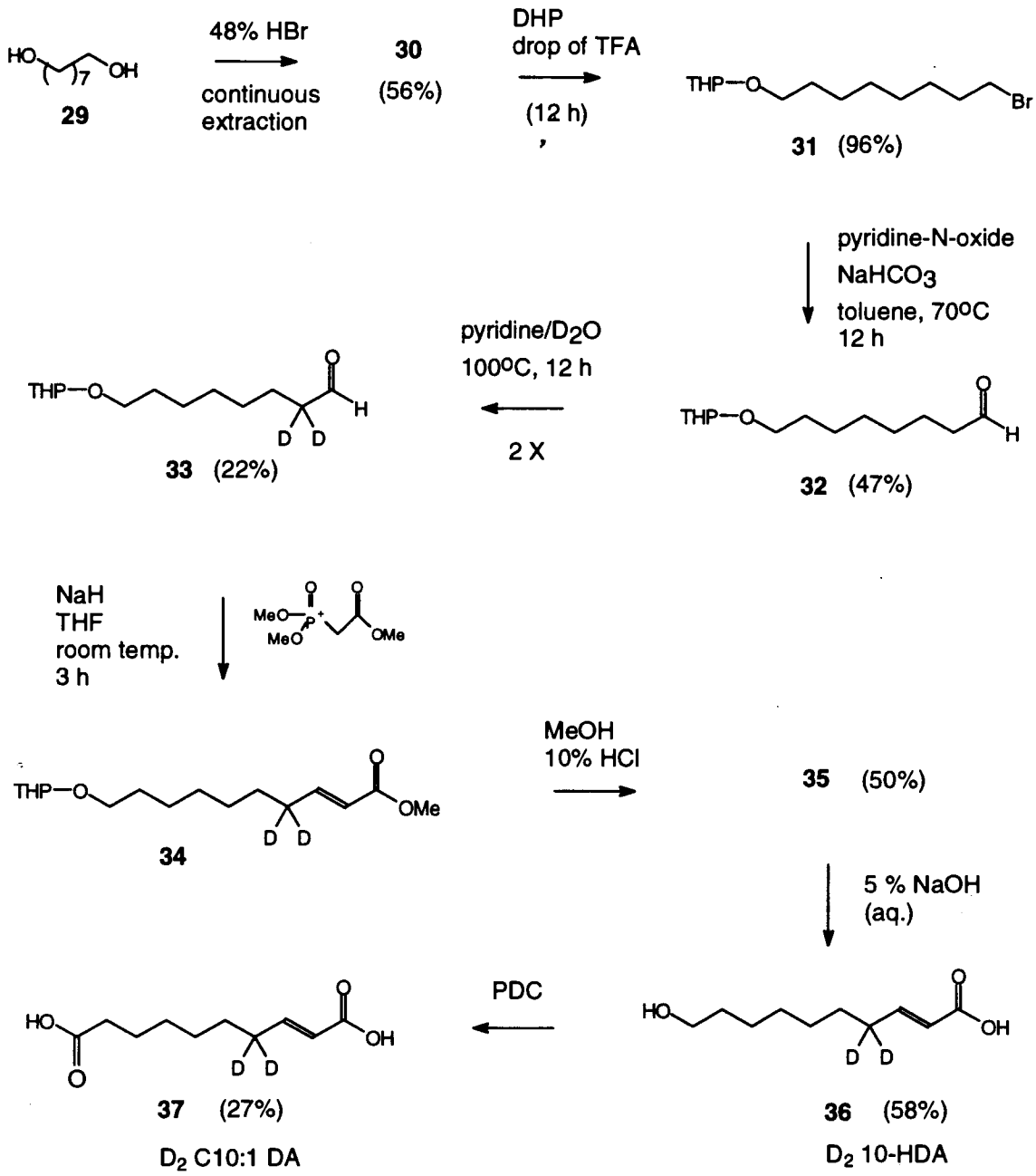
The MS(EI) of the TMS derivative of an unlabelled standard of 9-decenoic acid was: m/e (relative abundance) 243 (M+1, 2.0); 230 (0.8); 229 (2.2); 228 (5.5); 227 (M-15, 25); 226 (2.7); 199 (2.6); 185 (3.2); 147 (8.7); 129 (28); 117 (44); 93 (11); 75 (100); 73 (71).

4,4-D_2 10-hydroxy-(E)2-decenoic acid (D_2 10-HDA) and 4,4-D_2(E)2-decenedioic acid (D_2 C10:1 DA)

The synthesis of D_2 10-HDA proceeded through THP-protected 8-hydroxy octanal, as shown in Scheme 5.

8-bromo-1-octanol (**30**) and its THP derivative

1,8-octanediol (Aldrich, 10 g, 69 mmol) was treated with HBr, as described for the analogous reaction in Scheme 3. After workup and purification by chromatography on silica gel with hexane:ether (2:1), 7.99 g of pure **30** (100 % by GC) was obtained in 56 % yield. IR: 3349 (sb); 2929 (s); 2860 (m); 1464 (m); 1244 (s); 1054 (s) cm^{-1}. MS (EI) of the TMS derivative: m/e (relative abundance) 209 (0.3); 207 (0.3); 193 (0.4); 191 (0.3); 164 (5.5); 162 (5.9); 137 (4.7); 135 (5.3); 111 (5.6); 109 (5.9); 81 (11); 79 (3.9).



Scheme 5. Synthetic route to 4,4-D_2 10-hydroxy-(E)2-decenoic acid and the corresponding diacid.

8-Bromo-1-octanol (3.99g, 19.2 mmol) was mixed with dihydropyran (Aldrich, 3.22 g, 38.4 mmol) and a few drops of trifluoroacetic acid. The mixture was stirred at room temperature overnight. After workup and purification of the crude product by chromatography on silica gel with hexane:ether (2:1), 5.38 g of pure **31** (100 % by GC) was obtained in 96 % yield. IR: 2925 (s); 2856 (s); 1465 (m); 1366 (m); 1239 (sb); 1120 (s); 1034 (s); 967 (m) cm^{-1}. MS(EI): m/e (relative abundance) 252 (6); 250 (7); 163 (11); 161 (11); 83 (26).

Oxidation of **31** to the THP-derivative of 8-hydroxyoctanal (**32**) and deuteration of **32**

The bromo group of **31** was converted to an aldehyde group using a buffered pyridine-N-oxide reagent (Chiron, 1982). A mixture of **31** (5.38 g, 17.9 mmol), pyridine-N-oxide (3.44 g, 35.9 mmol) and $NaHCO_3$ (3.77 g, 44.8 mmol) in anhydrous toluene was refluxed at 120 °C for 16 h. The crude material obtained after workup was purified by chromatography on silica gel with hexane:ether (2:1), to give 1.91 g of pure product (100 % by GC) in 47 % yield. IR: 2934 (s); 2867 (s); 1726 (s); 1465 (m); 1352 (m); 1200 (w); 1122 (s); 1034 (s) cm^{-1}. MS(EI): m/e (relative abundance) 227 (M-1, 0.6); 137 (12); 135 (1.4); 109 (21); 85 (100); 44 (44). ^1H NMR (in $CDCl_3$): δ 1.31 (12 H, m, 4-6-H, 3-H of THP); 1.69 (20 H, m, 3- and 7-H, 2- and 4-H of THP); 2.32 (0.7, t $J_{2,3}$ = 8 Hz, 2-H of acid impurity); 2.40 (2.6 H, t $J_{2,3}$= 10 Hz, d $J_{1,2}$= 3 Hz, 2-H); 3.35 (1.7 H , d $J_{8,8}$ = 10 Hz, t $J_{7,8}$= 7.5 Hz, 8-H); 3.47 (1.7, m, 5'-H(THP)); 3.71 (1.7 H, d $J_{8,8}$= 10 Hz, t $J_{7,8}$= 7.5 Hz, 8-H); 3.83 (1.7, m, 5'-H(THP)); 4.55 (1.7 H, d J= 4 Hz, d J= 3 Hz, 1'-H(THP)); 9.72 (1 H, t $J_{2,1}$= 3 Hz).

The aldehyde **32** (1.55 g, 6.9 mmol) was treated twice with pyridine:D_2O (1:1, 10 mL) as described before for octanal in Scheme 2. Purification of the crude product by chromatography on silica gel with hexane:ether (2:1) afforded 0.35 g of pure **33** (97 % by GC) in 22 % yield. MS(EI): m/e (relative abundance) 229 (M-1, 0.6); 139 (0.3); 137 (1.4); 111 (14); 85 (100); 46 (13). ^1H NMR (in $CDCl_3$): δ 1.31 (12 H, m, 4-6-H, 3-H of THP); 1.69 (21 H, m, 3- and 7-H, 2'- and 4'-H of THP); 2.32 (0.13 H, m, 2-H of acid impurity); 2.40 (0.13, m, residual 2-H); 3.35 (1.6, d $J_{8,8}$ = 10 Hz, t $J_{7,8}$= 7 Hz, 8-H); 3.47 (1.7, m, 5'-

H(THP)); 3.71 (1.7 H, d $J_{8,8}$= 10 Hz, t $J_{7,8}$= 7 Hz, 8-H); 3.83 (1.7, m, 5'-H(THP)); 4.55 (1.7 H, d J= 4 Hz, d J= 2 Hz, 1'-H(THP)); 9.72 (1 H, s, 1-H). The material was 94 % enriched for 2 D as estimated from the ^{1}H NMR.

10-hydroxy-(E)2-decenoic acid (**35**)

The labelled aldehyde **33** (350 mg, 1.51 mmol) was condensed with TMPA (280 mg, 1.54 mmol) in the presence of NaH (120 mg, 60 % dispersion in mineral oil, 3.08 mmol), as described before for **8** in Scheme 2. The crude material obtained after workup contained 80 % of **34** and one major impurity (12 %) which was identified as the Z isomer; a second impurity (2%) had the same retention time as methyl 10-hydroxy-(E)2-decenoate which was known from a previous study. The crude material was dissolved in methanol (5 mL), water (20 mL) and 10% HCl (50 mL) and stirred at room temperature overnight. The crude methyl ester **35** obtained after workup was purified by chromatography on silica gel with ether:hexane (1:1), which gave 150 mg of **35** in 50 % yield. The purified material contained 92 % E and 7 % Z by GC. MS(EI) of the TMS derivative: m/e (relative abundance) 275 (3.7); 274 ($M^{+\cdot}$, 1.0); 262 (1.1); 261 (6.3); 260 (21); 259 (M-15, 100); 227 (36); 83 (26); 82 (39); 81 (22). ^{1}H NMR (in $CDCl_3$): δ 1.30 (9 H, m, 6-8-H); 1.45 (2.5 H, m, 5-H); 1.58 (5.2 H, m, 9-H); 2.20 (0.13 H, mb, 4-H); 3.62 (2.5 H, t $J_{9,10}$ = 7 Hz, 10-H); 3.73 (3 H, s, $-CH_3$); 5.77 (0.08 H, d $J_{3,2(Z)}$= 11 Hz, 2-H of Z); 5.81 (1 H, d $J_{3,2(E)}$ = 15 Hz, 2-H of E); 6.22 (0.08 H, d $J_{2,3(Z)}$ = 11 Hz, 3-H of Z); 6.96 (1 H, d $J_{2,3\,(E)}$= 15 Hz, 3-H of E). The material was 94 % enriched for 2 D, as calculated from the ^{1}H NMR.

For comparison, an unlabelled standard was synthesized the same way. MS(EI) of the TMS derivative: m/e (relative abundance) 273 (0.9); 272 ($M^{+\cdot}$, 0.7); 260 (0.7); 259 (5.4); 258 (19); 257 (M-15, 100); 256 (3.7); 225 (37); 81 (91). ^{1}H NMR (in $CDCl_3$): δ 1.30 (9 H, m, 6-8-H); 1.45 (2.5 H, m, 5-H); 1.58 (6 H, m, 9-H); 2.20 (2 H, mb, 4-H); 3.62 (2.5 H, t $J_{9,10}$ = 7 Hz, 10-H); 3.73 (3 H, s, $-CH_3$); 5.77 (0.06 H, db $J_{3,2(Z)}$= 11 Hz, 2-H of Z); 5.81 (1 H, d $J_{3,2(E)}$ = 16 Hz, t $J_{4,2}$= 2 Hz, 2-H of E); 6.22 (0.06 H, d $J_{2,3(Z)}$ = 11 Hz, t $J_{4,3}$= 7Hz, 3-H of Z); 6.96 (1 H, d $J_{2,3\,(E)}$= 16 Hz, t $J_{4,3}$= 7 Hz, 3-H of E).

The methyl ester **35** (123 mg, ca. 0.06 mmol) was hydrolyzed in 5% NaOH (30 mL) for 3 h at room temperature. After acidification with 10% HCl and workup, 100 mg of crude product (84 % pure by GC) were obtained. Purification on acidic silica gel with ether:hexane (1:1) afforded 66 mg of pure (96 % by GC) product in 58 % yield. MS(EI) of the TMS derivative: m/e (relative abundance) 334 (1.2); 333 (3.5); 332($M^{+\cdot}$, 2.0); 320 (1.3); 319(5.6); 318 (15); 317 (M-15, 55); 316 (7.5) 301 (13); 227 (24); 149 (34); 147 (69); 83 (33). ^{2}H NMR (in $CHCl_3$): δ 2.26 (sb).

An unlabelled 10-HDA standard was also synthesized. The material was a white solid m. p. 62-65 °C (lit. Chiron 1982, 64-65 °C). MS(EI) of the TMS derivative: m/e (relative abundance) 332 (2.8); 331 (10); 330 ($M^{+\cdot}$, 3.1); 317(7.0); 316(17.4); 315 (M-15, 64); 314 (1.7); 299 (13); 243(3.4); 225 (19); 149 (34); 147 (56); 81 (95). ^{13}C NMR (in $CDCl_3$): δ 152 (3-C); 120 (2-C); 62.9 (10-C); 32.7; 32.2; 29.1; 29.0; 27.8; 25.6.

Oxidation of D_2 10-HDA (**36**) to D_2 (E)2-decenedioic acid (**37**)

D_2 10-HDA (6.5 mg, ca. 0.04 mmol) was oxidized with PDC to give the diacid **37**. The crude product was purified on acidic silica gel with hexane:ether 3:1, which gave 1.9 mg of pure material in 27 % yield. MS(EI) of the TMS derivative: m/e (relative abundance) 347 (M+1, 3.7); 333 (3.3); 332 (7.3); 331(M-15, 25); 330 (2.0); 287 (13); 257 (2.8); 256 (); 241 (3.1); 213 (15); 165 (31); 137 (33); 129 (16); 120 (36); 107 (12); 82 (13); 75 (92); 73 (100).

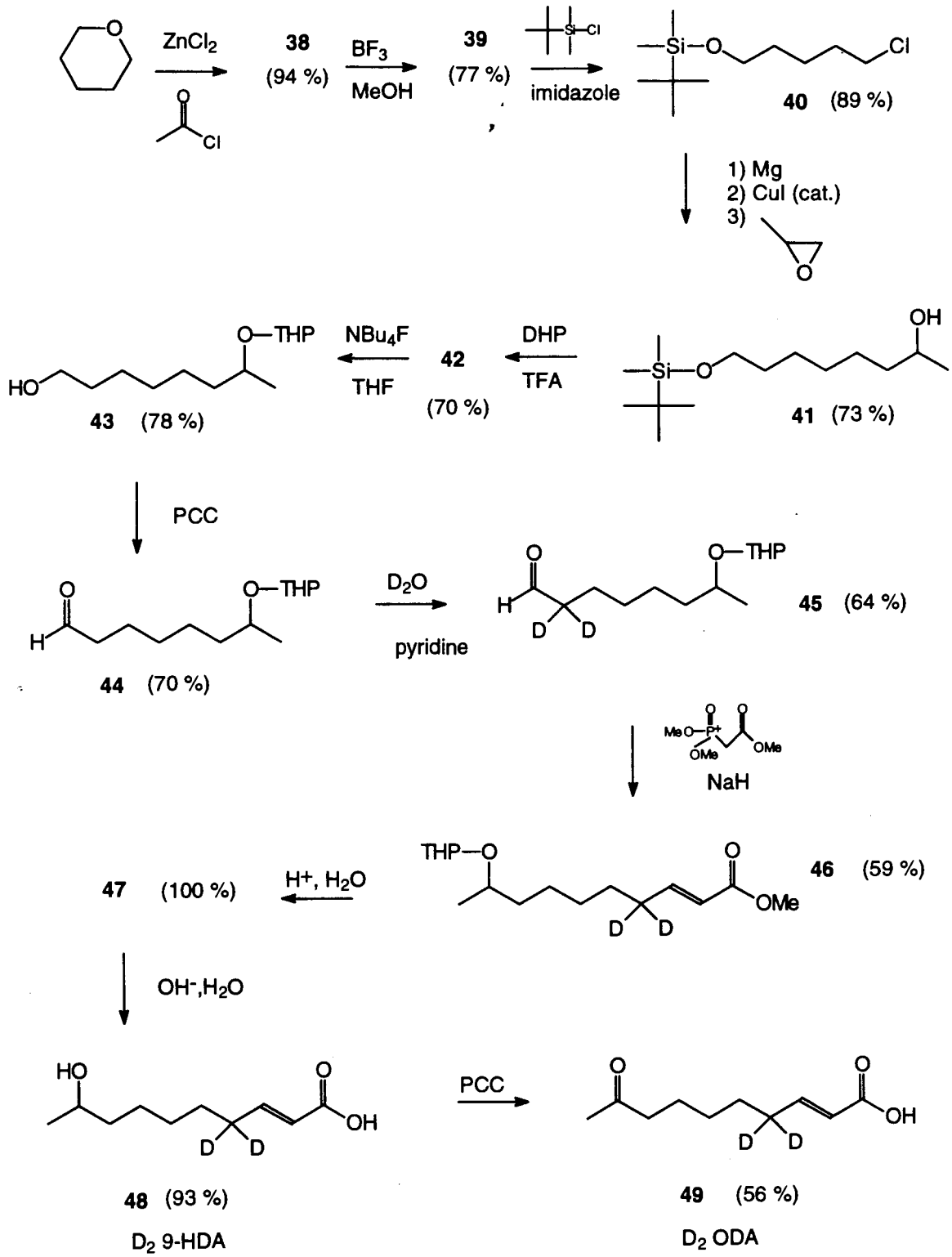
An unlabelled standard was prepared from 10-HDA. MS(EI) of the TMS derivative: m/e (relative abundance) 345 (1.9); 344 ($M^{+\cdot}$, 6.8); 331 (1.1); 330 (3.3); 329 (M-15, 30); 328 (3.0); 285 (15); 255 (3.8); 239 (3.0); 211 (13); 164 (34); 136 (41); 129 (15); 119 (51); 107 (15); 81 (18); 75 (100); 73 (81). ^{1}H NMR (in $CDCl_3$): δ 1.34 (5.7 H, m, 7- and 6-H); 1.48 (2.9 H, m, 5-H); 1.65 (2.3 H, m, 8-H); 2.19 (2.4 H, d $J_{3,4}$= 6 Hz, t $J_{5,4}$= 6 Hz, 4-H); 2.35 (2 H, t $J_{8,9}$ = 9 Hz, 9-H); 5.81 (1 H, d $J_{3,2}$= 17 Hz, 2-H); 7.06 (1 H, d $J_{2,3}$= 17 Hz, t $J_{4,3}$= 6 Hz, 3-H).

4,4-D_2 9-hydroxy- and 9-keto -(E)2-decenoic acids (D_2 9-HDA and D_2 ODA)

The key intermediate in the synthesis of D_2 9-HDA, the THP derivative of 7-hydroxyoctanal, was synthesized by a Grignard coupling of propylene oxide and protected 5-chloro-1-pentanol, followed by THP protection of the 7-hydroxy group of the product and oxidation to the aldehyde (Scheme 6). The rest of the synthesis was analogous to that of D_2 10-HDA.

1-acetoxy-5-chloropentane **38**

Tetrahydropyran (30 g, 348 mmol), acetyl chloride (25 g, 318 mmol) and $ZnCl_2$ (5.0 g, 37 mmol) were refluxed at 100 °C with vigorous stirring for 3.5 h. The reaction was quenched with cold water (150 mL) and benzene (75 mL). The layers were separated, and the benzene phase was washed with saturated $NaHCO_3$. The combined aqueous phase was extracted with ether (3X). The organic extract was washed with brine and dried over Na_2SO_4. Flash evaporation of the solvent afforded 51.2 g of a yellow oil. The crude product was distilled under vacuum (17 mm Hg). The main fraction, a colorless oil, distilled at 125 °C, and 45.5 g of pure **38** were collected (94 % yield). MS(EI): m/e (relative abundance) 149 (M-15, 0.4); 135 (0.6); 104 (1.6); 94 (1.6); 80 (1.8); 78 (2.8); 76 (3.0); 69 (3.2); 55 (4.2); 50 (5.0); 44 (100); 43 (13).



Scheme 6. Synthesis of 4,4-D_2 9-hydroxy- and 9-keto-(E)2-decenoic acids.

Conversion of **38** to the t-butyl dimethylsilyl derivative

To remove the acetyl group, **38** (5.3 g, 35 mmol) was dissolved in 10% BF_3 (Aldrich) in methanol (100 mL) and stirred overnight at room temperature. After workup, 5.1 g of a yellow oil was obtained. The crude material was purified by chromatography on silica gel with ether:hexane (3:2), which gave 3.3 g of pure **39**, a colorless oil, in 77 % yield. MS(EI): m/e (relative abundance) 105 (3.3); 104 (M-18, 6.5); 103 (13); 102 (4.3); 100 (2.2); 92 (6.0); 90 (18); 69 (11); 68 (53); 67 (100); 66 (18); 65 (6.5); 63 (6.0).

Imidazole (7.4 g, 109 mmol) and t-butyldimethylchlorosilane (Aldrich ,7.85 g, 52 mmol) were added to a solution of **39** from two lots (5.3 g, 43 mmol) in DMF. The mixture was stirred at room temperature overnight. The crude product obtained after workup was purified by chromatography on silica gel with hexane:ether (20:1) to give 9.19 g of pure **40** (99 % by GC) in 89 % yield. IR: 2950 (s); 2862 (s); 1688 (w); 1468 (m); 1400 (s); 1250 (s); 1106 (s); 837 (s); 763 (s) cm^{-1}. MS(EI): m/e (relative abundance) 234 (1.1); 200 (1.3); 178 (0.5); 124 (27); 123 (15); 122 (69); 121 (M-TBDMS, 11); 95 (28); 94 (7.6); 93 (68); 75 (46); 73 (35); 69 (100); 68 (82); 67 (50). ^1H NMR (in $CDCl_3$): δ 0.50 (6 H, s, Si-CH_3); 0.90 (9.4 H, s, t-butyl-H); 1.52 (4.3 H, m, 3- and 4-H); 1.80 (2 H, tt $J_{1,2} = J_{4,5} = 7$ Hz, 5-H); 3.62 (2 H, t $J_{1,2} = 7$ Hz, 1-H).

1-(t-butyldimethylsilyloxy)octan-7-ol (**41**)

The silylated chloropentanol **40** was condensed with propylene oxide following a procedure described by Kandil (1985). Crushed Mg turnings (2.50 g, 102 mmol) were added to a 3-neck flask. A portion of **40** (12.1 g, 50.9 mmol) in freshly distilled THF was added, along with a drop of 1,1,2,2 tetrabromoethane and a crystal of I_2. The mixture was stirred vigorously at room temperature and, when the yellow color disappeared, the remaining solution of **40** was added in small portions. The mixture was stirred at room temperature for 1.5 h, after which it was cooled to -30°C. Once cool, CuI (0.97 g, 5.1 mmol) was added and the dark mixture was stirred at -30°C for 40 min before a solution of

propylene oxide (Aldrich, 2.90 g, 50.9 mmol) in THF was added. After the addition, the mixture was warmed to 0°C and stirred at that temperature for 6 h. The reaction was quenched with saturated NH_4Cl (50 mL). The crude product obtained after workup was purified by chromatography on silica gel with ether:hexane (1:1) to give 9.71 g of **41** (92 % by GC) in 73 % yield. IR: 3337 (sb); 2938 (s); 2876 (s); 1475 (m); 1400 (m); 1263 (s); 1088 (s); 843 (s); 775 (s) cm^{-1}. ^1H NMR (in $CDCl_3$): δ 0.04 (6 H, s, Si-CH_3); 0.89 (11 H, s, t-butyl-H); 1.19 (3.6 H, d $J_{7,8}$ = 8.5 Hz, 8-H); 1.32 (7.8 H, m, 3-5-H); 1.43 (3 H, m, 6-H); 1.50 (2.6 H, m, 2-H); 3.6 (2 H, t $J_{1,2}$ = 7 Hz, 1-H); 3.79 (1 H, m, 7-H).

Conversion of **41** to 7-tetrahydropyranyloxy-1-octanol (**43**)

Treatment of **41** (9.71 g, 37.3 mmol) with dihydropyran (9.41 g, 112 mmol) and a drop of trifluoroacetic acid, followed by workup and purification on a column of silica gel with ether:hexane (1:1), gave 13.9 g of **42**, which showed two peaks 0.2 min apart in the GC (47 and 49 %), in 70 % yield. IR: 2940 (s); 2875 (s); 2341 (w); 1744 (w); 1463 (m); 1383 (m); 1255 (m); 1088 (s); 1023 (s); 836 (s); 775 (s) cm^{-1}. MS(EI): m/e (relative abundance) 314 (M-30, 0.5); 237 (7.5); 215 (8.0); 159 (50); 137 (15); 111 (14); 85 (100); 75 (46) . The ^1H NMR (in $CDCl_3$) had two sets of signals corresponding to diastereomers: δ 0.05 and 0.10 (6 H, s, -CH_3); 0.88 and 0.91 (10.4 H, s, t-butyl); 1.09 and 1.18 (3 H, d $J_{7,8}$= 6 Hz, 8-H); 1.31 and 1.53 (17 H, m, 3-6-H and 2'-4'-H(THP)); 1.70 and 1.82 (2.2 H, m, 2-H); 3.48 (2 H, m, 7-H); 2.58 (2 H, t $J_{1,2}$= 9 Hz, 1-H); 3.73 and 3.88 (2 H, m, 5'-H(THP)); 4.58 and 4.70 (1 H, m, 1'-H(THP)). The major impurity in the crude product was identified as the di-THP derivative of 1,7-octanediol.

The material from the previous reaction **42** (13.8 g, 40.1 mmol) was stirred 1M tetrabutylammonium fluoride in THF (Aldrich, 10 mL) at room temperature for 4 h. The crude product was chromatographed on a 250 g column of silica gel with ether:hexane (1:1), giving 7.15 g of **43**, which displayed two peaks 0.2 min apart in the GC (49 and 51 %), in 78 % yield. IR: 3396 (sb); 2950 (s); 2875 (s); 1742 (m); 1454 (s); 1374 (s); 1259 (w); 1134 (s); 1050 (s) cm^{-1}. ^1H NMR (in $CDCl_3$): δ 1.09 and 1.20 (3 H, d $J_{7,8}$ = 6.5 Hz, 8-H); 1.35 and 1.52 (17 H, m, 2-6-H and 3'-4'-H(THP)); 1.70 and 1.85 (2.6 H, m, 2'-

H(THP)); 3.45 and 3.53 (2.6 H, tb $J_{1,2}$ = 8 Hz, 1-H); 3.76 and 3.90 (2 H, m, 5'-H(THP)); 4.62 and 4.53 (1 H, m, 1'-H(THP)).

Oxidation of **43** to the corresponding aldehyde (**44**) and deuteration of **44**

The alcohol **43** (7.15 g, 31.1 mmol) was oxidized with PCC, and the crude product was chromatographed on a column of silica gel with ether:hexane (1:1) giving 5.00 g of **44** which displayed two peaks 0.2 min apart in the GC (49 and 49 %) in 70 % yield. IR: 2936 (s); 2880 (m); 1724 (s); 1454 (m); 1119 (m); 1022 (s) cm^{-1}. MS(EI): m/e (relative abundance) 227 (M-1), 0.5); 213 (1.1); 159 (7.7); 137 (28); 127 (10); 109 (53); 101 (22); 85 (100). ^1H NMR (in $CDCl_3$): δ 1.08 and 1.20 (3 H, d $J_{7,8}$ = 7.5 Hz, 8-H); 1.35 (6 H, m, 4-5-H and 3'-H(THP)); 1.61 (11 H, m, 6- and 3-H and 2'- and 4'-H(THP)); 2.35 (0.2 H, t $J_{2,3}$ = 10 Hz, 2-H of acid impurity); 2.42 (1.5 H, t $J_{2,3}$ = 10 Hz, d $J_{1,2}$ = 3 Hz, 2-H); 3.48 (1-H, m, 7-H); 3.74 and 3.90 (1.4 H, m, 5'-H(THP)); 4.64 (0.7 H, m, 1'-H(THP)); 9.73 (0.7 H, t $J_{1,2}$ = 3 Hz, 1-H) .

The aldehyde **44** was subjected to two rounds of deuteration with pyridine (14 mL) and D_2O (10 mL). The crude product (a yellow oil) was purified by chromatography on silica gel with ether:hexane (1:1), which afforded 3.20 g of **45** (96 % by GC, total of two peaks) in 64 % yield. MS(EI): m/e (relative abundance) 229 (M-1, 0.5); 215 (1.0); 159 (3.3); 137 (11); 129 (13); 111 (59); 101 (23); 85 (100). ^1H NMR (in $CDCl_3$): δ 108 and 1.20 (3 H, d $J_{7,8}$ = 8 Hz, 8-H); 1.32 and 1.50 (13 H, m, 4-5-H and 3'-4'-H(THP)); 1.69 and 1.82 (2-H, m, 2'-H(THP)); 2.23 (0.1, mb, 2-H of acid impurity); 2.40 (0.06 H, mb, residual 2-H); 3.49 (1 H, m, 7-H); 3.72 and 3.88 (2 H, m, 5'-H(THP)); 4.65 (1 H, m, 1'(THP)); 9.75 (0.65 H, s, 1-H). As estimated from the ^1H NMR, the material was 95 % D_2.

4,4-D_2 9-hydroxy-(E)2-decenoic acid (**48**)

The aldehyde **45** (3.21 g, 13.9 mmol) was coupled with TMPA using the same procedure as described for the analogous reaction in Scheme 2. After workup, 3.50 g of

crude product, which contained 80 % of the E and 14 % of the Z isomer, was obtained. The two isomers were separated by chromatography on a column of silica gel (250 g) with ether:hexane (1:1). The early-eluting fraction (0.14 g) contained mostly the Z isomer; the later-eluting one (2.88 g), mostly E. Because the second fraction still had 8 % of the Z isomer (by GC), it was rechromatographed using hexane:ether (3:2), which gave 2.34 g of pure **46** in 59 % yield.

Treatment of of **46** (2.34 g, 8.29 mmol) in THF (50 mL) with 3% HCl (15 mL), followed by chromatography on silica gel with ether:hexane (2:1), gave 1.66 g of methyl D_2 9-HDA **47** (96 % pure by GC) in 100 % yield. ^{1}H NMR (in $CDCl_3$): δ 1.18 (3.6 H, d $J_{9,10}$ = 8 Hz, 10-H); 1.47 (14.7 H, m, 5-8-H); 2.20 (0.12 H, mb, residual 4-H); 3.71 (3 H, s, $-CH_3$); 3.79 (1 H, m, 9-H); 5.81 (1 H, d, $J_{2,3}$ = 16 Hz, 2-H); 6.95 (1 H, d, $J_{2,3}$ = 16 Hz, 3-H).

The methyl ester **47** (1.64 g, 8.11 mmol) was hydrolyzed with 1M NaOH (30 mL). The resulting acid **48** was chromatographed on acidic silica with ether:hexane (3:1), giving 1.05 g of a colorless, very viscous oil (98 % pure by GC) in 69 % yield. MS(EI) of the TMS derivative: m/e (relative abundance) 334 (1.9); 333 (4.5); 332 ($M^{+\cdot}$,1.4); 331 (1.0); 319 (3.2); 318 (7.2); 317 (M-15, 27); 316 (3.1); 315 (0.1); 301 (10); 288 (22); 243 (12); 227 (11); 147 (34); 117 (77); 82 (27); 75 (64); 73 (100). ^{1}H NMR (in $CDCl_3$): δ 1.19 (4H, d $J_{9,10}$ = 7 Hz, 10-H); 1.38 (11 H, m, 5-8-H); 2.22 (0.14 H, m, residual 4-H); 3.80 (1 H, m, 9-H); 5.82 (1 H, d $J_{2,3}$ = 16 Hz, 2-H); 7.06 (1 H, d $J_{2,3}$ = 16 Hz, 3-H). ^{2}H NMR (in $CHCl_3$) : δ 2.26 (sb). According to the ^{1}H NMR the material was 93 % enriched for 2 D; the MS indicated that the label was distributed as 90 % D_2 and 10 % D_1.

An unlabelled 9-HDA standard was also synthesized. MS(EI) of the TMS derivative: m/e (relative abundance) 333 (1.8); 332 (3.8); 331 (14); 330 ($M^{+\cdot}$, 1.2); 329 (2.7); 317 (3.9); 316 (10: 315 (M-15, 37); 299 (13); 286 (24); 243 (9.3); 225 (7.5); 147 (23); 117 (71); 81 (44); 75 (63); 73 (100). ^{1}H NMR (in $CDCl_3$): δ 1.19 (4 H, d $J_{9,10}$ = 7 Hz, 10-H); 1.37 (10 H, m, 5-8-H); 2.24 (2 H, td(b) $J_{4,5}$=$J_{3,4}$ = 6.5 Hz, 4-H); 3.80 (1 H, m, 9-H); 5.82 (1 H, d(b) $J_{2,3}$ = 16 Hz, 2-H); 7.06 (1 H, d $J_{2,3}$ = 16 Hz, t $J_{3,4}$ = 6.5 Hz, 3-H).

4,4-D_2 9-keto-(E)2-decenoic acid (**49**)

A sample of D_2 9-HDA (5 mg,0.02 mmol) was oxidized with PCC to give 2.8 mg of D_2 ODA **49** in 56 % yield. MS(EI) of the TMS derivative: m/e (relative abundance) 258 ($M^{+\cdot}$, 6.3); 257 (2.7); 244 (3.2); 243 (M-15, 14); 242 (4.2); 225 (6.5); 215 (15); 201 (16); 185 (6.0); 167 (26); 156 (26); 144 (32); 125 (24); 109 (23); 96 (24); 82 (76); 81 (70); 75 (100); 73 (71).

The TMS derivative of unlabelled ODA (Phero Tech Inc.) gave the following MS (EI): m/e (relative abundance) 256 ($M^{+\cdot}$, 5.5); 255 (1.9); 242 (2.9); 241 (M-15, 12); 223 (7.7); 213 (15); 199 (16); 183 (7.1); 166 (23); 155 (35); 142 (28); 123 (23); 108 (24); 95 (31); 81 (100); 75 (93); 73 (63).

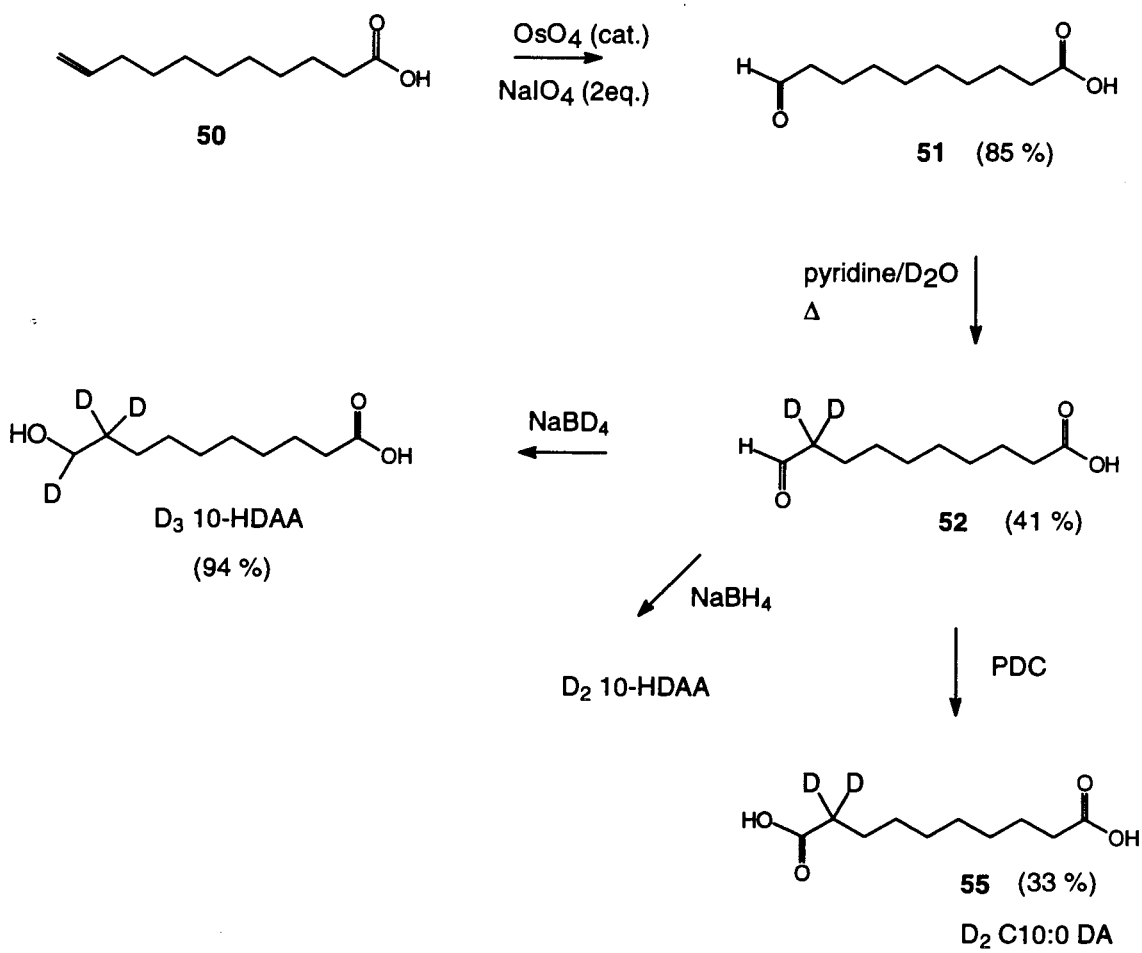
9,9,10-D_3, 9,9-D_2 10-hydroxydecanoic acids (D_3 and D_2 10-HDAA) and 2,2-D_2 decanedioic acid (C10:0 DA)

These compounds were synthesized *via* 10-oxodecanoic acid which was deuterated with D_2O/pyridine (Scheme 7).

10-oxodecanoic acid (**51**)

The terminal double bond of 10-undecenoic acid, **50**, was cleaved with $NaIO_4$ and OsO_4 according to the procedure of Graham and Williams (1966). OsO_4 (0.011 g, 0.045 mmol) was added to a solution of **50** (Matheson, 0.836 g, 4.5 mmol) in 15 mL of freshly distilled (over $LiAlH_4$) dioxane. The reaction mixture turned brown within 5 min. $NaIO_4$ (2.11 g, 9.8 mmol) was added in small portions over a period of 30 min, during which a yellow precipitate formed. The mixture was stirred at 25 °C for 1.5 h, after which ether (100 mL) was added and the organic extract filtered through some Na_2SO_4. The ether extraction was repeated, and evaporation of the solvent from the combined extract afforded 0.905 g of a green oil. The crude product was redissolved in ether and filtered through

some Florisil^{R}, giving a yellow filtrate. The solution was concentrated and the product recrystallized from ether:hexane 1:1, giving 0.752 g of a white solid, m. p. 52-53 °C (lit. 56-57 °C, Beilstein **3**, III, 1269a), in 85 % yield. IR: 3418 (s); 2934(s); 2825 (s) 1737 (s); 1700 (s); 1297 (s). MS(EI) of the TMS derivative: m/e (relative abundance) 259 (M+1, 3); 257 (1.5); 243 (M-15, 6); 225 (15); 224 (1); 223 (0.5); 169 (6); 117 (44); 75 (100). ^{1}H NMR (in $CDCl_3$): δ 1.30 (15 h, m, 4-7-H); 1.60 (7 H, m, 3- and 8-H); 2.35 (4 H, t $J_{1,2}$ = 9 Hz, 2-H); 2.43 (2.8 H, t $J_{8,9}$ = 10 Hz d $J_{9,10}$ = 3 Hz, 9-H); 9.78 (1 H, t $J_{9,10}$= 3 Hz, 10-H).



Scheme 7. Synthesis of 9,9,10-D_3 and 9,9-D_2 10-hydroxydecanoic acids and the corresponding diacid.

Deuteration of 10-oxodecanoic acid

10-Oxodecanoic acid (203 mg, 1.09 mmol) was subjected to two rounds of deuteration with pyridine:D_2O (1:1, 6 mL), which gave 194 mg of crude product. This material, which contained 20% of decanedioic acid (by GC), was chromatographed on acidic silica gel with ether:hexane (1:1), giving 84 mg of pure **52**, m. p. 48-49 °C, in 41 % yield. MS(EI) of the TMS derivative: m/e (relative abundance) 261(0.9); 260 ($M^{+\cdot}$, 0.7); 259 (1.2); 245 (M-15, 1.9); 227 (9.4); 226 (5.2); 225 (2.8); 171 (3.4); 117 (40); 75 (100). ^1H NMR (in $CDCl_3$): δ 1.30 (9.8 H, m, 4-7-H); 1.60 (4.8 H, m, 3- and 8-H); 2.35 (2.5 H, t $J_{2,3}$= 10 Hz, 2-H); 2.43 (0.1 H, mb, residual 9-H); 9.78 (1 H, s, 10-H). The material was 95 % enriched for 2 D, as estimated from the ^1H NMR.

9,9,10-D_3 and 9,9-D_2 10-hydroxydecanoic acids and 2,2-D_2 decanedioic acid

D_2 10-oxodecanoic acid, **52** (4.9 mg, 0.03 mmol), was added to a solution of $NaBD_4$ (1.1 mg, 0.03 mmol) in methanol. The mixture was stirred at room temperature for 3 h. Workup furnished 4.7 mg of pure (98 % by GC) D_3 10-HDAA. MS(EI) of the TMS derivative: m/e (relative abundance) 337 (1.5); 336 (M+1, 2.5); 334 (1.3);323(2.0); 322 (9.8) 321 (24.5); 320 (M-15, 86); 319 (4.9); 318 (2.0); 317 (6.0); 304 (40); 301 (2.5); 245 (2.7); 230 (20); 149 (40); 147 (33); 75 (82); 73 (100). ^1H NMR (in $CDCl_3$): δ 1.29 (9 H, m, 4-8-H); 1.57 (3.8 H, m, 3-H); 2.32 (2 H; t $J_{2,3}$ = 8 Hz, 2-H); 3.65 (1 H, m, 10-H). The material was 93 % D_3, as determined from the M-15 fragment in the MS.

Reduction of **52** with $NaBH_4$ gave D_2 10-HDAA. MS(EI) of the TMS derivative: m/e (relative abundance) 321 (6.7); 320 (17); 319 (66); 318 (12); 317 (1.2); 303 (42); 229 (27); 149 (41); 147 (41); 75 (91); 73 (100). The material was 92 % D_2, 6.6 % D_1 and 1.3 % unlabelled (calculated from the M-15 fragment in the MS).

An unlabelled 10-HDAA standard was prepared from **51**. M. p. 67-69 °C (lit. 72-73 °C, Beilstein **3**, IV, 896). MS(EI) of the TMS derivative: m/e (relative abundance) 319 (6); 318 (15); 317 (67); 316 (7.7); 301 (44); 227 (28); 149 (37); 147 (33); 75 (85); 73

(100). ^{1}H NMR (in $CDCl_3$): δ 1.29 (12 H, m, 4-8-H); 1.57 (6 H, m, 3-and 9-H); 2.32 (2 H, t $J_{2,3}$= 8 Hz, 2-H); 3.65 (2 H, t $J_{9,10}$= 6 Hz, 10-H).

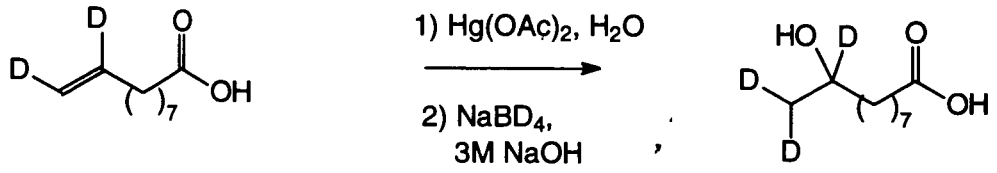
A sample of **52** (6.5 mg, 0.04 mmol) was treated with 44 mg (0.12 mmol) of PDC in DMF. Recrystallization of the crude product from ether:hexane (1:1) afforded 2.3 mg of pure D_2 C10:0 DA in 33 % yield. MS(EI) of the TMS derivative: m/e (relative abundance) 349 (9.5); 348 ($M^{+\cdot}$, 4.7); 333 (M-15, 100); 289 (3.8); 259 (11); 258 (3.1); 75 (55); 73 (75). The TMS derivative of an unlabelled decanedioic acid standard (Eastman) had: m/e (relative abundance) 347 (4.4); 346 (1.3); 331 (M-15, 100); 287 (5.2); 257 (6); 75 (83); 73 (80).

9,10,10-D_3 9-hydroxydecanoic acid (D_3 9-HDAA) and 17,18,18-D_3 17-hydroxyoctadecanoic acid (D_3 17-OH C18:0)

Conversion of terminal alkenoic acids to the corresponding ω-1 hydroxy acids was accomplished by solvomercuration/demercuration (Scheme 8).

Solvomercuration/demercuration of D_2 C10:1 (**28**)

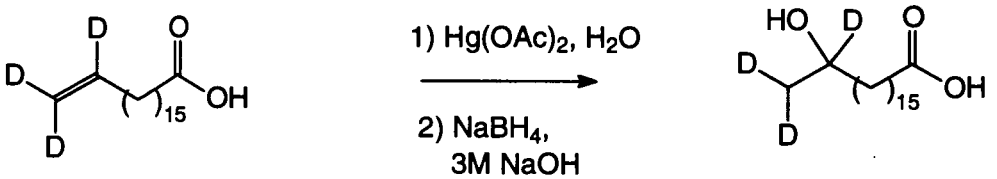
The Markovnikov hydration of **28** was accomplished by the procedure of Brown and Geoghegan (1967). Mercuric acetate (146 mg, 0.46 mmol) was dissolved in water (4 mL). Freshly distilled THF (0.5 mL) was added to the solution which turned into a bright yellow suspension. A solution of the alkenoic acid **28** (62 mg, 0.37 mmol) in THF was added to the suspension, and the mixture was stirred for 15 min, after which 3 M NaOH (1.5 mL) was added. Two min later, a solution of $NaBD_4$ (15 mg, 0.37 mmol) in 3 M NaOH (1.5 mL) was added. The mixture, which turned dark gray immediately, was stirred for another 5 min. The Hg was settled by centrifugation of the reaction mixture at 800 rpm for 30 min.



28

56 (62 %)

D_3 9-HDAA



16

57 (91 %)

D_3 17-OH C18:0

Scheme 8. Synthesis of 9,10,10-D_3 9-hydroxydecanoic- and 17,18,18-D_3 17-hydroxydecanoic acids.

The supernatant was acidified with 3 M HCl and extracted with ether (3 X) and the extract was dried over Na_2SO_4. The product was purified by chromatography on acidic silica (20 g) with hexane:ether (3:1), which gave 43 mg of D_3 9-HDAA (93 % by GC) in 62 % yield. ^{1}H NMR (in $CDCl_3$): δ 1.15 (1 H, m, 10-H); 1.32 (8 H, m, 4-7-H); 1.43 (2 H, m, 8-H); 1.64 (2 H, tt $J_{2,3} = J_{3,4} = 8$ Hz, 3-H); 2.35 (2 H, t $J_{2,3} = 8$ Hz, 2-H); 3.79 (0.25 H, m, residual 9-H). ^{2}H NMR (in $CHCl_3$): δ 1.17 (2 D, sb, 10-D); 3.75 (0.8 D, sb, 9-D). MS (EI) of the TMS derivative: m/e (relative abundance) 337 (1.8); 336 (5.2); 335 ($M^{+\cdot}$, 1.5); 334 (3.6); 333 (5.0); 323 (1.1); 322 (12); 321 (32); 320 (M-15, 77); 319 (48); 318 (27); 317 (7.4); 304 (68); 288 (43); 246 969); 217 (27); 204 916); 156 (26); 149 (13); 147 914); 137 (33); 120 (100). The material was 2.7 % D_5, 7.8 % D_4, 48 % D_3, 23 % D_2, 15 % D_1 and 3.1 % unlabelled (calculated from the M-15 ion in the MS).

An unlabelled standard was also synthesized. MS(EI) of the TMS derivative: m/e (relative abundance) 332 ($M^{+\cdot}$, 1.5); 331 (1.5); 319 (2.6); 318 (11); 317 (M-15, 37); 316 (6.6); 301 (35); 288 (20); 243 (26); 217 (21); 204 (13); 149 (12); 147 (8.9); 135 (17); 117 (100).

Solvomercuration/demercuration of D_3 C18:1 (**16**)

The alkenoic acid **16** (6 mg, 0.02 mmol) was converted to the 17-hydroxy acid **57** (2 mg), by the same procedure as outlined above[4], in 30 % yield. The product was 75 % pure by GC, the impurities being lower homologs: 17-C (11 %), 16-C (10 %), 15-C (3%) and 14-C (trace). ^{1}H NMR (in $CDCl_3$): δ 1.13 (1 H, m, 18-H); 1.26 (26 H, m, 4-15-H); 1.60 (32 H, m, 3-, 16- and 18-H); 2.34 (2 H, t $J_{2,3}$ = 8 Hz, 2-H); 3.55 (0.6 H, m, residual 17-H). ^{2}H NMR (in $CHCl_3$): δ 1.17 (1.3 D, sb, 18-D); 3.78 (1 D, sb, 17-D). MS (EI) of the TMS derivative[5]: m/e (relative abundance) 446 (M-1, 0.2); 434 (3.3); 433 (8.3); 432 (M-15, 22); 431 (5.0); 430 (7.9); 429 (1.1); 419 (1.5); 418 (5.7); 417 (14); 416 (38); 415 (5.6); 400 (25); 358 (11); 342 (11); 217 (27); 204 (28); 149 (7.7); 147 (7.1); 129 (16); 120 (100). The M-15 ion in the MS revealed that the material was 6.0 % D_4, 59 % D_3, 7.0 % D_2, 18 % D_1 and 10 % unlabelled.

7,7-D_2 8-hydroxyoctanoic acid (D_2 8-HOAA), 15,15-D_2 16-hydroxyhexadecanoic acid (D_2 16-OH C16:0) and 18-D_1 18-hydroxyoctadecanoic acid (D_1 18-OH C18:0)

Unlabelled 8-HOAA and 18-OH C18:0 were synthesized from the corresponding diols as outlined in Scheme 9.

[4] $NaBH_4$ was used for demercuration.
[5] Scanned from 90 - 450 amu.

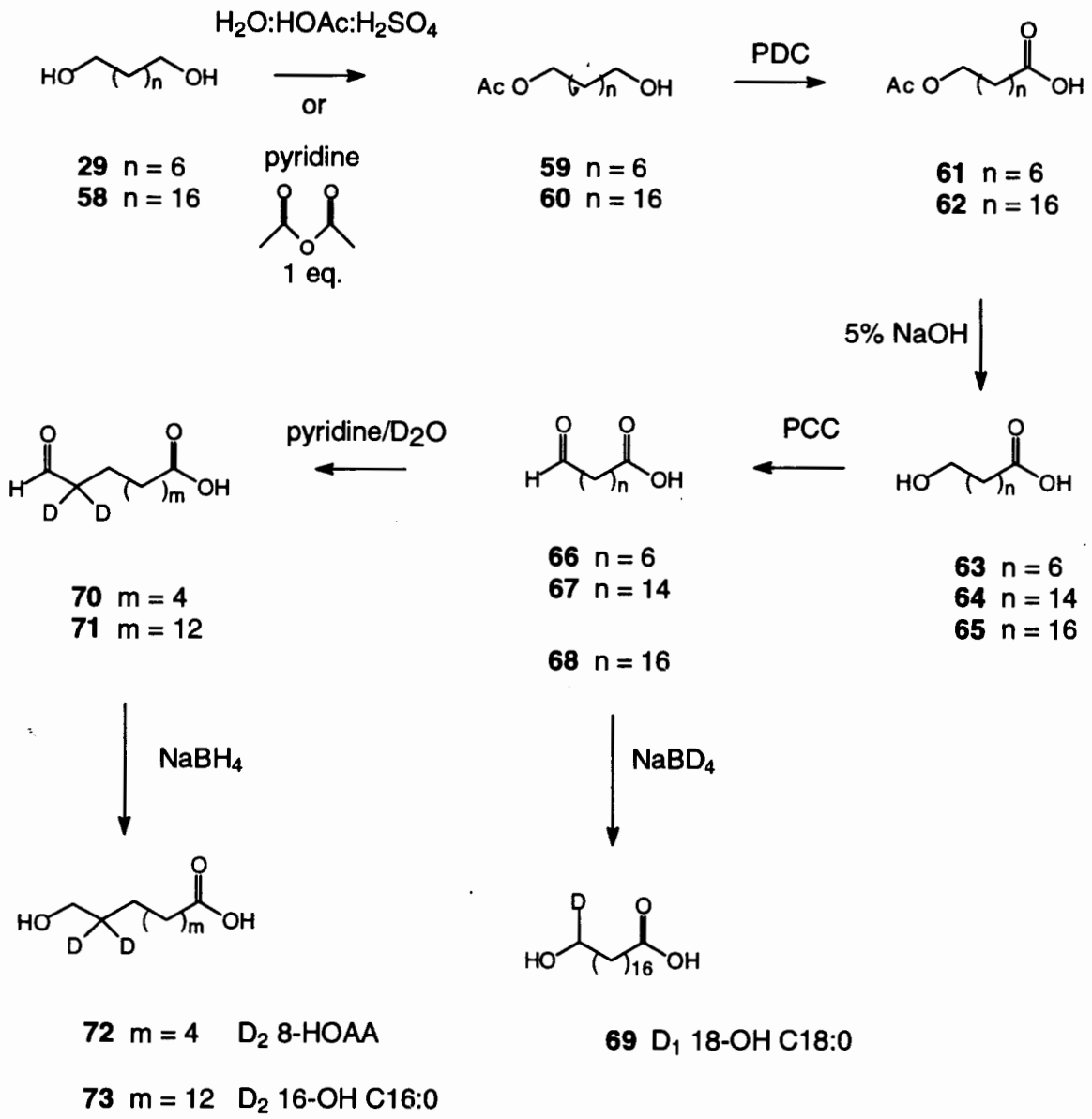
Acetylation of 1,8-octanediol and 1,18-octadecanediol

1,8-Octanediol (10 g, 69 mmol) and water:glacial acetic acid:H_2SO_4 (400:50:1, 100 mL) were warmed (60 °C) under continuous extraction with hexane (Babler and Coghlan, 1979). After 1 day, the hexane extract was collected and the solvent, evaporated. The crude product was purified by chromatography on silica gel with ether:hexane (2:1), which afforded 6.37 g of monoacetate **59**, a colorless oil, (100 % by GC) in 49 % yield. IR: 3453 (sb); 2932 (s); 2875 (m); 1740 (s); 1475 (m); 1375 (m); 1241 (s); 1037 (s). MS(EI) of the TMS derivative: m/e (relative abundance) 261 (2.2); 260 ($M^{+\cdot}$, 11); 244 (21); 185 (7); 117 (73); 135 (16); 75 (100); 73 (87).

A solution of 1,18-octadecanediol (Karl Ind., 369 mg, 1.29 mmol) in pyridine (30 mL) was treated with acetic anhydride (145 mg, 1.42 mmol) at 100 °C for 1.5 h. The mixture was worked up, and the crude product was purified by chromatography on silica gel with ether:hexane (1:1), giving 133 mg of the monoacetate **60** (72 % by GC) in 31 % yield. IR: 3386 (sb); 2925 (s); 2850 (m); 1727 (s); 1255 (s). MS(EI) of the TMS derivative: m/e (relative abundance) 403 (3.3); 402 (11); 401 (34); 400 ($M^{+\cdot}$, 2.1); 399 (2.3); 387 (8.3); 386 (25); 385 (M-15, 100); 384 (2.5); 325 (8.7); 250 (11).

Oxidation of the monoacetates **59** and **60** to the corresponding acetoxy acids and hydrolysis to the hydroxy acids **63** and **65**

The monoacetates **59** (2.02 g, 10.8 mmol) and **60** (133 mg, 0.41 mmol) were oxidized with PDC in DMF. The 8-acetoxyoctanoic acid **61** was separated from unreacted diol by redissolving the crude material in saturated $NaHCO_3$:water (1:1) and extracting the aqueous mixture with ether. Acidification of the aqueous layer with 10% HCl and extraction with ether, furnished 2.00 g of the acetoxy acid **61** (100% by GC) in 92 % yield. IR: 3050 (sb); 2940 (s); 1744 (s); 1706 (s) 1388 (s); 1256 (sb); 1038 (s). MS (EI) of the TMS derivative: m/e (relative abundance) 276 (4.8); 275 (15); 274 ($M^{+\cdot}$, 86); 273 (5.6); 259 (M-15, 57); 215 (18); 199 (32); 143 (17); 125 (36); 117 (100); 107 (14); 75 (88); 73 (83).



Scheme 9. Synthesis of labelled 8-hydroxyoctanoic 16-hydroxyhexadecanoic and 18-hydroxyoctadecanoic acids.

The acetoxy acid **62** was purified by chromatography on acidic silica gel with ether:hexane (1:1), which gave 161 mg of acetoxy acid (55 % by GC). IR: 3475 (sb); 2925 (s); 2848 (m); 1738 (m); 1700 (m); 1238 (m). MS (EI) of the TMS derivative: m/e (relative

abundance) 416 (2.4); 415 (7.8); 414 (M+., 1.3); 299 (M-15, 9.9); 355 (23); 339 (100); 283 (7.7); 265 (14).

The acetoxy acid **61** (2.00 g, 9.9 mmol) was hydrolyzed with 3% NaOH. On workup, 0.796 mg (51 % yield) of pure 8-HOAA (95 % by GC), m. p. 57-58 °C (lit. 61-63 °C, Weaver *et al.* 1968) was recovered. ^{1}H NMR (in $CDCl_3$): δ 1.33 (8.6 H, m, 4-6-H); 1.59 (5.5 H, m, 3- and 7-H); 2.35 (2 H, t $J_{2,3}$ = 7 Hz, 2-H); 3.65 (2 H, t $J_{7,8}$ = 5 Hz, 8-H). MS (EI) of the TMS derivative: m/e (relative abundance) 305 (35); 304 ($M^{+\cdot}$, 3.4); 303 (3.0); 292 (1.2); 291 (9.1); 290 (21); 289 (M-15, 97); 288 (2.9); 273 (58); 215 (23); 199 (28); 149 (30); 147 (33); 75 (94); 73 (100).

18-Acetoxyoctadecanoic acid **62** (161 mg, 55%, 0.26 mmol) was hydrolyzed with 3 % NaOH, and the product chromatographed on acidic silica with ether:hexane (1:1), giving a total of 62 mg of hydroxy acid in 79 % yield. Two fractions were obtained from chromatography. The first one (36 mg) contained 71 % 18-OH C18:0 by GC, and the impurities were lower homologs: 17-C (9.8 %), 16-C (10 %); 15-C (7.9 %) and 14-C (1.3 %). The second fraction (26 mg) contained 63 % 18-OH C18:0 and lower homologs: 17-C (12 %), 16-C (17 %), 15-C (5.0 %) and 14-C (3.7 %). Analysis of the starting diol revealed that it contained lower homologs. ^{1}H NMR (in $CDCl_3$, fr. 1): δ 1.36 (32 H, m, 4-16-H); 1.55 (3 H, m, 17 H); 1.64 (2 H, tt $J_{2,3}$=$J_{3,4}$ = 8 Hz, 3-H); 2.34 (2 H, t $J_{2,3}$ = 8 Hz, 2-H); 3.63 (2 H, t $J_{17,18}$ = 6.5 Hz, 18-H). MS (EI) of the TMS derivative: m/e (relative abundance) 446 (1.4); 445 (3.2); 444 ($M^{+\cdot}$, 1.2); 432 (4.0); 431 (15); 430 (35); 429 (M-15, 100); 428 (0.9); 413 (84); 354 (5.2); 339 (30); 217 (14); 204 (24).

Deuteration of 8-HOAA and 18-OH C18:0 *via* the corresponding oxo-acids

Oxidation of 8-HOAA (1.19 g, 7.42 mmol) with PCC was carried out as described before, except that the ether extract was filtered through acidic Florisil^R/silica. The product was chromatographed on acidic silica with ether:hexane (2:1), which gave 0.329 g of

product (100 % by GC) in 28 % yield. ^{1}H NMR (in $CDCl_3$): δ 1.34 (15.5 H, m, 4- and 5-H); 1.64 (13 H, m, 3- and 6-H); 2.35 (7.7 H, t $J_{2,3}$ = 6 Hz, 2-H + 2- and 7-H from diacid); 2.45 (3.2 H, t $J_{6,7}$ = 8 Hz, d $J_{7,8}$ = 2 Hz, 7-H); 4.05 (0.8 H, t $J_{7,8}$ = 5 Hz, 8 H of hydrated aldehyde); 9.75 (1 H, t $J_{7,8}$ = 2 Hz, 8-H).

The 8-oxooctanoic acid **66** (321 mg, 2.3 mmol) was deuterated twice with D_2O/pyridine as previously described. The crude product was chromatographed on acidic silica with ether:hexane (2:1), which afforded 287 mg of product in 89 % yield. ^{1}H NMR (in $CDCl_3$): δ 1.34 (13 H, m, 4-5-H); 1.64 (8 H, m, 3- and 6-H); 2.35 (6 H, t $J_{2,3}$ = 6 Hz, 2-H); 2.45 (0.8 H, m, residual 7-H); 4.05 (0.5 H, t $J_{7,8}$ = 5 Hz, 8-H of hydrated aldehyde); 9.75 (1 H, s, 8-H). This spectrum revealed that the oxo acid was 60 % labelled.

The deuterated 8-oxooctanoic acid (230 mg, 1.44 mmol) was reduced with $NaBH_4$ and the product was chromatographed on acidic silica with ether:hexane (1:1), which afforded 61 mg of D_2 8-HOAA (100 % by GC) in 26 % yield. ^{1}H NMR (in $CDCl_3$): 1.33 (5.8 H, m, 4-6-H); 1.59 (2.6 H, m, 3- and residual 7-H); 2.35 (2 H, t $J_{2,3}$ = 7 Hz, 2-H); 3.65 (2 H, m, 8-H). MS (EI) of the TMS derivative: m/e (relative abundance) 306 ($M^{+\cdot}$, 11); 305 (9.4); 294 (2.2); 293 (7.1); 292 (20); 291 (M-15, 76); 290 (66); 289 (41); 275 (39); 274 (38); 273 (26); 217 (8); 201 (15); 149 (45); 147 (78); 75 (100); 73 (97). The MS revealed that the 8-HOAA was 39 % D_2, 35 % D_1 and 25 % unlabelled.

The 18-OH C18:0 (10.4 mg, 0.03 mmol), from fraction 1, was converted to the oxo acid (8.3 mg), as described before, in 80 % yield. MS (EI) of the TMS derivative: m/e (relative abundance) 370 ($M^{+\cdot}$, 2.2); 355 (M-15, 100); 341 (19); 281 (39); 221 (40); 202 (80). The oxo acid **68** was reduced with $NaBD_4$, and the product was purified on acidic silica with hexane:ether 3:1. This afforded 7 mg of D_1 18-OH C18:0 (89 % by GC), m. p. 81-86 °C (lit. 99.3-99.5 °C, Beilstein **3**, IV, 946), which was enriched for 18-OH C18:0 but still contained lower homologs: 17-C (8.0 %), 16-C (3.0 %), in 83 % yield. ^{1}H NMR (in $CDCl_3$): δ 1.26 (26 H, m, 4-16-H); 1.56 (4.3 H, m, 17-H); 1.64 (4.3 H, m, 3-H); 2.35 (2 H, t $J_{2,3}$ = 8 Hz, 2-H); 3.63 (0.8 H, tb $J_{17,18}$ = 7 Hz, 18-H). ^{2}H NMR (in $CHCl_3$): δ 3.65 (sb). MS (EI) of the TMS derivative: m/e (relative abundance) 431 (26); 430 (M-15, 91); 429

(4.4); 414 (100); 355 (13); 340 (52); 281 (26); 265 (22); 217 (39); 204 (61). The material was 96 % D_1, as calculated from the M-15 ion in the MS.

15,15-D_2 16-hydroxyhexadecanoic acid (D_2 16-OH C16:0)

16-Hydroxyhexadecanoic acid **64** (Aldrich, 219 mg, 0.80 mmol) was treated with PCC, and the product was chromatographed on acidic silica with ether:hexane (1:1). This afforded 110 mg of the oxo-acid **67** in 50 % yield. ^{1}H NMR (in $CDCl_3$): δ 1.28 (58 H, m, 4-13-H), 1.61 (11 H, m, 3- and 14-H); 2.33 (6.5 H, t $J_{2,3}$ = 10 Hz, 2-H, hydroxy- and diacid 2-H); 2.41 (2.5 H, tb $J_{14,15}$ = 8 Hz, 15-H); 3.65 (0.5 H, t $J_{15,16}$ = 3 Hz, 16-H of hydroxy acid); 9.75 (1 H, t $J_{15,16}$= Hz, 16-H). The NMR sample contained 70 % oxo acid, 15 % hydroxy- and 15 % diacid by GC.

The oxo acid **67** (109 mg, 0.4 mmol) was subjected twice to deuteration with D_2O/pyridine, followed by two purifications on acidic silica with ether:hexane (1:1) and (3:1). The product **68** (43 mg, 39 % yield) contained 84 % oxo acid, 14 % diacid and 2 % hydroxy acid. ^{1}H NMR (in $CDCl_3$): δ 1.26 (30.5 H, m, 4-13-H); 1.61 (9.3 H, m, 3- and 14-H); 2.32 (3 H, t $J_{2,3}$ = 8 Hz, 2-H); 2.41 (1 H, tb $J_{14,15}$ = 7 Hz, residual 15-H); 9.75 (1 H, sb, 16-H). The material was *ca.* 50 % labelled in the 15 position.

This oxo acid **68** was reduced with $NaBH_4$, giving 31 mg of pure (97 % by GC) D_2 16-OH C16:0 in 74 % yield. M. p. 92-95 °C, original 97-99 °C. ^{1}H NMR (in $CDCl_3$): δ 1.28 (24.8 H, m, 4-14-H); 1.56 (6.6 H, m, 2- and residual 15 H); 2.35 (2 H, t $J_{2,3}$ = 8 Hz, 2-H); 3.63 (2 H, m, 16-H). MS (EI) of the TMS derivative: m/e (relative abundance) 419 (2.2); 418 (2.8); 417 (1.7); 405 (8.1); 404 (25); 403 (78); 402 (92); 401 (82); 387 (75); 386 (100); 385 (89); 313 (32); 312 (44); 311 (39); 217 (38);204 (55).

The TMS derivative of unlabelled 16-OH C16:0 gave the following MS (EI): m/e (relative abundance) 418 (1.4); 417 (3.9); 416 (M^+, 1.5); 405 (1.6); 404 (3.8); 403 (14); 402 (34); 401 (M-15, 100); 400 (1.0); 385 (86); 327 (4.9); 311 (33); 217 (18); 204 (24).

The labelled material was 23 % D_2, 34 % D_1 and 43 % unlabelled, as calculated from the M-15 ion in the MS.

II.2. Treatment of the bees

The bees for this work were obtained from the S. F. U. apiculture group and were from a North American strain of *A. mellifera*, most similar to *A. m. ligustica*. Young workers were obtained by allowing bees to emerge from a brood comb in an incubator. Newly emerged workers were kept in a cage with water, sugar syrup and pollen for one day before an experiment. Queens were reared at S. F. U.[6] and, in the case of virgin queens, were kept for one week after emergence in small cages in a queen bank. Mated queens were *ca.* 1 year old, laying queens taken from their colonies during the summer.

Semiochemical biosynthesis has been studied in three ways: *in vivo*, by topical application of labelled precursors onto the intact gland and *in vitro*. For instance, bark beetle pheromone biosynthesis was studied *in vivo* by exposing the insects to vapors of labelled volatile precursors (Vanderwel *et al.*, 1992), while topical application onto the pheromone gland was used in early studies of moth pheromone biosynthesis. Once the biosynthetic pathways for moth pheromones were known, it was possible to isolate and study individual enzymes from the pathway (Jurenka and Roelofs 1993). Early attempts in our laboratory to study incorporation of deuterated fatty acids into worker mandibular components *in vivo* failed because the fatty acids were probably metabolized in many other tissues besides the mandibular gland and because the labelled fatty acids were diluted by the endogenous ones. This approach was further complicated by a low survival rate of injected or topically applied bees.

To avoid the above problems, the labelled compound to be tested was applied in DMSO directly onto an intact mandibular gland. Freshly dissected glands were rinsed with buffered saline (5 mM Tris, pH 7, 0.02 % (w/v) $MgCl_2$, 0.02 % KCl, 0.02 % $CaCl_2$ and

[6] I thank Mr. P. Laflamme, Mr. S. Mitchell and Ms. H. Higo for rearing queens.

0.9 % NaCl), blotted with a piece of tissue and exposed to 0.5 µL of substrate solution (generally 40 µg/µL). The gland was placed in an Eppendorf tube and perfused for 10-20 min, after which it was rinsed with saline, blotted and extracted with methanol (2 X 12 µL for workers and 2 X 25 µL for queens). The methanol used for extraction contained 0.2 µg/µL of 10-undecenoic acid as internal standard. Two 2 µL aliquots of the extract were derivatized with 2 µL of BSFTA (Slessor *et al.* 1990). The samples were diluted with hexane (30 µL) and one was analyzed by GC, the other by GC-MS.

The treatments were generally done in 6 - 10 replicates, with one gland per replicate. Two types of control were included every time a new set of labelled compounds was tested: DMSO blanks and substrate blanks. For the former, mandibular glands were perfused with DMSO (0.5 µL) and extracted as described before. For the latter, 0.2 µL of the solution of labelled substrate was diluted with methanol containing internal standard (20 µL). In the results, only the DMSO blanks are shown. The substrate blanks were used to check the baseline at the retention times of interest in the GC-MS.

II.3. Analytical methods

3.1. Identification of compounds in the extracts

The compounds of interest in the extracts were identified by comparison of their GC retention time and MS fragmentation pattern with synthetic standards. However, for some compounds no synthetic standard was available. In such cases, the retention time was predicted using the Kovats Index (K. I.) calculated from the K. I. of a homolog. Under isothermal conditions, the retention times of the straight chain alkanes follow a logarithmic pattern, and their K. I. is the number of carbons X 100. For all other compounds the K. I. is calculated as follows:

$$K.\ I. = 100 \times (C_{A1} + \log(t_Y/t_{A1}) / \log(t_{A2}/t_{A1}))$$

where C is the number of carbons, t is the retention time, A1 and A2 are the straight chain alkanes eluting before and after the compound of interest, Y (Rooney, 1981). For a

homologous series, a one carbon increment in the chain length results in an increment of 100 in the K. I. Thus, as long as the retention time for one member of a series and for the alkanes are known, it is possible to predict the retention time for any higher or lower homolog. Even under non-isothermal conditions, this method gave good results. For example, no standard for 12-hydroxy-(E)2-dodecenoic acid (12-OH C12:1) was available. The K. I. of its lower homolog, 10-HDA, was 1867 corresponding to a retention time of 12.7 min. The predicted K. I. of 12-OH C12:1 was 2067 which gave a predicted retention time of 15.8 min.

The MS fragmentation pattern was used to locate a compound in the vicinity of the predicted retention time. Comparison of the mass spectra of synthetic standards revealed that the high molecular weight fragment ions in the mass spectra were shifted by the appropriate number of 28 mass unit increments within a series of even-numbered homologs. This knowledge was used to predict MS fragmentation patterns of compounds for which there was no standard. Furthermore, it was possible to distinguish ω from isomeric ω-1 hydroxy acids by the fragmentation pattern. The former had intense M-15 and M-31 fragments, while the latter had an additional M-44 fragment. Returning to the example, the expected pattern for 12-OH C12:1 was found at 15.7 min, which is in agreement with the predicted retention time. The isomer, 11-OH C12:1, came at 14.6 min (K. I. 1990) and had the pattern of an ω-1 hydroxy acid.

3.2. Quantitation of total material and incorporation of label

The compounds of interest were quantitated from the GC trace. However, some minor components were not detected in the GC. Their quantity was estimated from the M-15 ion current across the GC peak in the GC-MS trace. The responses of the FID and the GC-MS were calibrated with solutions of known concentration.

The percentage of labelled material found in treated samples was determined from the M-15 fragment ion. This ion arises by loss of a methyl group from one of the

trimethylsilyl moieties in the parent ion and was chosen because it is much more intense than the molecular ion and loss of label attached to the main chain is not possible in this particular fragmentation reaction (Pierce 1968). Furthermore, this ion is not prone to ion/molecule reactions over a wide range of concentrations (0.2 - 20 ng of injected material). This allowed the analysis of major and minor components, whose concentration varied over two orders of magnitude, in a single run. Separate runs for the analysis of major and minor components were only necessary in the case of mated queens, in which variation between components can span four orders of magnitude.

To determine the percentage of labelled material, the ion currents for the M-15 and M-15+n (n = the number of D) fragments were recorded for each scan across the GC peak without background subtraction, added, and the ratio

$FR = \Sigma(M\text{-}15+n)/(\Sigma(M\text{-}15) + \Sigma(M\text{-}15+n))$

was calculated. The FR value represents the fraction of the ion current due to an isotope peak of the M-15 fragment ion, relative to the total ion current of the fragment ion and the isotope peak. To correct for the natural isotope abundance, the ratio (FR_o) for all compounds of interest was calculated either from unlabelled standards or from extracts that had been prepared for that purpose. The percentage of label in a sample was calculated from a calibration line that had been prepared using mixtures of labelled and unlabelled standards. The calibration lines did not change significantly between 0.2 and 20 ng of injected material and for different compounds with the same labelling pattern (e. g., Figure II.1). Thus, it was valid to use the line of a homolog or isomer in cases where pairs of synthetic labelled and unlabelled standards were not available. A new set of calibration lines was determined every time the instrument was tuned as FR values varied slightly with tuning.

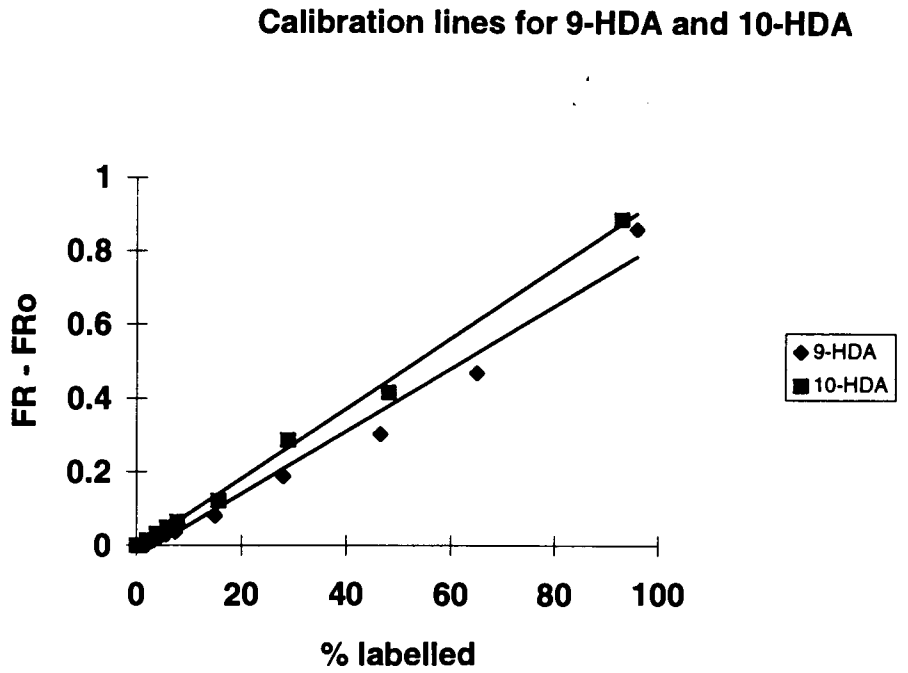


Figure II.1. Calibration of 9-HDA, D_2 9-HDA and 10-HDA, D_2 10-HDA. The line for 9-HDA was y = 0.0085x - 0.030 (R^2 = 0.979) and for 10-HDA, y = 0.0095x - 0.0081 (R^2 = 0.997).

When the percentage of labelled material was low (< 3%) the calculation did not always reflect that label was present, even though label was clearly visible in the spectra corresponding to the early portion of the GC peak. In such cases, only the front 5 % (3-4 scans) of the GC peak were added as described before. This approach was chosen because, in analyses of standard mixtures with a low content of labelled material (0 - 5 %), most of the label was found in the front portion of the GC peak (Figure II.2). Calibration lines for low percentage samples were constructed by this method and used to estimate the percentage of labelled material in the samples (Figure II.3).

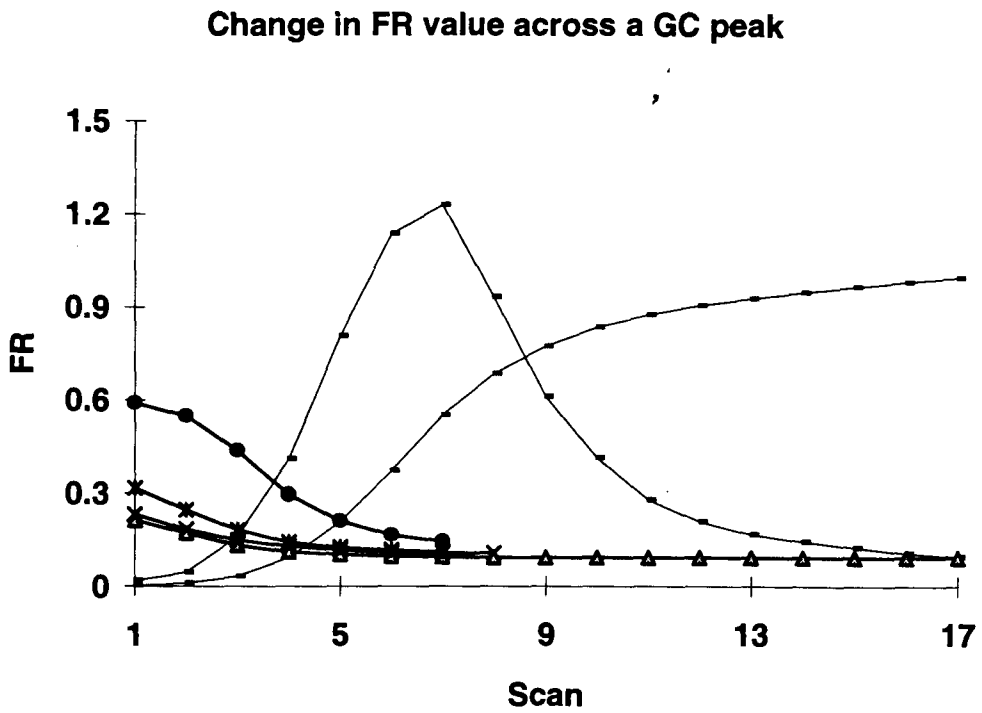


Figure II.2. Change in the FR value across a GC peak of 10-HDA. The scaled area counts and the proportion of the peak added up to a given scan are indicated with thin lines, the change in FR (see p. 66) value calculated by adding up to a given scan is shown for 0 % of D_2 10-HDA (triangles), 0.8 % (crosses), 1.4 % (*) and 3.6 % (•). The MS was operated at 1 scan/s.

A substrate blank was run for every set of treatments to correct for possible contributions from impurities which may have some ions of the same mass as a compound in the extract. Generally, the baseline in the substrate blanks did not show ions corresponding to the M-15 or M-15+n of the compounds of interest. In the few cases where a background was found, the contaminant ion current/ng of substrate was scaled to the substrate in the treatments and subtracted from the appropriate ion current. In spite of the substrate blank correction, systematic errors caused by baseline artifacts could still be introduced. For this reason, different forms of some key substrates with different labelling

patterns and/or from different sources, which were likely to contain different impurities, were tested.

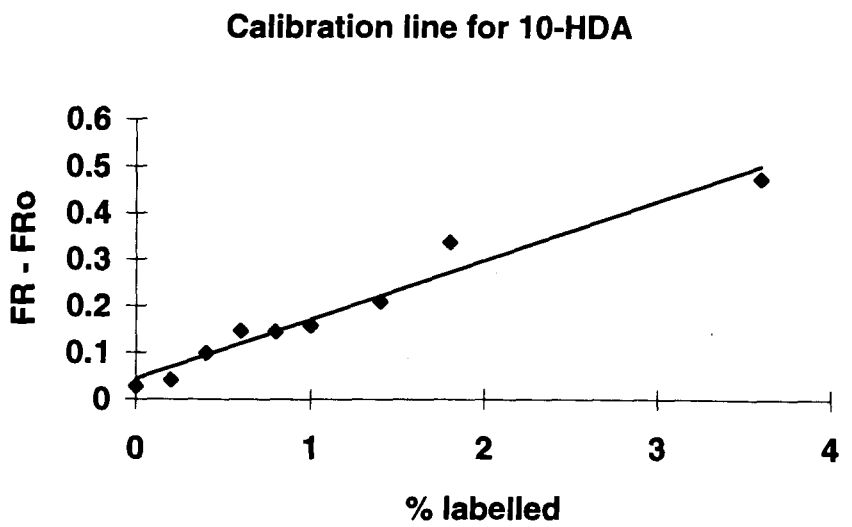


Figure II.3. Low percentage calibration line for 10-HDA, D_2 10-HDA. The FR values were calculated using the first three scans of the GC peak. The equation for the line was $y = 0.13x + 0.03$ ($R^2 = 0.96$).

II.4. Statistics

Data were subjected to analysis of variance (ANOVA). Means of treatments and blanks were grouped and compared pairwise by Tukey's multiple range test with $\alpha = 0.05$ (SAS Institute Inc.). Mean amounts of labelled ω- and ω-1-hydroxy acids accumulated during perfusions with D_1 C18:0 and 2-F C18:0 (p.114) and of labelled ω- and ω-1-functionalized acids formed from D_1 C18:0 (p. 116) were compared by the Kruskal-Wallis test (SAS Institute Inc.). Calibration lines were obtained by linear regression (Devore 1991).

Chapter III: Elucidation of the biosynthetic pathway of mandibular acids in workers and queens

III.1 Search for a fatty acid precursor

The objective of these experiments was to find a straight-chain fatty acid precursor to the functionalized acids produced by the bees. Decanoic acid applied topically to the bees gave sporadic incorporation, so decanoic, (E)2 decenoic, hexadecanoic and octadecanoic acids were tested directly on the glands in 10 min perfusions. Two trials were done on workers and the results were pooled.

The incorporation of the four acids into 9-HDA, 8-HOAA, 10-HDAA and 10-HDA is shown in Table III.1. Only D_1 C18:0 gave a significant incorporation into all four hydroxy acids analyzed. D_3 C16:0 was incorporated into 8-HOAA and D_4 C10:0, into 10-HDA. D_2 C10:1 was not incorporated.

Table III.1. Percentage of labelled hydroxy acids formed from decanoic, (E)2-decenoic, hexadecanoic and octadecanoic acids in workers.

treatment	N	(% labelled material) mean +/- S. E. [a] product			
		9-HDA	8-HOAA	10-HDAA	10-HDA
D_2 C10:1	6	5.0 +/- 0.7	3.0 +/- 0.4	2.3 +/- 0.3	2.9 +/- 0.4
blank	6	2.4 +/- 1.1	3.4 +/- 1.8	2.6 +/- 0.3	3.9 +/- 0.3
D_4 C10:0	9	1.0 +/- 0.5	1.8 +/- 1.2	0.3 +/- 0.1	**0.8 +/- 0.1**
blank	10	0.3 +/- 0.1	0.3 +/- 0.1	0.2 +/- 0.1	0.2 +/- 0.1
D_3 C16:0	14	7.5 +/- 2.2	**14 +/- 4**	3.3 +/- 0.4	3.6 +/- 0.6
blank	10	3.3 +/- 0.9	3.3 +/- 1.1	2.6 +/- 0.2	3.0 +/- 0.5
D_1 C18:0	16	**22 +/- 3**	**17 +/- 5**	**10 +/- 1**	**13 +/- 3**
blank	10	1.5 +/- 0.7	0.0 +/- 0.0	0.0 +/- 0.0	0.0 +/- 0.0

[a] For entries in bold type, treatments and blanks differed significantly ($P<0.05$) by Tukey's test.

Because D_1 C18:0 was incorporated most readily, the samples were analyzed for interconversion among the potential precursor acids. The results are shown in Table III.2. D_1 C18:0 was chain shortened to hexadecanoic and (E)2-decenoic acid, but not to decanoic acid. D_3 C16:0 was readily chain shortened to both 10-carbon acids and elongated to octadecanoic acid. D_4 C10:0 was neither desaturated to (E)2-decenoic acid, nor elongated to hexadecanoic acid, but it was elongated to octadecanoic acid to a small extent. Finally, (E)2-decenoic acid was not converted to decanoic acid, but was readily elongated to hexa- and octadecanoic acids.

Table III.2. Interconversion among potential precursors to hydroxy acids in worker mandibular glands.

substrate	N	(% labelled) mean +/- S. E. product			
		C10:1	C10:0	C16:0	C18:0
D_2 C10:1	6	[**94 +/- 4**]	9.7 +/- 2.2	**18 +/- 8**	**49 +/- 10**
blank	6	[50 +/- 11]	**39 +/- 10** *	2.8 +/- 1.8	0.6 +/- 0.3
D_4 C10:0	9	13 +/- 4	[**75 +/- 3**]	1.6 +/- 0.6	0.8 +/- 0.4 **
blank	10	5.0 +/- 1.5	[3.9 +/- 0.7]	2.0 +/- 0.7	0.20 +/- 0.02
D_3 C16:0	14	**32 +/- 4**	**24 +/- 4**	[**86 +/- 5**]	**52 +/- 8**
blank	10	9.6 +/- 3.6	8.2 +/- 3.0	[17 +/- 6]	0.0 +/- 0.0
D_1 C18:0	16	**27 +/- 8**	0.4 +/- 0.4	**3.7 +/- 1.5**	[**78 +/- 3**]
blank	10	0.8 +/- 0.5	0.0 +/- 0.0	0.0 +/- 0.0	[2.3 +/- 0.5]

Percentage of labelled material incorporated into straight-chain fatty acids from other fatty acids. Values shown in bold differ significantly from the blank (Tukey $P<0.05$).

* A baseline artifact gave apparent labelling in the blanks.
** Label was visible in the mass spectra.

The results suggest that the mandibular glands must be capable of fatty acid synthesis and β-oxidation, since the precursor acids were elongated and chain shortened.

Thus, it is possible that complete degradation to acetate and resynthesis competed with direct utilization in the case of D_4 C10:0, which may explain the low incorporation of this compound into octadecanoic acid and the hydroxy acids. On the other hand, (E)2-decenoic acid was elongated to octadecanoic acid but was not incorporated into the hydroxy acids. This behavior may be due to an inhibition of β-oxidation, which would prevent complete degradation to acetate and incorporation into the hydroxy acids. The source of inhibition was not identified, but was most likely not the (E)2-decenoic acid itself because its CoA derivative is an intermediate in β-oxidation. The acids shorter than 18 carbons were incorporated into the hydroxy acids to a lesser extent than octadecanoic acid, but were elongated to octadecanoic acid. This suggests that incorporation of D_3 C16:0 and D_4 C10:0 into the hydroxy acids proceeds *via* octadecanoic acid, the entry point to the pathway.

III.2. *De novo* biosynthesis from acetate

The objective of this experiment was to determine whether the precursor and hydroxy acids can be synthesized *de novo* from acetate. Labelled acetate (1-^{13}C) was applied to the glands in DMSO:saline 1:1 (1 µL of a 70 µg/µL solution), and the glands were perfused for 10 and 20 min. The results are shown in Tables III.3 and 4[1].

One mass unit from 1-^{13}C acetate was incorporated into 9-HDA, 8-HOAA, 10-HDAA and 10-HDA at one or both perfusion times, which indicates that these compounds can be synthesized *de novo* from acetate in the mandibular glands. If octadecanoic acid is the precursor, then labelled acetate should be incorporated into this compound. Analysis of the saturated fatty acids revealed that this was the case. Of the fatty acids analyzed, only hexa- and octadecanoic acids incorporated label from 1-^{13}C acetate, which suggests that the products of the fatty acyl synthetase in this tissue are hexa- and octadecanoic acids. This is consistent with the patterns of fatty acid synthesis observed in other insects such as *T. ni* and *M. domestica* (Stanley-Samuelson *et al.* 1988). The

[1] I thank Ms. K. Bray for helping with this experiment.

failure of the shorter acids to incorporate 1-^{13}C acetate, indicates that they do not arise by premature release during fatty acids synthesis, but by chain shortening of hexa- and octadecanoic acids.

Table III.3. Incorporation of one 1-^{13}C acetate into hydroxy acids in workers.

treatment	N	(% labelled) mean +/- S. E.[a] product			
		9-HDA	8-HOAA	10-HDAA	10-HDA
20 min	12	**52 +/- 6**	**17 +/- 5**	**20 +/- 6**	3.9 +/- 0.8
10 min	8	**44 +/- 8**	1.8 +/- 1.4	5.5 +/- 4.2	**5.3 +/- 1.1**
blank	8	2.3 +/- 0.9	0.0 +/- 0.0	1.3 +/- 0.9	2.2 +/- 0.6

Table III.4. Incorporation of one 1-^{13}C acetate into straight-chain fatty acids in workers.

treatment	N	(% labelled) mean +/- S. E.[a] product				
		C10:0	C12:0	C14:0	C16:0	C18:0
20 min	12	7.7 +/- 1.1	3.1 +/- 1.2	0.8 +/- 0.6	**6.6 +/- 1.1**	**5.5 +/- 0.6**
10 min	8	11 +/- 3	0.7 +/- 0.7	2.7 +/- 2.0	**5.4 +/- 1.6**	**4.3 +/- 0.8**
blank	8	9.7 +/- 0.9	2.7 +/- 1.1	1.7 +/- 0.8	1.5 +/- 0.7	0.0 +/- 0.0

[a] For entries in bold type, treatments and blanks differed significantly ($P<0.05$, Tukey).

The results from the first two experiments indicate that the hydroxy acids are synthesized in the mandibular gland from acetate *via* octadecanoic acid.

III.3. Lipid-bound fatty acid profile

The objectives of lipid analysis were to determine the chain-length distribution of lipid-bound fatty acids and to ascertain whether the major queen and worker mandibular acids (ODA, 9-HDA, 10-HDA and 10-HDAA) can be lipid-bound.

To extract the lipid, a pair of glands that had been thoroughly rinsed with saline were soaked overnight at 4°C in $CHCl_3$:methanol 2:1 (500 µL). The solution was withdrawn and the solvent evaporated to dryness under Ar. The lipids were separated on a column of DEAE Sephadex[2], which adsorbs phosphatidic acid (PA), phosphatidylserine (PS), phosphatidylinositol (PI) and free acids, but not phosphatidylcholine (PC), phosphatidylethanolamine (PE), mono-, di- and triglycerides (Wood *et al.*, 1989). The lipid was resuspended in $CHCl_3$:methanol:water 30:60:8 (10 mL) and loaded onto the column which had been equilibrated with this solvent. Additional solvent (6 mL) was passed through the column, and the eluate was collected. This solution contained the non-acidic lipids. To elute the acidic lipids, the column was rinsed with $CHCl_3$:methanol: 0.8 M sodium acetate 30:60:8. Sodium acetate was removed from the acidic fraction by evaporating the solvent to dryness under Ar. The residue was resuspended in $CHCl_3$ (1 mL) and the suspension was centrifuged to settle the sodium acetate particles. The supernatant was washed twice with water and three times with $CHCl_3$:methanol:water 3:48:47 (Wood *et al.* 1989). Both fractions were subjected to a second column separation.

TLC analysis of the lipid fractions by the method of Wolf and Roelofs (1989) revealed that the non-acidic fraction contained mostly PC and PE and the acidic one, mostly PA. More than 90% of the free acid present in the gland was removed during the saline wash prior to lipid extraction. The remaining free acid eluted in the acidic fraction and was removed along with the sodium acetate.

Both extracts were dried under Ar and treated with acetic anhydride:glacial acetic acid 3:2 (0.3 mL) at 150°C for 5 h. Once the samples had cooled to room temperature, water (2 mL) was added and the mixture, extracted with $CHCl_3$ (2 mL). The organic extract was washed 4 times with 5 % $NaHCO_3$ and once with water, followed by drying over Na_2SO_4. By this procedure, the phospholipids were converted to triglycerides with acetate in place of the phosphate-containing group (Kumar *et al.* 1983). The lipid-bound fatty

[2] The column was prepared in a silylated Pasteur pipette with a glass wool plug at the bottom. DEAE Sephadex (Sigma, 0.5-0.6 mL wet) was layered on the glass wool.

acids were freed in the form of methyl esters by transesterification of the material recovered from acetolysis with 0.5 M KOH in methanol (50 µL). The mixture was reacted at room temperature for one h, after which the reaction was quenched with 1 N HCl (60 µL). The methyl esters were extracted with hexane (3 X 50 µL), and the hexane extract was washed with 5% $NaHCO_3$ (50 µL) and dried over Na_2SO_4.

Table III.5. Amounts of fatty acids bound in acidic and non-acidic lipids from queen and worker mandibular glands.

compound	amounts (ng/bee) queen		worker	
	acidic	non-acidic	acidic	non-acidic
C10:0	96	9	8	16
C12:0	244	44	56	60
C14:0	1230	31	119	224
C16:1 (Z)9	2880	619	254	171
C16:0	5240	476	556	1860
C18:3 and/or :2 *	4450	not detected	366	not detected
C18:1 (Z)11	5390	4870	1880	6170
C18:1 (Z)9	387	10	9	52
C18:0	1480	639	16	300
C20:0	2650	46	17	295
C20:1 (Z)9	4220	5	21	33

Abbreviations: C10:0 = decanoic acid, C12:0 = dodecanoic acid, etc.
The acids were analyzed as the corresponding methyl esters.

* Standards of methyl (all Z) 9,12,15-octadecatrienoate and 9,12-octadecadienoate eluted at the same retention time.

The hexane extract containing the methyl esters was analyzed by GC. Compounds were identified by comparison of their retention time with standards. To find compounds with free hydroxyl groups, a sample of the hexane solution was derivatized with BSTFA and analyzed by GC. Compounds with free hydroxyl groups are silylated in the BSTFA

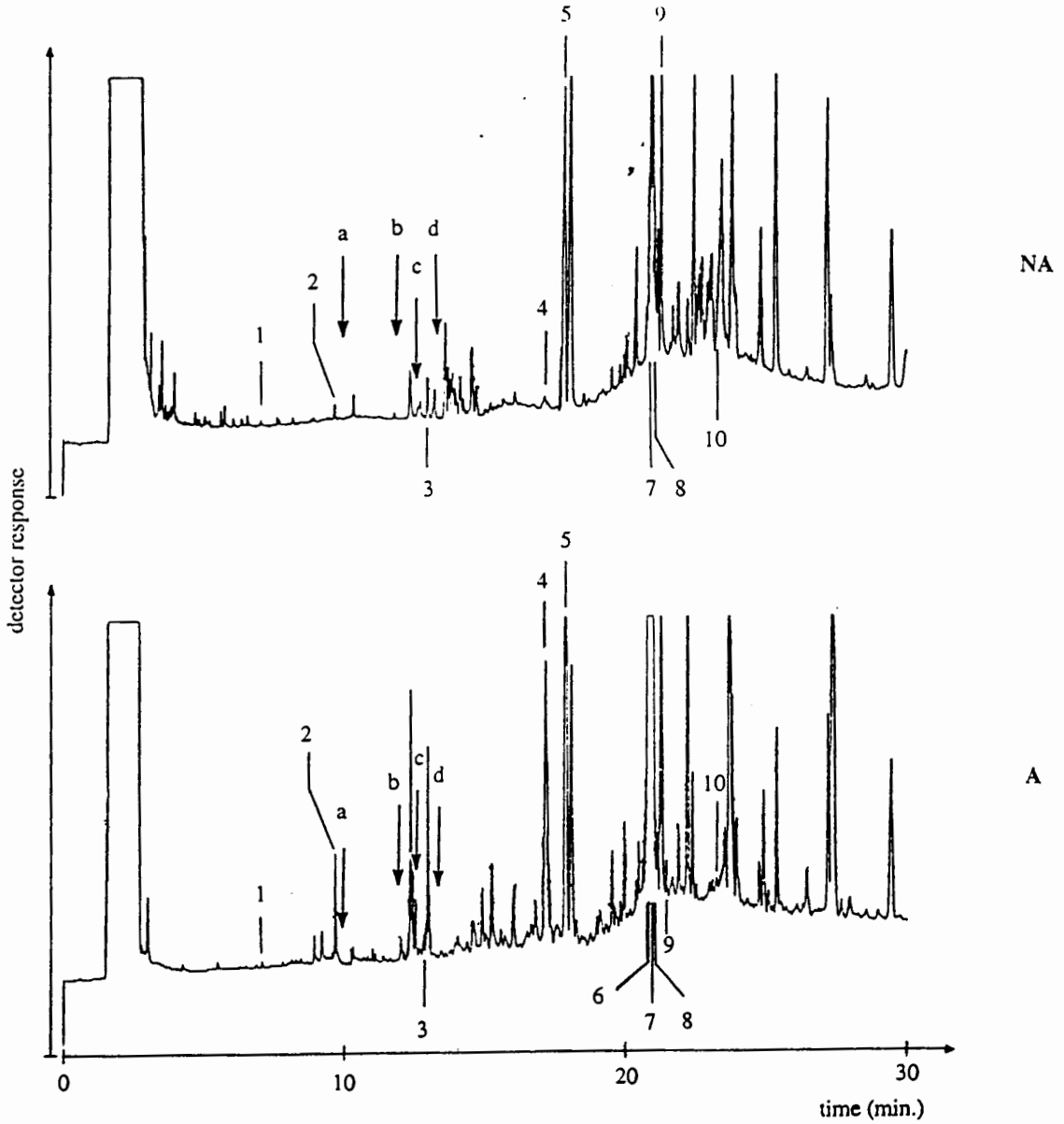


Figure III.1. GC traces of methyl esters obtained through acetolysis and transesterification of non-acidic (NA) and acidic (A) lipids from worker mandibular glands. Arrows indicate the retention times of the methyl esters of a) ODA, b) 9-HDA, c) 10-HDAA and d) 10-HDA. Numbers indicate the methyl esters of 1) decanoic, 2) dodecanoic, 3) tetradecanoic, 4) (Z)9-hexadecenoic, 5) hexadecanoic, 6) (all Z) 9,12,15-octadecatrienoic and/or 9,12-octadecadienoic, 7) (Z)11-octadecenoic, 8) octadecanoic, 9) (Z)9-octadecenoic and 10) eicosanoic acids.

treatment and, therefore, come at a later retention time. To verify the identity of peaks whose retention time matched that of a standard methyl ester, an aliquot of the hexane solution was transesterified with KOH in ethanol. Finally, to determine whether keto- or aldehydo acid esters were present, an aliquot of the original hexane solution was treated with $NaBH_4$ in methanol. Samples from the worked up transesterification and reduction were run directly and as BSTFA derivatives. Methyl esters whose identity was known were quantitated from the GC trace of the original hexane solution (Figure III.1).

Even though PC and PE appeared to be major constituents of the mandibular gland lipid by TLC, the total quantity of fatty acid methyl esters recovered from the non-acidic lipid fraction was low compared to the amount recovered from the acidic lipid (Table III.5). A possible explanation is that some PC and PE was lost during the column separation or the acetolysis of these compounds was incomplete.

Queens and workers had similar fatty acid profiles (Table III.5). In both castes, the most abundant lipid-bound fatty acids were 16 and 18 carbons long. Octadecanoic acid was one of the most abundant fatty acids in non-acidic lipid and one of the least abundant in acidic lipid. Hexadecanoic acid was abundant in both types of lipid. The possibility that the lipid may serve as an additional source of octadecanoic acid for mandibular acid biosynthesis was not pursued. More importantly, none of the mandibular acids nor any other hydroxy- or keto acids were found to be lipid-bound in either caste. Thus, the lipid in the mandibular gland is not a reservoir for oxygen-functionalized acids. Furthermore, failure to find lipid-bound hydroxy- or keto acids suggests that the biosynthesis of mandibular acids does not proceed through a lipid-bound intermediate.

III.4 Interconversion among major components

The objective of these experiments was to determine whether the major components in the queen and worker blends can interconvert. In the first set of treatments, the major hydroxy acids (10-HDAA, 10-HDA and 9-HDA) were tested for oxidation to the

corresponding di- and keto acids. In the second set, the 8- and 10-carbon ω-hydroxy acids were tested for interconversion, and in the third set the analogous treatments with the 10-carbon ω-1-hydroxy acids were done in both castes. Finally, 9- and 10-HDA were tested for isomerization with respect to the hydroxy group.

4.1 Hydroxy group oxidation

To test for oxidation of hydroxy acids, worker and queen mandibular glands were perfused with D_3 10-HDAA, D_2 10-HDA and D_2 9-HDA, and the resulting extracts were analyzed for the corresponding oxidation products, C10:0 DA, C10:1 DA and ODA. The results are shown in Table III.6.

Table III.6. Oxidation of 10-carbon hydroxy acids in workers and queens.

caste		substrate oxidized		
		D_3 10-HDAA	D_2 10-HDA	D_2 9-HDA
worker	tr.	**24 +/- 2** (9)	**78 +/- 14** (8)	not detected
	bl.	6.4 +/- 0.1 (10)	5.1 +/- 0.2 (10)	
queen (virgin)	tr.	**46.5 +/- 0.2** (8)	**71 +/- 2** (8)	13 +/- 13 (8)
	bl.	11.0 +/- 2.3 (8)	24.6 +/- 6.8 (8)	2.9 +/- 1.2 (8)
queen (mated)	tr.	not determined	not determined	**6.6 +/- 2.8** (8)
	bl.			0.5 +/-0.3 (8)

The values represent the mean +/- S. E. of the percentage of labelled oxidized product formed from the corresponding substrate (C10:0 DA from 10-HDAA, C10:1 DA from 10-HDA, ODA from 9-HDA). Entries corresponding to treatments (tr.) shown in boldface are significantly different from the blank (bl.), Tukey $P<0.05$. The number of replicates is indicated in parenthesis after each entry.

Workers and virgin queens both readily oxidized ω-hydroxy acids to the corresponding diacids. This conversion was found to proceed *via* the corresponding oxo-acids (data not shown). Neither workers nor virgin queens oxidized 9-HDA to ODA, but mated queens did (Figure III.2). Mated queens were not tested for oxidation of the ω-hydroxy acids.

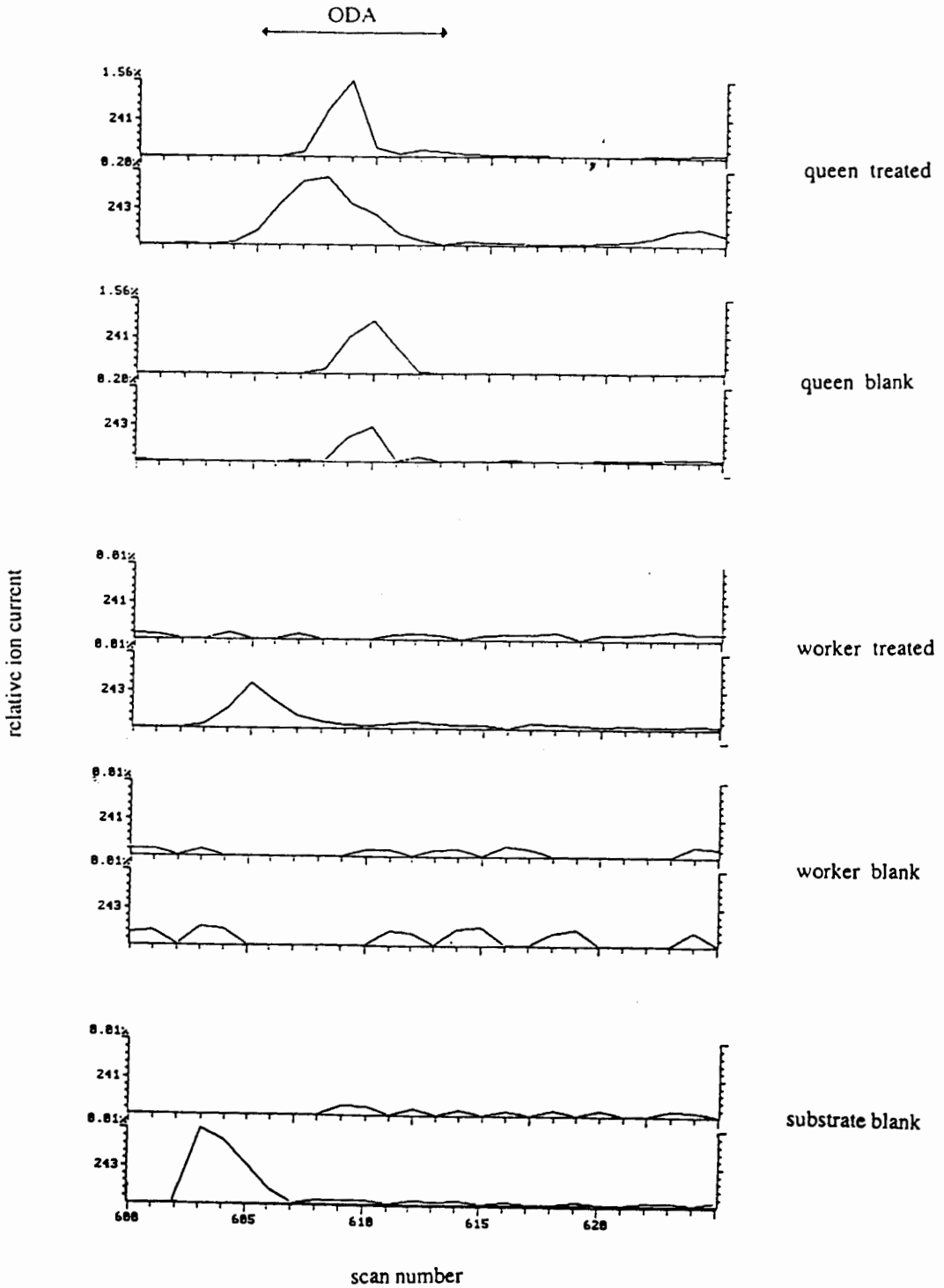


Figure III.2 GC-MS single ion displays from tests for oxidation of 9-HDA to ODA in workers and mated queens. The chromatograms correspond to the M-15 ion of the unlabelled (241) and labelled (243) material. The 243 ion for the worker samples and the blank is displayed at a 20-fold gain compared to the 243 ion in the queen samples.

4.2 Interconversion among ω-hydroxy acids

Labelled 10-HDAA and 10-HDA were tested for interconversion in virgin queens and workers, and 8-HOAA was tested for elongation and further chain shortening in workers. The results are listed in Table III.7.

Table III.7. Interconversion among ω-hydroxy acids in queens and workers.

caste	substrate	(% labelled material) mean +/- S. E. product		
		10-HDAA	10-HDA	8-HOAA
queens (virgin)	D_3 10-HDAA	[**83 +/- 3**]	1.1 +/- 0.1	1.4 +/- 0.1
	blank	[1.1 +/- 0.1]	1.3 +/- 0.4	1.3 +/- 0.1
	D_2 10-HDA	1.3 +/- 0.5	[**53 +/- 2**]	**30 +/- 5**
	blank	0.5 +/- 0.1	[0.2 +/- 0.1]	3.6 +/- 0.9
workers	D_3 10-HDAA	[**86 +/- 4**]	**17 +/- 7**	**20 +/- 10**
	blank	[0.3 +/- 0.1]	0.5 +/- 0.1	1.3 +/- 0.7
	D_2 10-HDA	**27 +/- 4**	[**90 +/- 5**]	**48 +/- 4**
	blank	0.3 +/- 0.2	[0.5 +/- 0.2]	1.3 +/- 0.7
	D_2 8-HOAA	1.5 +/- 0.1	4.0 +/- 1.4	[**73 +/- 1**]
	blank	3.6 +/- 0.2	4.1 +/- 0.4	[6.3 +/- 1.8]

Percentage of labelled material found in mandibular glands treated with labelled ω-hydroxy acids. Bracketed entries on the diagonal correspond to the applied substrates. Bold entries indicate a significant difference between treatments and blanks (Tukey $P<0.05$). For queens, N = 8; for workers, N = 10.

Virgin queens did not convert 10-HDAA to 10-HDA or 8-HOAA, but they chain shortened 10-HDA to 8-HOAA. Workers chain shortened 10-HDAA and 10-HDA to 8-HOAA and they converted 10-HDA to the saturated form. They did not elongate or further chain-shorten 8-HOAA. These data suggest that the ω-hydroxy acids can be

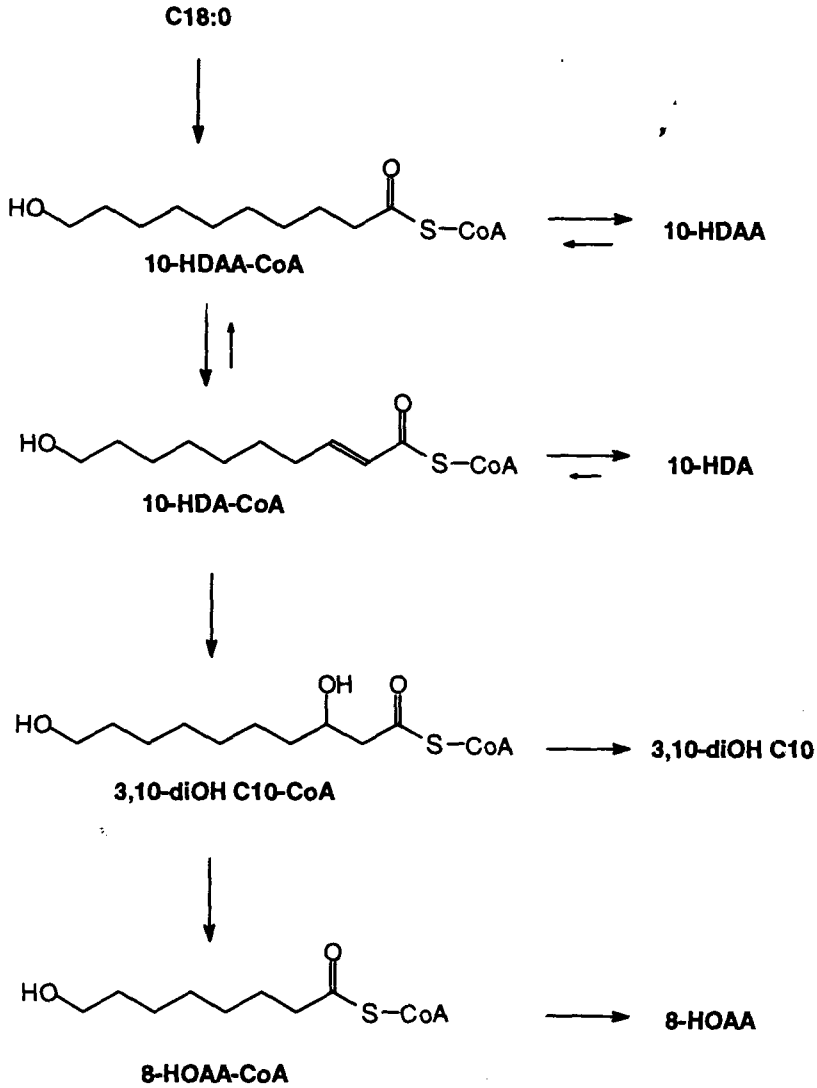


Figure III.3. Interconversion among ω-hydroxy acids.

produced from 10-HDAA by limited β-oxidation. The process would start with the conversion of 10-HDAA to its CoA ester, followed by desaturation at the second position to give 10-HDA-CoA, hydration of the (E)2 double bond to give 3,10-dihydroxydecanoyl-CoA, oxidation of the 3-hydroxy group to give the 3-keto acyl-CoA and attack by coenzyme A to give acetyl-CoA and 8-HOAA-CoA (Figure III.3). The identification of 3,10-dihydroxydecanoic acid in worker mandibular secretions (Weaver *et al.*, 1968) further supports the idea that the blend of ω-hydroxy acids arises by limited β-oxidation of 10-HDAA-CoA, with hydrolysis of intermediate CoA esters.

Workers chain shortened 10-HDAA and 10-HDA more efficiently than virgin queens. Furthermore, workers converted 10-HDA to 10-HDAA, which suggests that the (E)2 desaturation of 10-HDAA is reversible in that caste. This process was not seen in the virgin queens. The observed difference between the castes may be due to differences in the specificity of β-oxidation, the ability to convert the hydroxy acids to their CoA esters or a combination.

4.3 Interconversion among ω–1-hydroxy acids

The ω-1-hydroxy acids, D_3 9-HDAA and D_2 9-HDA, were tested for interconversion, and the results are shown in Table III.8.

Table III.8. Interconversion among ω-1-hydroxy acids in queens and workers.

caste	substrate	(% labelled) mean +/- S. E. product 9-HDAA	9-HDA	7-HOAA
queens (mated)	D_3 9-HDAA	[**100 +/- 0**]	**0.8 +/- 0.3**	3.8 +/- 3.8
	blank	[0.5 +/- 0.5]	0.05 +/- 0.05	1.9 +/- 1.0
	D_2 9-HDA	0.6 +/- 0.6	[**27 +/- 9**]	16 +/- 9
	blank	0.0 +/- 0.0	[0.0 +/- 0.0]	0.1 +/- 0.1
workers	D_3 9-HDAA	[**100 +/- 0**]	**24 +/- 3**	not determined
	blank	[5.6 +/- 3.7]	3.8 +/- 0.9	
	D_2 9-HDA	**58 +/- 4**	[**82 +/- 3**]	not determined
	blank	21 +/- 3	[9.3 +/- 1.5]	

Percentage of labelled material found in mandibular glands treated with labelled ω-hydroxy acids. Bracketed entries on the diagonal correspond to the applied substrates. Bold entries indicate a significant difference between treatments and blanks (Tukey $P<0.05$). For queens, N = 8; for workers, N = 10.

Queens were able to convert 9-HDAA to 9-HDA, and they may have been able to chain-shorten 9-HDA to 7-HOAA to a small extent. As with the ω-hydroxy acids, (E)2-desaturation of 9-HDAA was not reversible in queens. In workers, 9-HDAA and 9-HDA readily interconverted, just like 10-HDAA and 10-HDA did. These data suggest that, like the ω-hydroxy acids, the blend of ω-1-hydroxy acids also arises by limited β-oxidation. Queens and workers must have a β-oxidation system able to chain-shorten ω– and ω-1-hydroxy acids, but this β-oxidation system differs between the castes. This difference manifests itself in different abilities to chain-shorten the 10-carbon acids and in different reversibilities of the desaturation step.

4.4 Interconversion between ω- and ω-1-hydroxy acids

The possibility that the ω-hydroxy acids can interconvert with their ω-1-isomers was investigated with D_2 9-HDA and D_2 10-HDA which were tested for incorporation into 10- and 9-HDA, respectively (Table III.9).

The data reveal that there was no interconversion between 9- and 10-HDA. If the conversion of ω-1-hydroxy acids to the ω-isomers were to occur by dehydration to the terminal alkenoic acid followed by rehydration, an anti-Markovnikov hydration would be required. This reaction is unlikely because it would preceed through a primary carbocation intermediate which is less stable than the secondary carbocation formed during a Markovnikov hydration (Carey and Sundberg, 1983). The opposite conversion, ω to ω-1, is more likely, but was not detected.

Table III.9. Check for isomerization between 9- and 10-HDA in queens and workers

caste	substrate	N	(% labelled) mean +/- S. E. product 9-HDA	10-HDA
queens (virgin)	D_2 9-HDA	8	----------	0.6 +/- 0.3
	blank	8		0.1 +/- 0.1
	D_2 10-HDA	8	0.1 +/- 0.1	----------
	blank	8	**3.7 +/- 1.4**	
workers	D_2 9-HDA	6	----------	0.9 +/- 0.8
	blank	6		1.0 +/- 0.5
	D_2 10-HDA	8	0.0 +/- 0.0	----------
	blank	8	0.0 +/- 0.0	

Percentage of labelled material found in glands treated with D_2 9-HDA or D_2 10-HDA. Except for the entry in boldface, there were no significant differences between treatments and blanks. In the case of the queen 9-HDA blanks, nonanedioic acid, which elutes before 9-HDA and has a 317 ion (the same as M-15 ion for D_2 labelled 9-HDA), overlapped with the front part of the 9-HDA peak.

The experiments described so far indicate that the biosynthesis of ω- and ω-1-functionalized acids starts with octadecanoic acid which is synthesized *de novo* from acetate and is incorporated into both types of functionalized acid. The hydroxy acids are derived from 9- and 10-HDAA by limited β-oxidation, and the keto- and diacids are formed from the corresponding hydroxy acids by oxidation. Finally, the pools of ω- and ω-1-functionalized acids appear to be separate.

III.5. Chain shortening of higher homologs

The conversion of octadecanoic acid to 9- and 10-HDAA requires at least two processes: hydroxylation and chain shortening to the 10-carbon length. These steps could occur in either order: octadecanoic acid (or its CoA ester) could be hydroxylated and the

resulting 18-carbon hydroxy acid, chain shortened, or octadecanoyl-CoA could be shortened to decanoyl-CoA which could be hydroxylated to 10-HDAA-CoA. The first experiment ruled out the possibility that free decanoic acid is hydroxylated, but not that decanoyl-CoA is. If decanoyl-CoA only formed by chain shortening of longer CoA esters, the second proposed pathway would still be consistent with the results from the first experiment.

If hydroxylation preceded chain shortening, then the glands should contain higher homologs of the mandibular acids and should be able to chain-shorten them. Thus, mandibular extracts from both castes were screened for 12-, 14-, 16- and 18-carbon homologs of the 10-carbon hydroxy acids by the method described in chapter II. The 12-carbon homologs were easily detected in both castes (20-50 ng). The 14- and 16-carbon compounds were present in trace amounts (< 5 ng); 18- and 17-hydroxyoctanoic acids were detectable in some samples (< 5 ng).

To determine whether 18- and 17-hydroxyoctadecanoic acids are chain shortened in the mandibular glands, D_1 18-OH C18:0 and D_3 17-OH C18:0 were assayed in both castes. In workers, D_2 16-OH C16:0 was also tested. Control runs with the corresponding 10-carbon homologs, 10-HDAA and 9-HDAA were done to assess whether the higher homologs can arise from the 10-carbon acids by elongation.

D_1 18-OH C18:0 was chain shortened to the 12-, 10- and 8-carbon length in both castes (Table III.10). Workers incorporated label from D_2 16-OH C16:0 into all the shorter hydroxy acids analyzed, and they converted D_2 10-HDAA to 10-HDA and 8-HOAA, as observed before. The 10-HDAA was also elongated to 12-OH C12:1, but not to 12-OH C12:0 or further.

D_3 17-OH C18:0 was chain shortened to the 10-carbon length in both castes (Table III.11). Queens also showed label in the 14-carbon homolog, but this result was probably due to a trace contamination of the substrate with 14-OH C14:0 (Chapter II, p 57).

Table III.10. Chain shortening and elongation of ω-hydroxy acids in workers and queens.

caste	treatment	(% labelled material) mean +/- S. E. [a] product 14-OH C14:0	12-OH C12:0	12-OH C12:1	10-HDAA	10-HDA	8-HOAA
workers	D_1 18-OH C18:0	**0.0 +/- 0.0**	**5.8 +/- 1.1**	**7.3 +/- 1.2**	4.5 +/- 1.1	**4.7 +/- 1.2**	**6.8 +/- 1.1**
	blank	2.0 +/- 0.8	1.9 +/- 1.2	2.9 +/- 0.9	2.5 +/- 0.7	1.2 +/- 0.6	1.7 +/- 0.9
	D_2 16-OH C16:0[b]	**17 +/- 1**	**20 +/- 2**	**49 +/- 5**	**9.3 +/- 1.9**	2.8 +/- 0.4 *	**12 +/- 2**
	blank	2.0 +/- 0.8	2.1 +/- 1.4	2.9 +/- 0.9	2.6 +/- 0.7	1.2 +/- 0.6	1.9 +/- 0.7
	D_2 10-HDAA	0.7 +/- 0.5	2.9 +/- 2.4	**12 +/- 4**	[**70 +/- 3**]	**0.3 +/- 0.1**	5.6 +/- 2.6 *
	blank	0.0 +/- 0.0	0.2 +/- 0.2	0.0 +/- 0.0	[0.0 +/- 0.0]	0.0 +/- 0.0	0.1 +/- 0.1
queens	D_1 18-OH C18:0	15 +/- 8 *	**5.5 +/- 1.0**	3.5 +/- 0.8 *	0.0 +/- 0.0	**1.9 +/- 0.5**	**1.0 +/- 0.3**
	blank	0.0 +/- 0.0	2.3 +/- 0.9	1.5 +/- 0.7	0.0 +/- 0.0	0.2 +/- 0.1	0.09 +/- 0.04

Percentage of labelled material found in glands treated with labelled ω-hydroxy acids of different chain length.
For workers, N = 10 and for queens N = 8.

[a] For entries in bold type, treatments and blanks differed significantly ($P<0.05$) by Tukey's test. For entries marked *, $P<0.07$.

[b] This substrate contained 34 % of D_1 and 23 % of D_2. Therefore, compounds were analyzed for the percentage of one and two D present and the values shown are the total of D_1 and D_2.

Table III.11. Chain shortening and elongation of ω–1-hydroxy acids in workers and queens.

caste	treatment	(% labelled material) mean +/- S. E. [a] product 13-OH C14:0	11-OH C12:0	11-OH C12:1	9-HDAA	9-HDA	7-HOAA
workers	D_3 17-OH C18:0	n. d.	2.5 +/- 1.3	**75 +/- 9**	7.8 +/- 2.5	**13 +/- 4**	46 +/- 23
	blank		1.5 +/- 1.1	5.2 +/- 2.9	5.6 +/- 3.7	3.8 +/- 0.9	2.1 +/- 1.4
	D_3 9-HDAA	n. d.	1.1 +/- 0.8	12 +/- 3	[**100 +/- 0**]	**24 +/- 3**	not determined
	blank		1.5 +/- 1.1	5.2 +/- 2.9	[5.6 +/- 3.7]	3.8 +/- 0.9	
queens	D_3 17-OH C18:0	**48 +/- 12**	**10 +/- 3**	6.6 +/- 1.1	2.5 +/- 0.9 *	0.9 +/- 0.5 *	5.2 +/- 2.7
	blank	1.4 +/- 1.4	0.6 +/- 0.6	3.6 +/- 1.6	0.5 +/- 0.5	0.1 +/- 0.1	1.9 +/- 1.0
	D_3 9-HDAA	0.0 +/- 0.0	0.0 +/- 0.0	0.7 +/- 0.7	[**100 +/- 0**]	0.8 +/- 0.3	3.8 +/- 3.8
	blank	1.4 +/- 1.4	0.6 +/- 0.6	3.6 +/- 1.6	[0.5 +/- 0.5]	0.1 +/- 0.1	1.9 +/- 1.0
	D_2 9-HDA	0.0 +/- 0.0	0.0 +/- 0.0	0.0 +/- 0.0	0.6 +/- 0.6	[**27 +/- 9**]	16 +/- 9
	blank	0.3 +/- 0.2	0.6 +/- 0.6	0.0 +/- 0.0	0.0 +/- 0.0	[0.0 +/- 0.0]	0.1 +/- 0.1

Percentage of labelled material found in glands treated with labelled ω–1-hydroxy acids of different chain length.
For workers, N = 10 and for queens N = 8. Bracketed entries correspond to applied substrates.
n. d. = not detected

[a] For entries in bold type, treatments and blanks differed significantly ($P<0.05$) by Tukey's test. For entries marked *, $P<0.07$.

However, some label was incorporated into 11-OH C12:0 in queens. Neither caste showed significant incorporation into 7-HOAA. Furthermore, 9-HDA and 9-HDAA were not elongated in either caste.

D_3 17-OH C18:0 was incorporated into ODA (Table III.12). Given that ODA is derived from 9-HDA, this result confirms that 17-OH C18:0 is chain shortened to 9-HDA. Incorporation of D_3 17-OH C18:0 into ODA was significant, while the analogous incorporation of D_1 18-OH C18:0 into C10:1 DA was not. The ODA was D_2 and the diacids were not labelled because one deuterium from the corresponding substrates was lost during the oxidation. Two processes are required to convert the 18-carbon hydroxy acids to the 10-carbon oxidized products: chain shortening and hydroxy group oxidation. The data reveal the expected labelling pattern for the two processes combined, for both substrates, which confirms the validity of the deuterium labelling method used.

Table III.12. Incorporation of 17- and 18-hydroxyoctadecanoic acids into the 10-carbon keto acid and diacid in mated queens.

		substrate			
		D_3 17-OH C18:0		D_1 18-OH C18:0	
queen (mated)	tr.	**3.0 +/- 1.2**	(8)	4.3 +/- 2.1	(8)
	bl.	0.5 +/- 0.3	(8)	3.8 +/- 0.6	(8)

Values represent the mean +/- S. E. of the percentage of labelled ODA (from 17-OH C18:0) and C10:1 DA (from 18-OH C18:0). The bold entry is significantly different from the blank Tukey, $P < 0.06$.

The amounts of labelled ω- and ω-1-hydroxy acids that formed from D_1 18-OH C18:0 and D_3 17-OH C18:0, respectively, were estimated and are shown in Figure III.4. Queens chain shortened 18- and 17-OH C18:0, the former mostly to 8-HOAA and the latter mostly to 9-HDA. Workers chain shortened 18-OH C18:0 mostly to 10-HDA, but 17-OH C18:0 gave rise to very low quantities of chain shortened product. Thus, even when provided with 17-OH C18:0, workers exhibited very low synthesis of 9-HDA. These data suggest that queens and workers have different specificities in β-oxidation and that queens

are able to chain-shorten both, ω- and ω-1-hydroxy acids, while the workers preferentially chain-shorten ω-hydroxy acids to the 10-carbon length. It is not clear whether workers have a very low chain shortening activity for ω-1-hydroxy acids, or whether they degrade 17-OH C18:0 beyond the 8-carbon length.

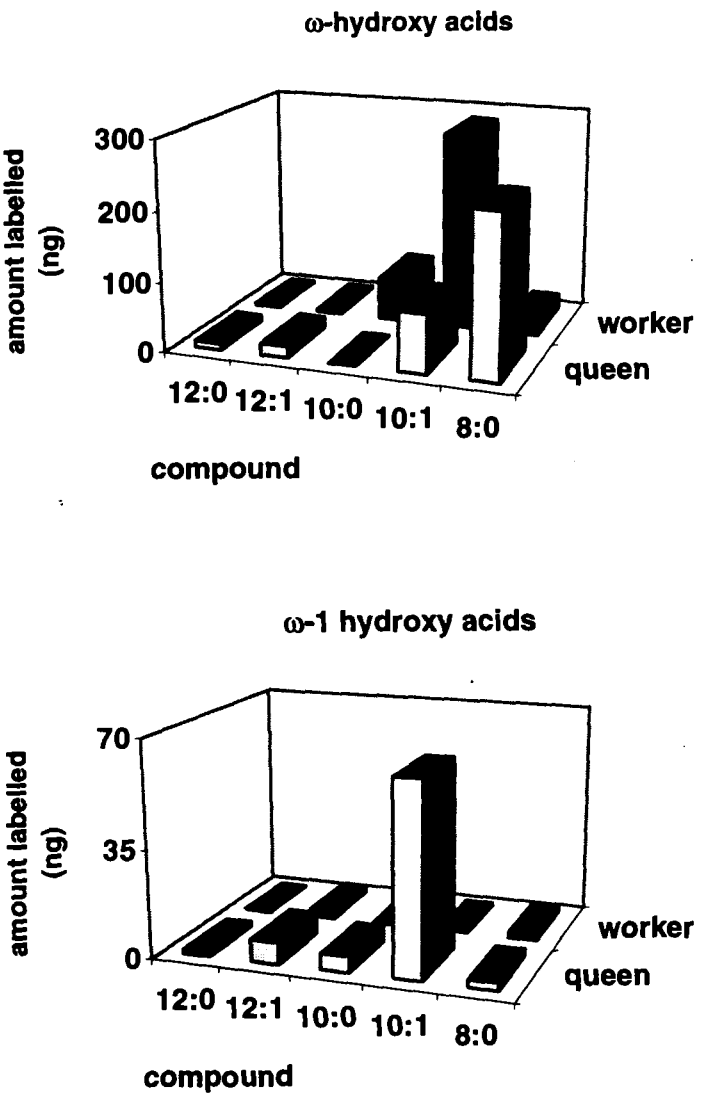


Figure III.4. Amounts of labelled ω- and ω-1-hydroxy acids formed from D_1 18-OH C18:0 and D_3 17-OH C18:0, respectively. The abbreviations indicate the chain-length of the hydroxy acids and the presence (:1) or absence (:0) of an (E)2-double bond.

To summarize, worker and queen mandibular extracts contain higher homologs of the 10-carbon hydroxy acids. These homologs arise from 18- and 17-octadecanoic acid by chain shortening, not from the 10-carbon hydroxy acids by chain elongation. Thus, hydroxylation of octadecanoic acid at the 18^{th} and 17^{th} position is most likely the first step in the conversion of octadecanoic acid to the 10-carbon hydroxy acids. This is followed by β-oxidation which differs in queens and workers.

III.6 Order of the steps in the pathway

To verify that hydroxylation precedes β-oxidation in the conversion of octadecanoic acid to the 10-carbon hydroxy acids, workers and queens were treated with labelled octadecanoic acid and 2-fluorooctadecanoic acid (2-F C18:0), an inhibitor of β-oxidation. The CoA ester of 2-F C18:0 is a competitive inhibitor of acyl CoA dehydrogenase (Fendrich and Abeles, 1982; Rosell *et al.* 1992). If hydroxylation occurs before chain shortening, the inhibited samples should show incorporation of label into the 18-carbon hydroxy acids. If hydroxylation occurs after chain shortening and the 18-carbon hydroxy acids arise by chain elongation from shorter homologs, no labelled 18-carbon hydroxy acids should be present in the inhibited samples.

6.1 Workers

Workers incorporated D_1 C18:0 into the 8- and 12-carbon ω-hydroxy acids and 18-OH C18:0 (Table III.13, Figure III.5), which indicates that the glands were able to incorporate the substrate in the absence of a β-oxidation inhibitor. The 14-OH C14:0 showed no incorporation of label; 16-OH C16:0 was not detected. In the presence of 2-F C18:0, there was no incorporation into 12-OH C12:1 or any of the shorter hydroxy acids, which suggests that β-oxidation was inhibited from the 12-carbon length onward. The 18-OH C18:0 was labelled in both treatments.

Table III.13. Incorporation of label from 12-D_1 octadecanoic acid into ω-hydroxy acids in workers, in the absence and presence of a β-oxidation inhibitor (2-fluorooctadecanoic acid).

treatment	N	(% labelled material) mean +/- S.E. [a] product			
		18-OH C18:0	14-OH C14:0	12-OH C12:0	12-OH C12:1
D_1 C18:0	8	**11 +/- 4**	7.6 +/- 2.5	**15 +/- 4**	**24 +/- 6**
blank	8	0.0 +/- 0.0	6.3 +/- 1.2	3.0 +/- 0.9	1.3 +/- 0.8
D_1 + 2-F C18:0	8	**32 +/- 8**	3.8 +/- 1.7	**13 +/- 1**	0.8 +/- 0.8
2-F C18:0	8	1.9 +/- 1.9	3.3 +/- 1.3	5.8 +/- 0.4	1.1 +/- 1.1
		(% labelled material) mean +/- S.E. [a] product			
		10-HDAA	10-HDA	3,10di-OH C10	8-HOAA
D_1 C18:0	8	**8.5 +/- 1.2**	**10 +/- 3**	**5.3 +/- 2.4**	**4.9 +/- 0.3**
blank	8	1.1 +/- 0.7	0.4 +/- 0.4	0.0 +/- 0.0	2.2 +/- 0.8
D_1 + 2-F C18:0	8	0.9 +/- 0.6	2.2 +/- 1.1	3.2 +/- 1.0	4.0 +/- 0.3
2-F C18:0	8	0.0 +/- 0.0	3.3 +/- 1.3	4.4 +/- 0.7	5.6 +/- 0.9

[a] For entries in bold type, treatments and blanks differed significantly ($P<0.05$, Tukey).

Incorporation of label into 18-OH C18:0 in the inhibited treatment indicates that hydroxylation precedes β-oxidation. The observations that inhibition with 2-F C18:0 manifested itself from the 12-carbon length onward and that the 14-OH C14:0 did not show incorporation of label, suggests that β-oxidation of 18-OH C18:0 to 12-OH C12:0 is tightly coupled with little release or exchange of intermediates. Because of this, 2-F C18:0 does not interfere with chain shortening of 18-OH C18:0 to the 12-carbon length. At that point a new set of β-oxidation enzymes, specific for shorter chain lengths, may continue the chain shortening process, which would make it possible for 2-F C18:0-CoA to compete with 12-OH C12:0-CoA and, therefore, inhibit further β-oxidation of the hydroxy acid.

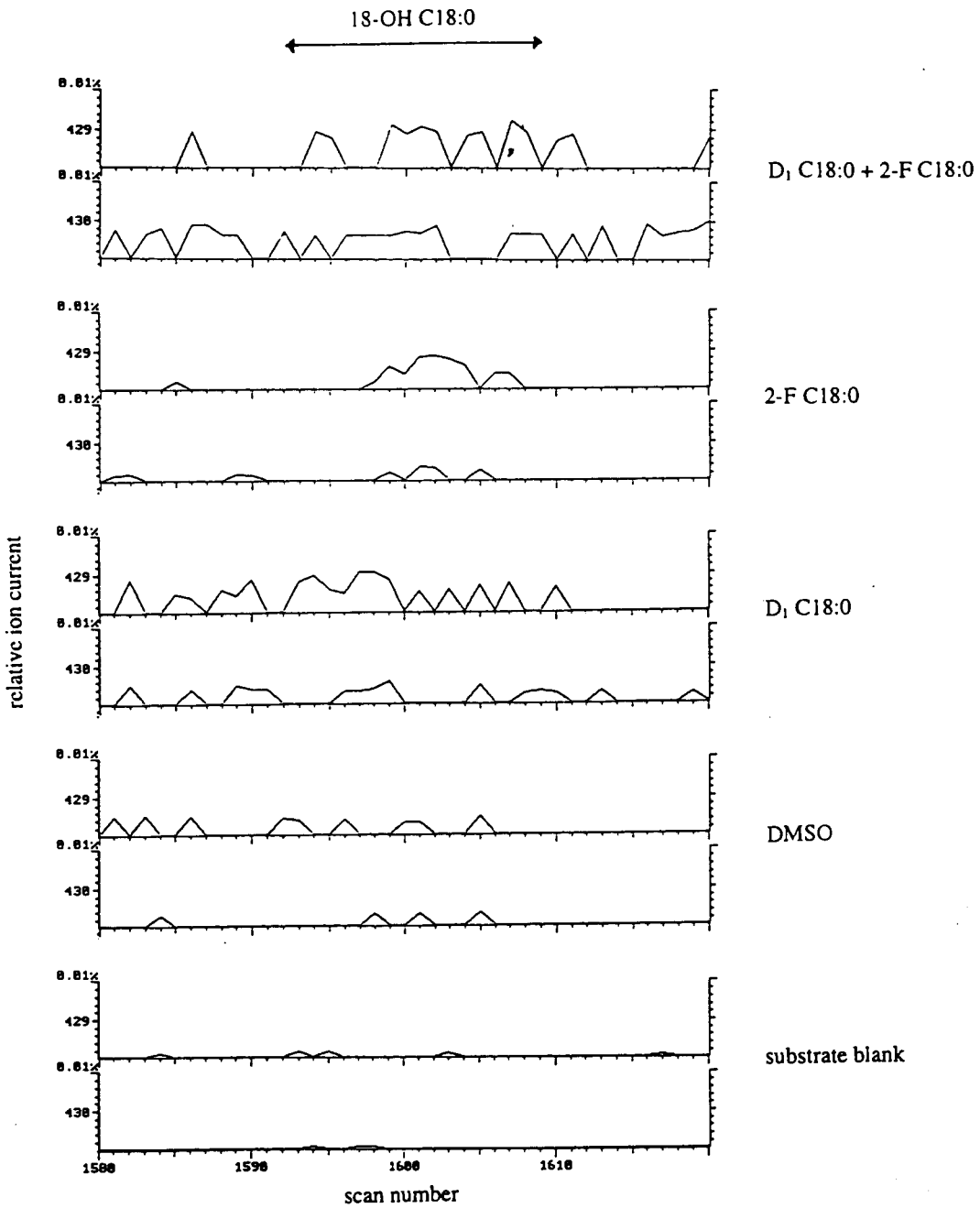


Figure III.5. Single ion displays at the retention time of 18-OH C18:0 from the GC-MS traces of worker mandibular extracts. The glands were treated with 2-F C18:0 and D_1 C18:0, 2-F C18:0, D_1 C18:0 and DMSO. The substrate blank for D_1 C18:0 is also shown. The ions displayed correspond to the M-15 ion of labelled (430) and unlabelled (429) 18-OH C18:0.

The results for the ω-1-hydroxy acids were similar (Table III.14), except that labelling of 17-OH C18:0 in the inhibited treatments was not significantly different from the blanks. This may have been due to dilution of the labelled substrate with 2-F C18:0 (which may also get hydroxylated). Nevertheless, some labelled 17-OH C18:0 was visible in some of the samples. The 9-HDAA showed some label in the inhibited runs, which could be due either to incomplete inhibition or to an artifact. Since the blanks also showed some apparent labelling, an artifact may have been present at the retention time of 9-HDAA Furthermore, 11-OH C12:1 and 9-HDA did not incorporate label in the inhibited runs, which means that inhibition was successful. As with the ω-hydroxy acids, the substrate was incorporated into the 12- and 10-carbon hydroxy acids in the absence of inhibitor and the 17-hydroxy acid was labelled in both treatments. Inhibition by 2-F C18:0 manifested itself from 11-OH C12:0 onward.

The experiment was repeated with D_3 C18:0 as the substrate, and similar results were obtained (Table III.15). The solution of D_3 C18:0 and 2-F C18:0 used for the inhibited treatments was too viscous for application and had to be diluted 2X. This may explain the low percentage of label in 18-OH C18:0 in the inhibited treatment. The amounts of labelled 9-HDA, 10-HDAA and 10-HDA formed from both substrates in the non-inhibited treatments were estimated from the percentage of labelled and the total amount of these acids (Table III.16).

The amount of each compound formed from both substrates was similar, which suggests that the terminal deuterium in D_3 C18:0 did not interfere with incorporation of this substrate into ω-1-hydroxy acids. Furthermore, the labelled materials formed in approximately the same ratio as the total acids present, namely, 9-HDA:10-HDAA:10-HDA, 1:40:110. This indicates that the pathway was not affected by the addition of exogenous C18:0.

Table III.14. Incorporation of label from 12-D_1 octadecanoic acid into ω–1-hydroxy acids in workers, in the absence and presence of a β-oxidation inhibitor (2-fluorooctadecanoic acid).

treatment	N	(% labelled material) mean +/- S.E. [a] product					
		17-OH C18:0	13-OH C14:0	11-OH C12:0	11-OH C12:1	9-HDAA	9-HDA
D_1 C18:0	8	**63 +/- 7**	0.7 +/- 0.7	**19 +/- 3**	**41 +/- 11**	9.0 +/- 4.3 *	**14 +/- 2**
blank	8	15 +/- 8	0.6 +/- 0.6	0.0 +/- 0.0	2.1 +/- 1.0	0.0 +/- 0.0	2.7 +/- 1.4
D_1 C18:0/2-F C18:0	8	27 +/- 12	0.0 +/- 0.0	**16 +/- 3**	1.1 +/- 1.1	11 +/- 4 *	5.9 +/- 2.4
2-F C18:0	8	5.3 +/- 3.0	0.0 +/- 0.0	1.8 +/- 0.9	0.0 +/- 0.0	3.4 +/- 1.0	4.1 +/- 1.6

Table III.15. Incorporation of label from 18,18,18-D_3 octadecanoic acid into ω-hydroxy acids in workers, in the absence and presence of a β-oxidation inhibitor (2-fluorooctadecanoic acid).

treatment	N	(% labelled material) mean +/- S.E. [a] product					
		18-OH C18:0	14-OH C14:0	12-OH C12:0	12-OH C12:1	10-HDAA	10-HDA
D_3 C18:0	8	**13 +/- 6**	18 +/- 8 *	**3.7 +/- 1.4**	**4.2 +/- 1.4**	**5.3 +/- 1.4**	**0.5 +/- 0.1** [b]
blank	8	0.0 +/- 0.0	2.6 +/- 1.2	0.0 +/- 0.0	0.0 +/- 0.0	0.0 +/- 0.0	0.0 +/- 0.0 [b]
D_3 C18:0/2-F C18:0	8	1.0 +/- 0.5	4.6 +/- 1.7	0.0 +/- 0.0	0.0 +/- 0.0	0.0 +/- 0.0	0.0 +/- 0.0 [b]
2-F C18:0	8	0.0 +/- 0.0	1.9 +/- 0.7	0.0 +/- 0.0	0.0 +/- 0.0	0.0 +/- 0.0	0.0 +/- 0.0 [b]

[a] For entries in bold type, treatments and blanks differed significantly ($P<0.05$, Tukey); for entries marked *, $P<0.07$.
[b] Analyzed by only using the front of the GC-MS peak to calculate the percentage labelled material.

Table III.16. Amount of labelled hydroxy acids formed from D_1 and D_3 C18:0 in workers.

treatment	N	(amount of labelled material (ng)) mean +/- S. E. [a] product		
		9-HDA	10-HDAA	10-HDA
D_1 C18:0	8	**3.7 +/- 0.6**	42 +/- 8	194 +/- 94
blank	8	0.9 +/- 0.5	17 +/- 12	24 +/- 24
D_3 C18:0	8	**1.8 +/- 0.3**	**49 +/- 17**	**128 +/- 55**
blank	8	0.2 +/- 0.2	0.0 +/- 0.0	0.0 +/- 0.0
total (μg)[b]	8	0.04 +/- 0.01	1.6 +/- 0.2	4.2 +/- 0.5

[a] For entries in bold type, treatments and blanks differed significantly ($P<0.05$, Tukey).
[b] Total amount of material/pair of glands for workers of the same age.

6.2 queens

An experiment with D_1 C18:0 and 2-F C18:0 was undertaken with mated queens. The results are shown in Table III.17.

In the non-inhibited treatment, label was incorporated into the 14- and 10-carbon acids, which indicates that the glands used for the experiment were active. The inhibited treatments showed label in 17-OH C18:0, the 14-carbon acids and 9-HDAA. Neither treatment gave labelled 12-carbon hydroxy acids. Furthermore, the inhibited samples showed a significant increase in the total amount of 14- and 12-carbon hydroxy acids (data not shown). This accumulation may be due to an inhibition of β-oxidation from 9-HDAA onward. The observation that the labelling patterns were parallel for both treatments from 13-OH C14:0 to 9-HDAA also supports the idea that β-oxidation from 17-OH C18:0 to 9-HDAA is tightly coupled with little exchange of intermediates. The last cycle of β-oxidation from 9-HDAA to 9-HDA and further to 7-HOAA may be completed by a different set of enzymes.

Table III.17. Incorporation of label from 12-D_1 octadecanoic acid into ω–1-hydroxy acids in queens, in the absence and presence of a β-oxidation inhibitor (2-fluorooctadecanoic acid).

treatment	(% labelled material) mean +/- S.E. [a] product			
	17-OH C18:0	13-OH C14:0	13-OH C14:1	11-OH C12:0
D_1 C18:0	0.0 +/- 0.0	**7.7 +/- 1.9**	0.0 +/- 0.0	4.9 +/- 1.7
D_1 C18:0/2-F C18:0	**5.9 +/- 2.5**	**6.3 +/- 0.4**	1.8 +/- 0.9 *	2.9 +/- 1.2
blank	0.0 +/- 0.0[c]	1.5 +/- 0.8	0.0 +/- 0.0	2.3 +/- 0.9
	(% labelled material) mean +/- S.E. [a] product			
	11-OH C12:1	9-HDAA	9-HDA	ODA
D_1 C18:0	1.7 +/- 0.5	**2.3 +/- 0.7**	**1.8 +/- 0.7**	5.7 +/- 1.8[b]
D_1 C18:0/2-F C18:0	0.9 +/- 0.6	**2.9 +/- 0.6**	0.7 +/- 0.5[c]	2.1 +/- 0.8
blank	1.4 +/- 0.7	0.0 +/- 0.0	0.0 +/- 0.0	2.7 +/- 0.9

[a] For bold entries, treatments and blanks differed significantly ($P<0.05$, Tukey); for entries marked *, $P<0.07$.
[b] The amount of labelled material was significantly higher than in the blanks.
N = 8, except for entries marked [c], for which N = 7.

The 18-carbon hydroxy acids were more difficult to detect in queens than in workers, which may reflect a faster utilization of these compounds in queens. Thus, 17-OH C18:0 was probably formed in the non-inhibited runs, but was chain shortened immediately and, hence did not accumulate. In the inhibited runs, some labelled 17-OH C18:0 accumulated. A similar effect was observed for 18-OH C18:0 (Table III.8): it accumulated only in the inhibited runs. The 14-carbon ω-hydroxy acids were present at trace levels and were not analyzed. The only other ω-hydroxy acid that incorporated label in the inhibited treatments was 12-OH C12:1.

Table III.18. Incorporation of label from 12-D_1 octadecanoic acid into ω-hydroxy acids in queens, in the absence and presence of a β-oxidation inhibitor (2-fluorooctadecanoic acid).

treatment	(% labelled material) mean +/- S.E. [a] product			
	18-OH C18:0	12-OH C12:0	12-OH C12:1	10-HDAA
D_1 C18:0	7.8 +/- 5.4	1.0 +/- 0.9	2.1 +/- 0.8	0.4 +/- 0.4
D_1 C18:0/2-F C18:0	18 +/- 5 *	2.3 +/- 1.2	**3.8 +/- 0.8**	0.0 +/- 0.0
blank	7.2 +/- 4.2	2.3 +/- 0.9	1.5 +/- 0.7	0.0 +/- 0.0

[a] For bold entries, treatments and blanks differed significantly ($P<0.05$, Tukey); for entries marked *, $P<0.07$. N = 8 for all treatments.

The amounts of labelled 9-HDAA, 9-HDA, ODA and 10-HDAA formed in the non-inhibited treatment were estimated and are shown in Table III.19. As in the workers, the amounts of labelled material paralleled the total amounts suggesting that the queen pathway is not affected by the addition of exogenous octadecanoic acid.

Table III.19. Amount of labelled mandibular acids formed from D_1 C18:0 in queens.

treatment	(amount of labelled material (ng)) mean +/- S. E.[a] product			
	9-HDAA	9-HDA	ODA	10-HDAA
D_1 C18:0	15 +/- 9	**269 +/- 94**	**1880 +/- 370**	0.9 +/- 0.9
blank	0.0 +/- 0.0	0.0 +/- 0.0	560 +/- 370	0.0 +/- 0.0
total (μg)[b]	1.2 +/- 0.4	27 +/- 7	50 +/- 12	1.3 +/- 0.3

[a] For entries in bold type, treatments and blanks differed significantly ($P<0.05$, Tukey).
[b] Amount of acid found in one gland from queens of the same age. N = 8 for all treatments.

The incorporation of label into the 18-carbon hydroxy acids shows that hydroxylation precedes β-oxidation in the conversion of octadecanoic acid to the 10-carbon

hydroxy acids in both castes. Furthermore, the different patterns of inhibition of β-oxidation by 2-F C18:0 in queens and workers are consistent with the hypothesis that the castes have different specificities in β-oxidation. Finally, workers and queens produced labelled ω- and ω-1-hydroxy acids in the ratio found in their normal blends which indicates that the exogenous octadecanoic acid did not alter the caste-specificity of the pathway.

III.7 The functionalization reaction

The hydroxylation reaction is the first point at which the key structural difference between the caste-specific compounds, the ω- and ω-1-hydroxy group, is introduced. Thus, the mechanism of this important step was investigated using substrates labelled at or next to the hydroxylation site and determining the loss or retention of label. Hydroxylation can occur by two routes (Figure III.6): direct introduction of the hydroxy group by a cytochrome-P450 fatty acid hydroxylase (1-center reaction) or by desaturation, followed by hydration of the double bond (2-center reaction).

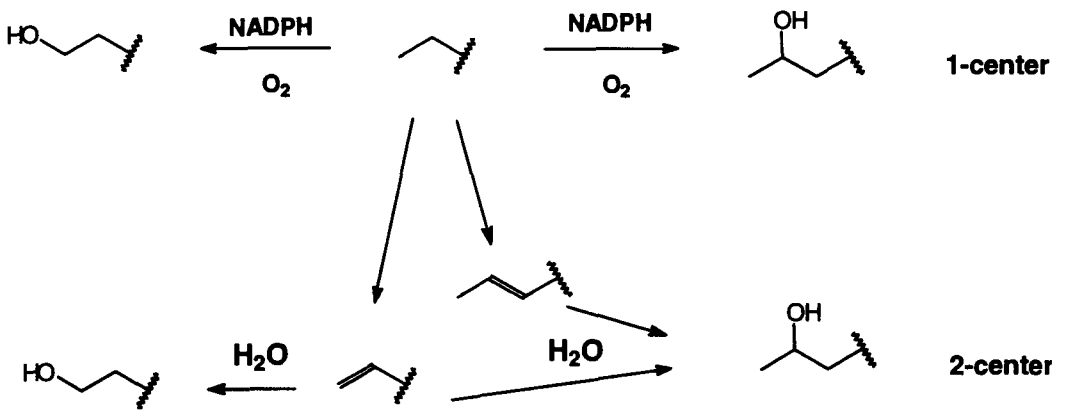


Figure III.6. Possible routes of hydroxylation.

To distinguish the two routes of hydroxylation for the ω-hydroxy acids, worker glands were treated with 18,18,18,17,17-D_5 C18:0. If hydroxylation takes place by a 1-center reaction, the products should be D_4 and if the 2-center route is operative, the products should be D_3. The results are shown in Table III.20.

Table III.20. Hydroxylation at the ω-position in workers

treatment	(% labelled material) mean +/- S. E. [a] product			
	10-HDAA		10-HDA[b]	
labelling pattern	D_3	D_4	D_3	D_4
D_5 C18:0	0.0 +/- 0.0	**4.1 +/- 1.3**	0.02 +/- 0.01	**0.33 +/- 0.11**
blank	0.0 +/- 0.0	0.2 +/- 0.1	0.00 +/- 0.00	0.04 +/- 0.01
D_5 C18:0/2-F C18	0.0 +/- 0.0	0.4 +/- 0.2	0.00 +/- 0.00	0.02 +/- 0.02
2-F C18	0.0 +/- 0.0	0.1 +/- 0.1	0.00 +/- 0.00	0.04 +/- 0.02

[a] For entries in bold type, treatments and blanks differed significantly ($P<0.05$, Tukey).
[b] Analyzed by only using the front of the GC-MS peak to calculate the % labelled material.
N = 8 for all treatments.

Incorporation of 4 D into 10-HDAA and 10-HDA was significant; incorporation of 3 D was not. This suggests that hydroxylation at the ω-position occurs by a 1-center reaction, possibly catalyzed by a cytochrome P450. Labelled substrate was incorporated into 10-HDAA and 10-HDA only in the absence of 2-F C18:0 which indicates that incorporation of the D_5 C18:0 occurred by the same pathway as that of D_1 and D_3 C18:0 in previous experiments and that the results were not due to impurities in the D_5 C18:0.
To verify the previous result, worker glands were perfused with D_3 C18:1 and D_3 C10:1Δ^9. No incorporation of terminal alkenoic acids was observed (Table III.21), which confirms that ω-hydroxylation does not proceed by hydration of a terminal double bond.

Table III.21. Assay of terminal alkenoic acids for incorporation into ω- hydroxy acids.

treatment	(% labelled) mean +/- S. E. product		
	8-HOAA	10-HDAA	10-HDA
D_3 C18:1Δ^{17}	2.3 +/- 1.9	0.0 +/- 0.0	0.4 +/- 0.3
D_3 C10:1Δ^{9}	2.5 +/- 1.5	1.7 +/- 0.3	5.8 +/- 4.1
blank	0.4 +/- 0.5	0.0 +/- 0.0	4.7 +/- 2.1

None of the treatments differed significantly from the blank ($P<0.05$)
N = 4 for all treatments

To distinguish the hydroxylation routes for the ω-1-hydroxy acids, worker glands were perfused with D_3 C18:0 and the 9-HDA was analyzed. If hydroxylation involves a terminal double bond, the product should be D_2 and if hydroxylation is direct or involves an ω-2/ω-1 double bond, the product should be D_3. Worker glands were chosen because in queen glands, the large amount of endogenous material made analysis for the two labelling patterns difficult. The results are shown in Table III.22.

Table III.22. Hydroxylation at the ω–1-position in workers

treatment	(% labelled material) mean +/- S. E. [a] product 9-HDA	
labelling pattern	D_2	D_3
D_3 C18:0	2.1 +/- 1.2	**12 +/- 1**
blank	0.0 +/- 0.0	0.9 +/- 0.6
D_3 C18:0/2-F C18	0.0 +/- 0.0	3.2 +/- 0.8
2-F C18	0.0 +/- 0.0	4.0 +/- 1.0

[a] For entries in bold type, treatments and blanks differed significantly ($P<0.05$, Tukey).
N = 8 for all treatments.

Incorporation of 3 D into 9-HDA was significant and incorporation of 2 D was not. Therefore, ω-1-hydroxylation does not involve a terminal double bond. To verify this

assertion, worker and queen glands were perfused with D_3 C18:1. Neither showed a significant incorporation of label into 9-HDA (Table III.23).

Table III.23. Assay of terminal alkenoic acids for incorporation into 9-HDA

treatment	queens [1]	workers [1]
D_3 C18:1 Δ^{17}	0.0 +/- 0.0	10 +/- 2
blank	0.0 +/- 0.0	5.4 +/- 1.9

[1] Percentage of labelled material. Mean +/- S. E.
None of the treatments differed significantly from the blank ($P<0.05$).
N = 4 for all treatments.

To conclude, the biosynthetic pathway of the acids found in the mandibular glands of queens and workers starts with the hydroxylation of octadecanoic acid at the 17th and 18th position. The resulting 18-carbon ω-1- and ω- hydroxy acids are chain shortened to the 10- and 8-carbon length. The 10-carbon hydroxy acids are further oxidized to keto- and diacids (Figure III.7). In experiments with labelled octadecanoic acid, workers produced more of the ω-hydroxy acids and queens more of the ω-1-hydroxy acids.

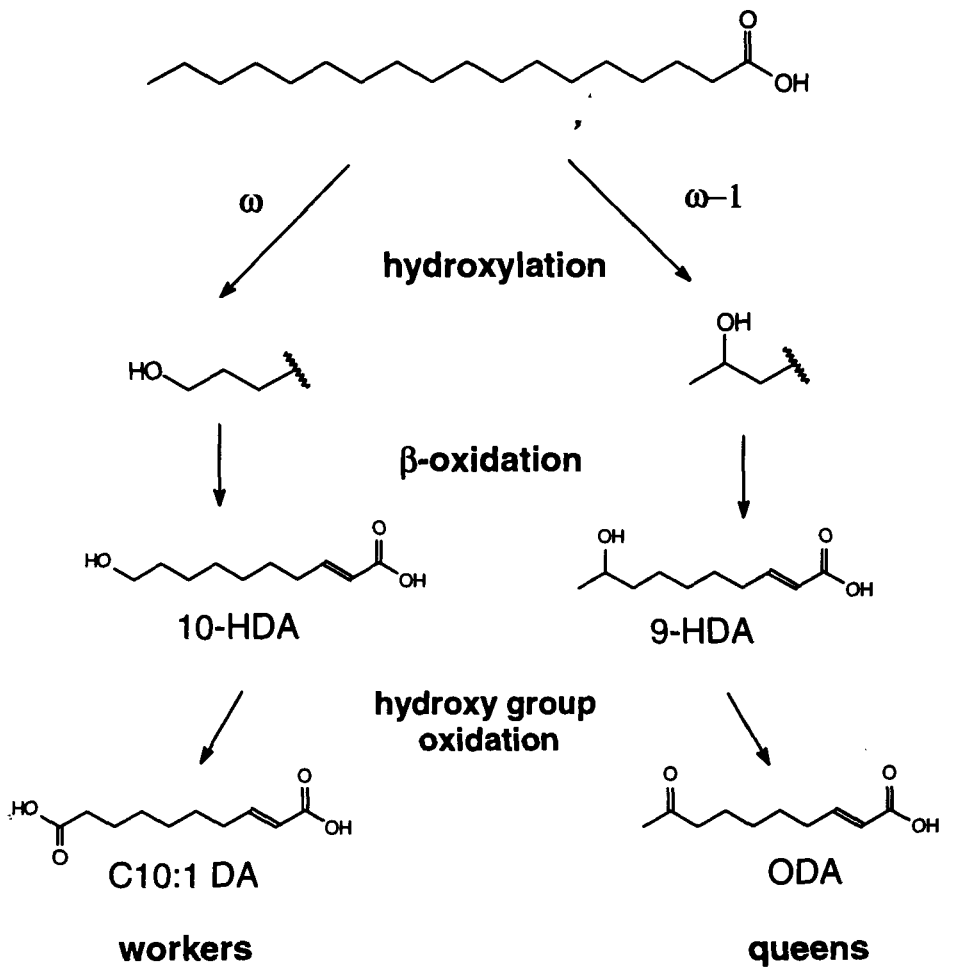


Figure III.7. Biosynthetic pathway of the mandibular acids in workers and queens. Both castes synthesize both types of acid, but workers produce mainly 10-carbon ω-functionalized acids and queens produce 10-carbon ω-1-functionalized acids.

Chapter IV: Discussion

IV.1. Rates of biosynthesis of functionalized acids in both castes

The rates of mandibular acid biosynthesis can be estimated from the quantities of labelled material formed in the experiments previously described. These rates are composites which cover several steps such as substrate uptake and conversion of FFA to CoA esters. The error in these estimates is large because of variablilty in substrate uptake, enzyme levels and reaction volumes between glands. However, a comparison between the overall rate of product formation from C18:0 and the rates for the individual steps is valid within the limits of error because the same method was used for all the experiments. The objectives of the rate analysis were to find the fastest and slowest steps and to determine whether rates estimated for the individual steps were consistent with the overall rate of product formation from C18:0.

1.1 Biosynthesis of ω-functionalized acids in workers

Table IV.1. Rates of incorporation of labelled substrates into ω-functionalized acids in workers.

substrate	product	N	glands/rep.	perfusion time (min)	amount of product (ng) [1]	rate (ng/min) [1]
D_3 C18:0 [2]	10-HDA	8	2	10	130 +/- 60	13 +/- 6
	10-HDAA				49 +/- 17	4.9 +/- 1.7
D_1 18-OH C18:0	10-HDA	10	1	5	280 +/- 90	57 +/- 17
	10-HDAA				62 +/- 20	12 +/- 4
D_3 10-HDAA	10-HDA	9	1	15	9.2 +/- 2.8	0.6 +/- 0.2
	C10:0 DA				470 +/- 90	30 +/- 6
D_2 10-HDA	8-HOAA	8	1	20	83 +/- 14	4.1 +/- 0.7
	C10:1 DA				600 +/- 130	30 +/- 7

[1] Mean +/- S. E.

[2] The amounts of labelled 10-HDA and 10-HDAA formed from D_1 C18:0 were similar (Table III.16).

The rates of formation of 10-HDAA and 10-HDA from labelled C18:0 and from D_1 18-OH C18:0 and the rate of diacid formation from labelled 10-HDAA and 10-HDA are shown in Table IV.1. Rates in nmol/min/gland for β-oxidation, the last round of β-oxidation and hydroxy group oxidation were estimated directly from the data in Table IV.1 and the rate of hydroxylation was estimated from the accumulation of labelled ω-hydroxy acids in the presence of 2-F C18:0 and D_1 C18:0. Inhibition of β-oxidation in the presence of 2-F C18:0 manifested itself from the 12-carbon length onward (section III.6), so the amounts of labelled 18-OH C18:0 and 12-OH C12:0 were added to obtain an estimate of the total amount of octadecanoic acid hydroxylated during the perfusion[1] (Table IV.2).

To assess how the rates of the three steps scale relative to each other, the rate of product formation was divided by the substrate titer in a young worker (Table IV.2). However, because the actual substrate concentration at the reaction site is not known, this approach gives a rough estimate, and only values one or more orders of magnitude apart can be considered different. The rate constant for β-oxidation is larger than the constant for hydroxylation, which suggests that β-oxidation is faster than hydroxylation. This is consistent with the observation that the 10-carbon hydroxy acids accumulate and 18-OH C18:0 does not. The low values for the last desaturation and for the last round of β-oxidation suggest that the rate of β-oxidation slows down once the 10-carbon length has been reached. Hydroxy group oxidation is slower than β-oxidation, which may explain why the diacids are not the major products of the pathway.

[1] The amount of labelled 18-OH C18:0 formed from D_1 C18:0 in 10 min was 5.0 +/- 1.5 ng (1.7×10^{-2} nmol) and the amount of 12-OH C12:0 was 23.7 +/- 5.6 ng (0.11 nmol). Since all the 12-OH C12:0 came from 18-OH C18:0, the total 18-OH C18:0 formed during the perfusion of two glands must have been 0.13 nmol, giving a rate of 6.3×10^{-3} nmol/min/gland.

Table IV.2. Rate constants for the three steps in the biosynthetic pathway of ω-functionalized acids in workers.

process	substrate	titer of substrate (ng/gland) [1]	titer of substrate (nmol/gland)	rate of product formation (nmol/min/ gland)	rate constant (min^{-1})
hydroxylation	C18:0	120 +/- 4 [12]	4.2×10^{-1}	6.3×10^{-3}	1.5×10^{-2}
β-oxidation[3]	18-OH C18:0	16 +/- 3 [8] [2]	5.4×10^{-2}	3.7×10^{-1}	6.8
last desat. [4]	10-HDAA	508 +/- 23 [8]	2.7	3.3×10^{-3}	1.2×10^{-3}
last β-ox. [5]	10-HDA	1460 +/- 340 [8]	7.8	8.5×10^{-3}	1.1×10^{-3}
OH gr. ox. [6]	10-HDA	1460 +/- 340 [8]	7.8	1.5×10^{-1}	1.9×10^{-2} [7]
	10-HDAA	508 +/- 23 [8]	2.7	1.5×10^{-1}	5.5×10^{-2} [7]

[1] Amount per gland in 2 day old untreated workers. The number of replicates is indicated in brackets after each entry.
[2] Total amount of 18-OH C18:0 found in an experiment with 2-F C18:0. In samples not treated with 2-F C18:0, this compound was often not detectable.
[3] To 10-HDA and 10-HDAA (total).
[4] Desaturation of 10-HDAA to 10-HDA.
[5] Shortening of 10-HDA to 8-HOAA.
[6] Hydroxy group oxidation.
[7] The constant for the combined formation of C10:0 DA and C10:1 DA is 2.9×10^{-2} min^{-1}.

To compare the rate of the three steps with the overall rate of mandibular acid formation from C18:0, the increase in the amount of hydroxy acids and diacids in workers was calculated using the rate constants in Table IV.2. Hydroxylation was assumed to provide a constant amount of 18-OH C18:0 (S) for β-oxidation. Thus, the amount (nmol) of 10-carbon hydroxy acid formed by β-oxidation during a time interval Δt is

$$\Delta P_\beta = 6.8 \times S \times \Delta t,$$

where S = (rate of hydroxylation)*Δt. Some of the hydroxy acid formed by β-oxidation is continuously removed by hydroxy group oxidation and by chain shortening to 8-HOAA, so the amount of hydroxy acid (10-HDA + 10-HDAA) formed during time Δt is

$$\Delta P_{OH} = \Delta P_\beta - \Delta P_{OX} - \Delta P_8,$$

where the amount of 10-carbon hydroxy acid chain shortened to 8-HOAA is $\Delta P_8 = 0.0011 \times \Delta P_\beta \times \Delta t$ and the amount oxidized to diacid is ΔP_{OX}. The amount of

hydroxy acid available for oxidation is not known, so two alternative hypotheses were tested using models: all the accumulated hydroxy acid is available for oxidation (model I), or only the newly formed hydroxy acid (ΔP_β) is available for oxidation (model II). Thus, for model I, the amount of hydroxy acid oxidized is $\Delta P_{OX\,i} = 0.029 \times P_{OH\,i} \times \Delta t$, where $P_{OH\,i} = P_{OH\,i-1} + \Delta P_{\beta\,i}$, the total hydroxy acid accumulated from the start to the i^{th} time interval. For model II the amount of hydroxy acid oxidized is $\Delta P_{OX} = 0.029 \times \Delta P_\beta \times \Delta t$.

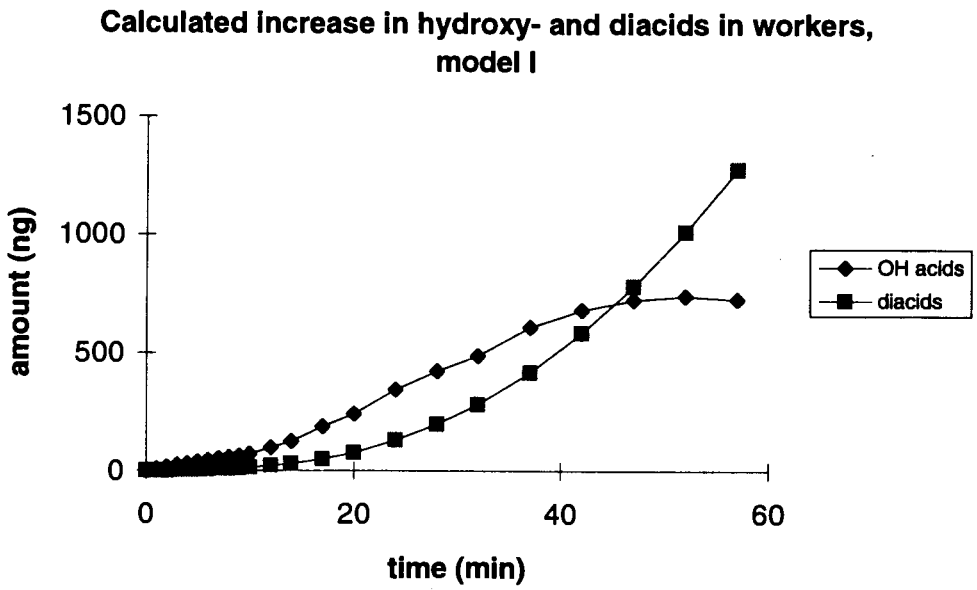


Figure IV.1. Increase in 10-HDA + 10-HDAA (◆) and in C10:0 DA + C10:1 DA (■) in workers, calculated using model I.

The amount of 10-HDA + 10-HDAA accumulated at the i^{th} time interval is $P_{OH\,i} = P_{OH\,i-1} + \Delta P_{OH\,i}$ and that of C10:0 DA + C10:1 DA is $P_{OX\,i} = P_{OX\,i-1} + \Delta P_{OX}$. The amount obtained after each iteration was converted from nmol to ng to allow comparison of the results from the model with results from experiments with labelled C18:0. In model I, the amount of hydroxy acid initially increases but eventually decreases, while the amount of diacid rises (Figure IV.1). This would mean that workers accumulate mostly diacids in their mandibular glands. Since this is not consistent with experimental observation, the

assumption that all the accumulated hydroxy acid is available for hydroxy group oxidation is not correct. The reactions may occur in different subcellular compartments and the hydroxy acids may continuously be removed for secretion. Both of these factors would limit the amount of substrate available for a given step.

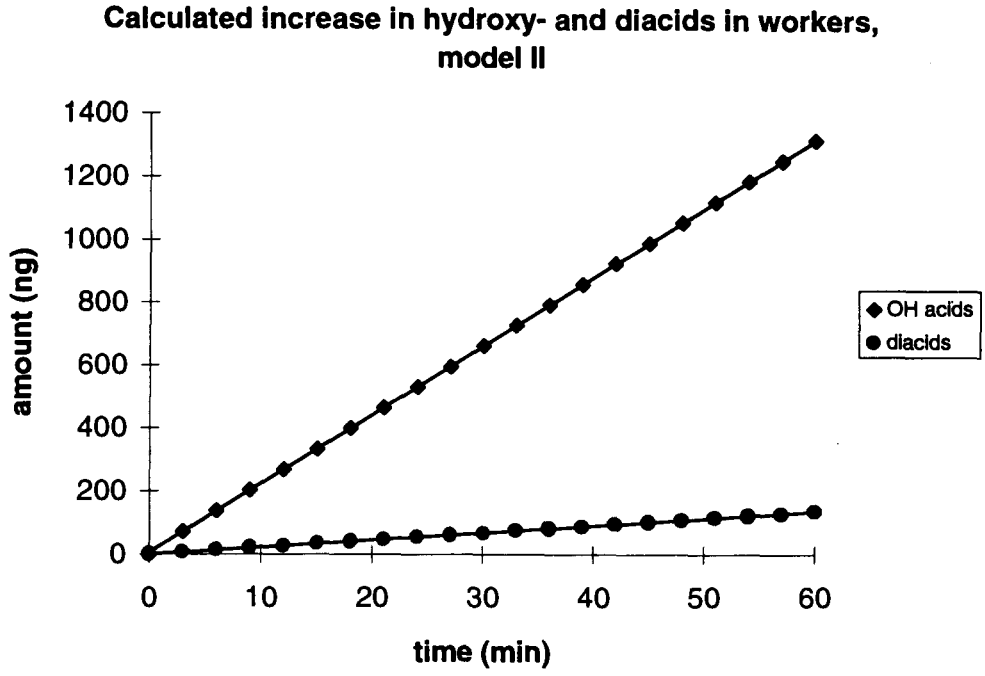


Figure IV.2. Increase in the amount of 10-HDA + 10-HDAA (◆) and of C10:0 DA + C10:1 DA (•) in workers, calculated using model II. The increase is linear giving $y = 22x + 5.2$ for the hydroxy acids and $y = 2.3x$ for the diacids.

The outcome of model II depends on the time interval, Δt, chosen. The larger the interval, the smaller the ratio of hydroxy acid:diacid formed. The experimentally observed ratio is *ca.* 10, and the time interval at which hydroxy acid:diacid is close to 10 in model I is 3 min, so $\Delta t = 3$ min was used for the iterations in model II. This calculation gave a linear pattern (Figure IV.2) which allowed to estimate the amount of total hydroxy acids and diacids formed per day. The calculated values were within the range of estimates obtained from experiments with labelled C18:0 (Table IV.3). This suggests that the independent rate

estimates for the three steps are consistent with the overall rate of formation of hydroxy- and diacids from C18:0.

Table IV.3. Comparison of observed and calculated daily rates of appearance of hydroxy and diacids in young workers.

Source of estimate	rate of appearance of 10-HDA + 10-HDAA (µg/day/worker)	rate of appearance of C10:0 DA + C10:1 DA (µg/day/worker)
experiment with D_1 C18:0 [2]	133 +/- 86 [N = 6] [1]	12 +/- 5 [N = 6] [1]
" D_1 C18:0 [3]	34 +/- 15 [N = 8] [1]	N. D.
" D_3 C18:0 [3]	26 +/- 10 [N = 8] [1]	N. D.
model II	63	6.5

[1] mean +/- S. E.
[2] Treatment of individual worker glands for 20 min with D_1 C18:0.
[3] See Table III.16.
N. D. = not determined

To conclude, β-oxidation is the fastest step in the pathway, which means that the total product accumulated is limited by the rate of hydroxylation. Furthermore, only the hydroxy acid formed during a short time interval appears to be accessible to oxidation. The hydroxy acids accumulate away from the site of biosynthesis, possibly in the central reservoir of the mandibular glands. Biosynthesis may occur in the glandular cells that are embedded in the glandular epithelium. Ducts connect the glandular cells to the reservoir and may provide an avenue for collection of glandular products (Vallet *et al.* 1991). The contents of the central reservoir are secreted through a pore in the mandible (Winston 1987). The increase in total hydroxy acid titer in the mandibular glands as workers age (Figure I.1) is *ca.* 2 µg/day/worker[2], but 63 µg/day/worker are biosynthesized. This means

[2] Estimated from the slopes of the lines obtained for the increase in 10-HDA and 10-HDAA with age in workers (Figure I.1, p. 5).

that secretion may be slightly slower than biosynthesis: *ca.* 61 µg/day of hydroxy acid (of which *ca.* 46 µg is 10-HDA) must be secreted in young workers.

The functions of the worker-produced acids in the colony are not fully understood. If these acids were only components of brood food, one would expect their production to cease when workers shift from brood tending to other tasks. However, foragers have the highest levels of hydroxy acids in their mandibular glands (Boch and Shearer 1967, Robinson *et al.*, unpublished observation). Unlike the hypopharyngeal glands (which are associated with brood care), the mandibular glands do not diminish in size as workers age, although their microscopic appearance and enzyme profiles change (Vallet *et al.* 1991, Costa-Leonardo 1980). One possible role of 10-HDA produced by older workers may be inhibition of precocious foraging among younger nestmates. Young workers progress through age polyethism faster when they are kept isolated from older workers than when workers of all ages are present. Conversely, old workers assume tasks of younger ones when they are kept in isolation from other age groups (Winston and Fergusson 1985). This effect could be mediated by an inhibitor of JH III biosynthesis that is produced by older workers. There are two reasons why 10-HDA is a good candidate inhibitor. First, this compound accumulates in the mandibular glands as workers age, which would be consistent with the observation that old workers inhibit the onset of foraging in younger nestmates and among each other when no young bees are present. Second, 10-HDA structurally resembles ODA which is known to inhibit JH III biosynthesis (Kaatz *et al.* 1992). The potential primer effect of 10-HDA has been suggested previously in the context of larval development (Kinoshita and Shuel 1975).

The experiments described in this thesis were done with young workers. Therefore, it is not known whether the rates of biosynthesis and secretion change as workers age. Possibilities for future work include studying the ontogeny of biosynthesis, following the fate of 10-HDA and the other worker acids in the hive and determining the role of the mandibular acids produced by foragers.

1.2 Biosynthesis of ω-1-functionalized acids in queens

The estimated rates of formation of 9-HDA and ODA from labelled C18:0 and 17-OH C18:0, and of oxidation of 9-HDA to ODA in queens are summarized in Table IV.4. As with workers, the rates and rate constants for the three steps in the biosynthesis of ODA and 9-HDA were calculated to find the fastest and the slowest step (Table IV.5).

Table IV.4. Rates of incorporation of labelled substrates into ω-1-functionalized acids in queens.

substrate	product	perfusion time (min)	amount of labelled product (ng) [1]	rate (nmol/min) [1, 2]
D_1 C18:0	ODA	10	1880 +/- 370	10 +/- 2
	9-HDA		270 +/- 90	1.4 +/- 0.5
D_3 17-OH C18:0	ODA	10	230 +/- 210	1.2 +/- 1.2
	9-HDA		62 +/- 42	0.3 +/- 0.2
D_2 9-HDA	ODA	10	180 +/- 70	1.0 +/- 0.4

[1] Mean +/- S. E.
[2] Rate of product formation.
All these experiments were done with 1 gland/replicate. N = 8 for all treatments.

The rate of hydroxylation was estimated from the experiment with 2-F C18:0[3]. The rate of β-oxidation was estimated as the rate of appearance of labelled 9-HDA and ODA with D_3 17-OH C18:0 as substrate. The rate of oxidation of 9-HDA was obtained from the experiment with D_2 9-HDA (Table IV.5). Rate constants were estimated by dividing the rate by the substrate titer. As in workers, β-oxidation in queens was faster than hydroxylation, and hydroxy group oxidation was slower than β-oxidation.

[3] During the 10 min perfusion, 17-OH C18:0 (1.3 +/- 0.6 ng, 0.004 nmol), 13-OH C14:0 (11 +/- 2 ng, 0.045 nmol), 11-OH C12:0 (12 +/- 5 ng, 0.056 nmol) and 11-OH C12:1 (15 +/- 11 ng, 0.072 nmol) accumulated. Since all the shorter hydroxy acids were derived form 17-OH C18:0, the total amount of C18:0 hydroxylated was 0.177 nmol, which gave a rate of 1.77×10^{-2} nmol/min/gland.

The rate constants were used to model the increase in 9-HDA and ODA in queens in the same way as the ω-functionalized acids were modelled in workers, except that no chain shortening of 9-HDA to 7-HOAA was included. The experimentally observed ratio of total ODA:9-HDA is *ca.* 2.5 in mated queens (Pankiw *et al.* in preparation). Model II was run with a time interval of 6 min, which gave a ratio of ODA:9-HDA close to 2.5 and a linear increase with time for both compounds (Figure IV.3).

Table IV.5. Rate constants for the three steps in the biosynthetic pathway of ω-1-functionalized acids in queens.

process	substrate	titer of substrate (ng/gland) mean +/- S. E.[1]	titer of substrate (nmol/gland)	rate of product formation (nmol/min/gland)	rate constant (min^{-1})
hydroxylation	C18:0	118 +/- 20 [7][2]	0.42	1.8×10^{-2}	4.2×10^{-2}
β-oxidation	17-OH C18:0	1.4 +/- 0.4 [8][3]	4.7×10^{-2}	1.6×10^{-1}	3.3
OH gr. ox.	9-HDA	140 +/- 30 [8][4]	0.75	9.8×10^{-2}	1.3×10^{-1}

[1] The number of replicates is indicated in brackets after each entry.
[2] Titer in untreated queens of the same age.
[3] Titer of 17-OH C18:0 in queens treated with 2-F C18:0. In untreated queens this compound was not detectable.
[4] Titer in a newly emerged queen.

The ratio of final products was used to find the optimum time interval for the iterations in the model. Therefore, the model only indicates whether the calculated overall rate of mandibular acid biosynthesis from C18:0 is consistent with the experimentally determined rate. It does not give insight into how the ratio of ODA:9-HDA arises. Even if oxidation of 9-HDA is slightly slower than β-oxidation, ODA can be the major product of the pathway, if removal of 9-HDA is slower than oxidation and therefore a large proportion of the 9-HDA is available for oxidation.

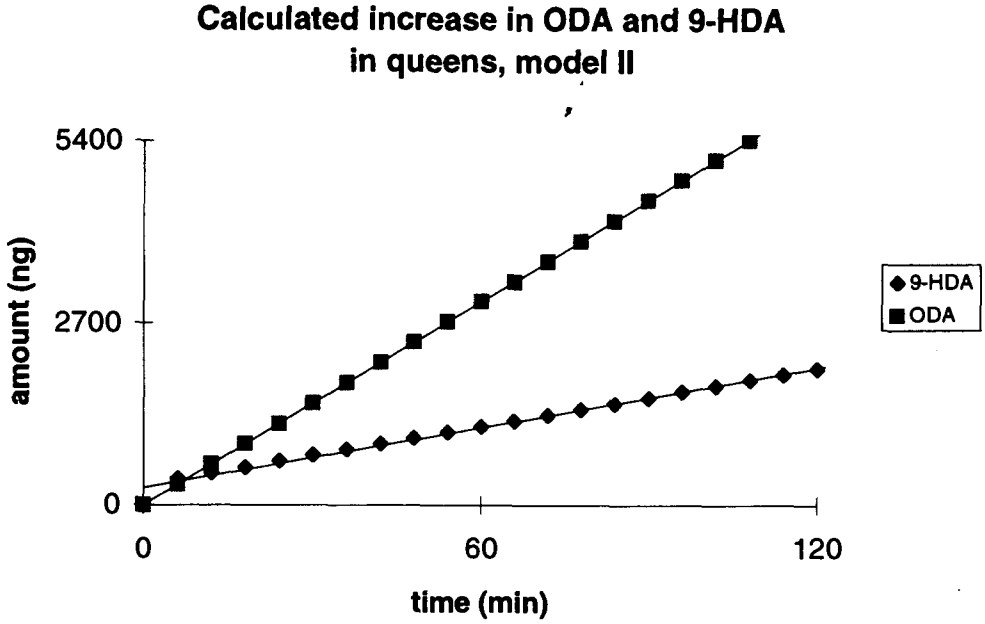


Figure IV.3. Increase in the amount of ODA (■) and 9-HDA (◆) in queens, calculated using model II with $\Delta t = 6$ min. The increase is linear giving $y = 19x + 155$ for 9-HDA and $y = 35x$ for ODA.

Table IV.6. Comparison of observed and calculated daily rates of formation of 9-HDA and ODA in queens.

Source of estimate	rate of formation of 9-HDA (µg/day/queen)	rate of formation of ODA (µg/day/queen)
experiment with D_1 C18:0	77 +/- 27 [1]	541 +/- 105 [1]
model II	55	144
Naumann *et al.* (1991)	N. D.	204 +/- 192 [1]

[1] Mean +/- S. E.

Daily production of 9-HDA and ODA was estimated from model II and from the experiment with D_1 C18:0 to allow comparison with data from Naumann *et al.* (1991). The calculated rate of 9-HDA formation was close to the experimental value; the rate calculated

for ODA was lower (Table IV.6). However, the blanks in the experiment with D_1 C18:0 showed some apparent labelling in the ODA (Table III.19, p. 97) and this artifact may have also affected the treatments, thus giving an overestimate. Correcting for this apparent label in the blanks, 380 +/- 210 µg/day/queen were obtained, which overlaps with the range obtained by Naumann *et al.* (1991). Thus, queens produce between 1 and 2 queen equivalents (Qeq) per day, where 1 Qeq consists of 200 µg of ODA and 80 µg of 9-HDA (Pankiw *et al.* in preparation). One Qeq/day is the best dose in practical applications of synthetic queen mandibular pheromone, such as suppression of emergency queen rearing (Winston *et al.* 1991), delaying of swarming (Winston *et al.* 1990) and stimulation of pollen foraging in small colonies (Higo *et al.* 1992).

IV.2. Determination of caste-specificity in mandibular acid biosynthesis

The final objective of this work was to determine which step(s) in the pathway control the caste-specific pattern of mandibular acids in workers and queens. This information can be obtained from the experiments described in Chapter III by comparing the conversion of ω– and ω-1-functionalized acids at each step in both castes.

2.1 Hydroxylation

The accumulation of hydroxy acids with more than 12 carbons in the presence of 2-F C18:0 allows to estimate of the amount of 17- and 18-hydroxyoctadecanoic acid formed during the perfusion. Comparison of these two amounts should give an indication of the hydroxylation preference. The results of this calculation are presented in Table IV.7.

In workers, ω-hydroxylation was slightly, but not significantly, higher than ω-1-hydroxylation and the opposite was true in queens. Therefore, even though hydroxylation is the step at which the functionalization pattern is introduced, no significant bias towards ω– or ω-1-hydroxylation was observed in workers or queens.

Table IV.7. Amounts of labelled hydroxy acids, 12-18-carbons long, accumulated during 10 min perfusions with D_1 C18:0 and 2-F C18:0.

caste	glands/rep.	amount accumulated (nmol) [1]	
		hydroxy group position	
		ω	ω-1
workers	2	0.13 +/- 0.03	0.09 +/- 0.03
queens	1	0.73 +/- 0.52	0.77 +/- 0.54

[1] Mean +/- S. E.
N = 8 for both castes.
The amounts of accumulated ω and ω-1 hydroxy acids did not differ significantly within each caste (Kruskal-Wallis P>0.05).

2.2 β-Oxidation

Both queens and workers were able to chain-shorten 18-OH C18:0 (Section III.5). Queens preferentially chain shortened 18-OH C18:0 to the 8-carbon length, while workers shortened it to the 10-carbon length. The observed ability of mated queens to chain-shorten 18-OH C18:0 is consistent with their high 8-HOAA titer. For example, the queens whose 9-HDA, ODA and 10-HDA titer was shown in Figure I.2, had 76 +/- 8 μg (N = 29) of 8-HOAA in their mandibular glands. Chain shortening of 17–OH C18:0 to 10-carbon hydroxy acids occurred to a much larger extent in queens than in workers (Table IV.8). However, it was not possible to determine whether workers have a very low chain shortening activity for ω-1-hydroxy acids or whether they channel 17-OH C18:0 into other products that were not analyzed.

Table IV.8. Rates of β-oxidation of 18- and 17-hydroxyoctadecanoic acids in workers and mated queens.

caste	rate of formation of the major chain shortened products (nmol/min/gland)	
	hydroxy group position	
	ω	ω-1
workers	3.7×10^{-1} [1]	7.0×10^{-4} [3]
queens	1.4×10^{-1} [2]	1.6×10^{-1} [4]

[1] Rate of appearance of labelled 10-HDA and 10-HDAA from D_1 18-OH C18:0.
[2] Rate of appearance of labelled 8-HOAA from D_1 18-OH C18:0.
[3] Rate of appearance of labelled 9-HDAA and 9-HDA from D_3 17-OH C18:0.
[4] Rate of appearance of labelled 9-HDA and ODA from D_3 17-OH C18:0.

The caste-specific pattern in β-oxidation can arise gradually or at one point in the chain shortening sequence. The amount of ω- and ω-1-hydroxy acids formed from D_1 C18:0 was compared at the 18-, 12- and 10-carbon length (Figure IV.3), to find the point at which different specificities in β-oxidation became apparent. There was no significant difference in the amounts of ω- and ω-1-hydroxy acids (Kruskal-Wallis, $P<0.05$) at the 18- and 12-carbon length, but at the 10-carbon length differences in amounts were pronounced in both castes. Workers had significantly more labelled 10-HDAA than 9-HDAA and 10-HDA than 9-HDA. In queens the pattern was reversed. This suggests that workers preferentially release ω-hydroxy acids from further β-oxidation at the 10-carbon length and queens release ω-1-hydroxy acids at that length.

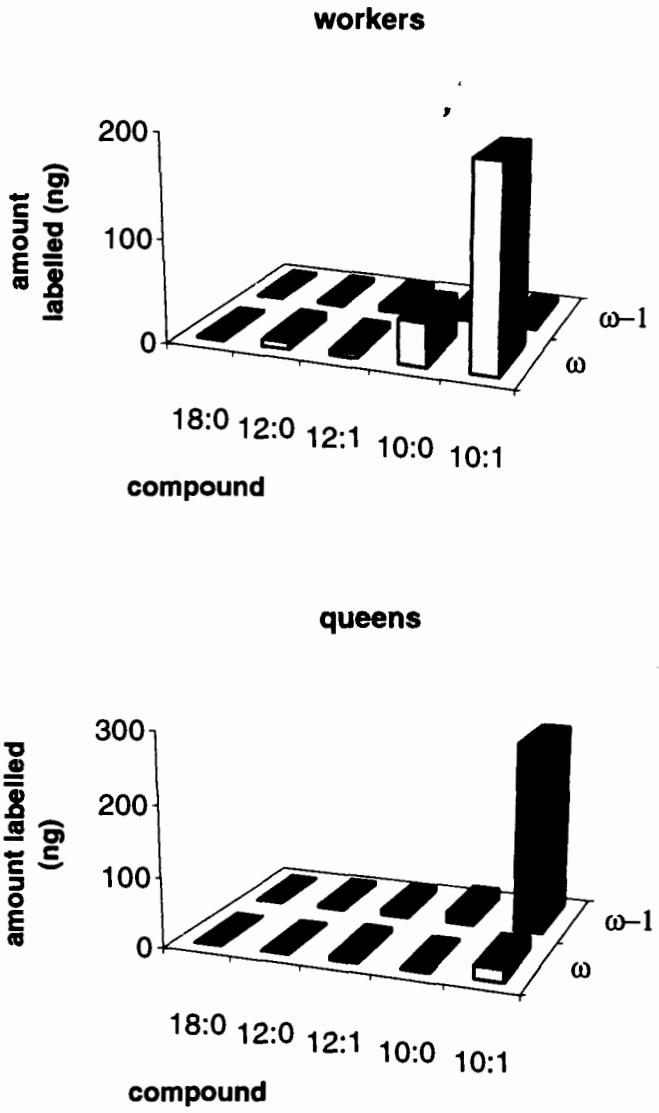


Figure IV.4. Amount of labelled 18-, 12- and 10- carbon ω- and ω-1-hydroxy acids formed from D_1 C18:0 in workers and queens. The 16-carbon hydroxy acids were not detected and the 14-carbon hydroxy acids showed no significant incorporation of label. Each datum point represents the mean of 8 replicates. The compound names indicate the chain length and the presence (:1) or absence (:0) of an (E)2 double bond.

Inhibition by 2-F C18:0 (section III.6) manifested itself at the same chain length as specificity in β-oxidation. This observation suggests that β-oxidation from the 18- to the

12-carbon length is tightly coupled and not specific for hydroxy group position. From that point onward, β-oxidation may be continued by a different set of enzymes specific for shorter chain lengths and for hydroxy group position.

2.3 Hydroxy group oxidation

Workers were able to oxidize ω-hydroxy acids to diacids, but not ω-1-hydroxy acids to keto acids. Preliminary *in vitro* studies with worker mandibular gland homogenates revealed that the hydroxy group oxidation activity is specific for 10-carbon ω-hydroxy acids (G. Sutherland, unpublished observation). These results ruled out the possibility that intact worker mandibular glands did not oxidize 9-HDA because of poor substrate uptake. Thus, hydroxy group oxidation enhances the caste-specific pattern that is established by β-oxidation of the hydroxy acids in workers (Figure IV.4).

In queens, the specificity of hydroxy group oxidation appeared to change with age. Young virgin queens oxidized ω-hydroxy acids to diacids, but they did not significantly oxidize 9-HDA to ODA (Table III.6). However, young queens must have some 9-HDA oxidizing activity because they always have low levels of ODA in their mandibular glands (Slessor *et al.* 1990). This activity may have been too low to detect by the method used in this study. Mated queens oxidized 9-HDA, but oxidation of ω-hydroxy acids was not detected. Mated queens have traces of the diacids, so they probably retain some ω-oxidizing activity as they age. The picture gained from these observations is that young queens preferentially oxidize ω-hydroxy acids, while older mated queens oxidize mainly 9-HDA.

2.4 Biosynthesis of mandibular acids in workers and queens

Biosynthesis of mandibular acids in workers begins with the hydroxylation of octadecanoic acid at the 17^{th} and 18^{th} position (Figure IV.4). There appears to be no

preference for one or the other position. The 18-carbon hydroxy acids are chain shortened by β-oxidation to the 10-carbon length. Workers chain-shorten 18-OH C18:0 much faster

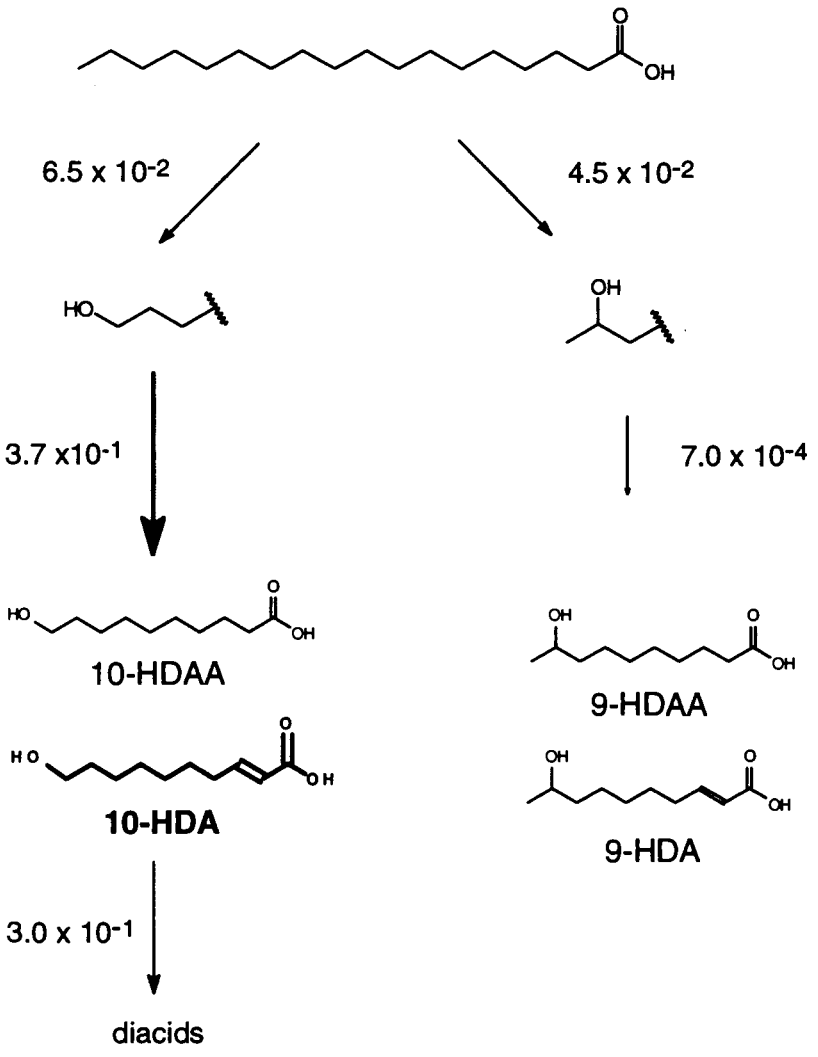


Figure IV.5. Caste-specific biosynthesis of mandibular acids in workers. Estimated rates (nmol/min/gland) for the three steps of the pathway are indicated next to the arrows (hydroxylation p. 114, β-oxidation p. 115, hydroxy group oxidation p. 105). The major component of the blend is shown in bold type.

than 17-OH C18:0, as judged by the conversion of these compounds into 10-carbon hydroxy acids. The ω-hydroxy acids are oxidized to the corresponding diacids, but the ω-1-hydroxy acids are not detectably oxidized to keto acids. Thus, β-oxidation and hydroxy group oxidation determine the caste-specific biosynthesis of ω-functionalized acids in workers.

Queens begin their biosynthesis like workers do, with the unbiased hydroxylation of octadecanoic acid (Figure IV.5). Unlike workers, queens β-oxidize both 17- and 18-hydroxyoctadecanoic acids; the former to the 10-carbon length and the latter to the 8-carbon length. The pattern of hydroxy group oxidation in queens is opposite to the pattern in workers. Queens oxidize 9-HDA to ODA, but they probably oxidize ω-hydroxy acids to diacids only to a small extent. Therefore, β-oxidation and hydroxy group oxidation are responsible for the preponderance of 10-carbon ω-1-functionalized acids in mature queens.

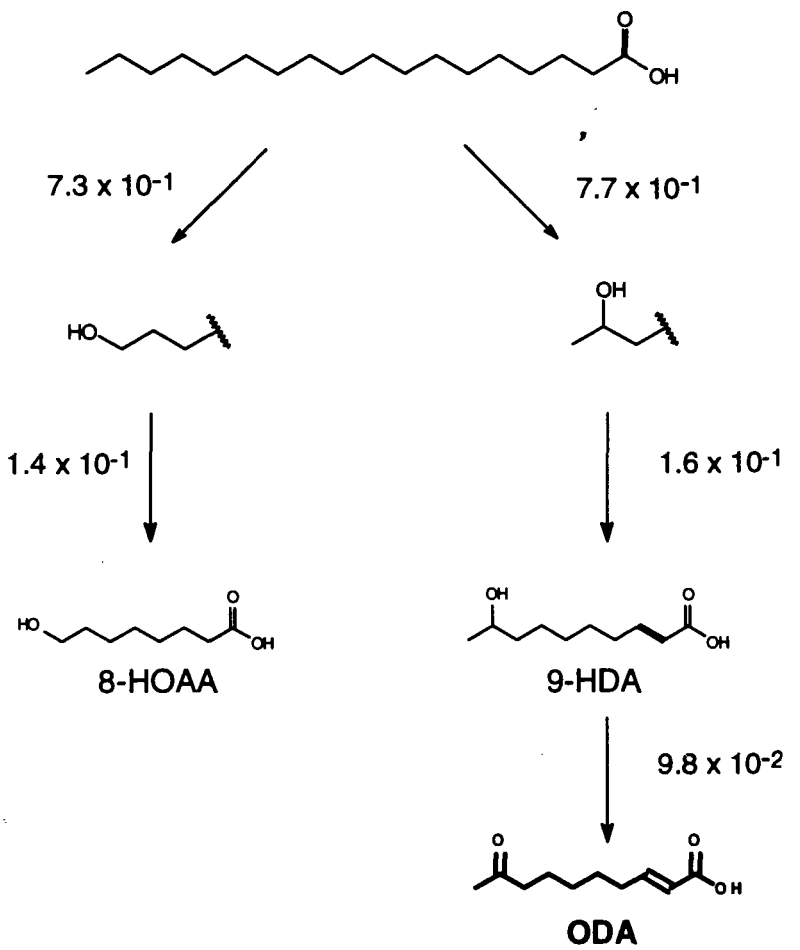


Figure IV.6. Caste-specific biosynthesis of mandibular acids in mated queens. Estimated rates (nmol/min/gland) for the three steps of the pathway are indicated next to the arrows (hydroxylation p. 114, β-oxidation p. 115, hydroxy group oxidation p. 111). The major component of the blend is shown in bold type.

2.5 The order of the steps in the pathway and the high output of the mandibular glands

The total production of mandibular acids in queens and workers is higher than the production of semiochemicals in other insects. For example, females of *T. ni* have 225 +/-

17 ng of (Z)7-dodecenyl acetate in their pheromone glands (Jurenka *et al.* 1994), *ca.* three orders of magnitude less material than the amount of ODA in queen mandibular glands. Furthermore, the biosynthetic pathway of mandibular acids in honey bees is longer than other semiochemical biosyntheses. These observations lead to the question why the bees do not use a shorter pathway, such as hydroxylation of decanoic acid to 9- and 10-HDAA, followed by desaturation. Precursor availability and the hydroxylation reaction may help to explain why mandibular acids are synthesized by the observed pathway. The precursor, octadecanoic acid, is one of the most abundant free and lipid-bound fatty acids in the gland. Furthermore, the results from the experiments with labelled acetate and other fatty acids suggest that hexa- and octadecanoic acids are synthesized directly from acetate and that shorter acids are derived from hexadecanoic acid by chain shortening. Therefore, a pathway in which decanoic acid was hydroxylated would be nearly as long as the observed pathway. Product inhibition of the hydroxylation reaction may be a possible reason why decanoic acid is not the hydroxylation substrate in the mandibular glands.

The ω-hydroxylation of octadecanoic acid is a one-center reaction, most likely catalyzed by cytochrome P-450 fatty acid hydroxylase. The ω-1-hydroxylation does not proceed by an intermediate with a terminal double bond and may be catalyzed by a similar hydroxylase. Some cytochromes P-450 are known to be inhibited by their product. For example, ecdysone 20-monooxygenase is inhibited by 20-hydroxyecdysone (Mitchell and Smith 1986). A polysubstrate monooxygenase preparation from houseflies, which converts (Z)9-tricosene to 10-keto-(Z)14-tricosene *via* the corresponding alcohol, is inhibited by 10-keto-(Z)14-tricosene (Guo *et al.* 1991). A microsomal fatty acid hydroxylase preparation from rat liver, which hydroxylates dodecanoic acid at the 11 and 12 position, is inhibited by 12-OH C12:0 (Ellin *et al.* 1973). If ω and ω-1 fatty acid hydroxylation in the mandibular glands is inhibited by one or both products, glandular output would be curtailed as products accumulate. Continuous removal of 17- and 18-OH C18:0 by rapid β-oxidation prevents the accumulation of these compounds and may thereby minimize product inhibition of hydroxylation.

Future *in vitro* studies of hydroxylation, β-oxidation and hydroxy group oxidation with gland homogenates will give insights into the subcellular location of these activities, their cofactor requirements, kinetic parameters and inhibition patterns. The hydroxylation reaction is of interest because few fatty acid hydroxylases, whose function is known, have been studied in insects. Studies of β-oxidation and hydroxy group oxidation will delineate how the caste-specific pattern of mandibular acid biosynthesis arises in queens and workers.

2.6 Changes in caste-specificity with age and colony state

The composition of the MC changes in queens during their ontogeny and in some workers as a consequence of queenlessness. In newly emerged queens, the major ω-hydroxy acid is 10-HDA; 8-HOAA is present in smaller quantities. In mated queens, this pattern is reversed: 8-HOAA is the major ω-hydroxy acid (Figure IV.7). Furthermore, in young queens the quantity of ω-hydroxy acids is equal to the quantity of ω-1-functionalized acids. As queens age, the quantity of the latter increases relative to the ω-hydroxy acids. These changes may be due to a shift in β-oxidation from a worker-like specificity to the characteristic queen specificity and an increase in the ability to oxidize 9-HDA.

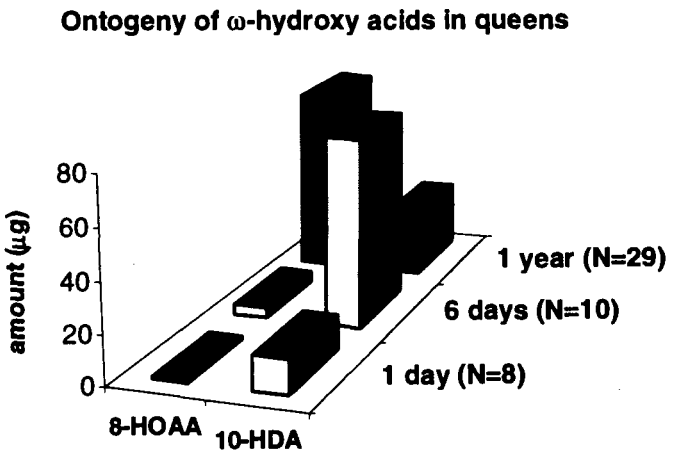


Figure IV.7. Ontogeny of 8-hydroxyoctanoic (8-HOAA) and 10-hydroxy-(E)2-decenoic acid (10-HDA) in queens (Plettner *et al.* unpublished observation).

In contrast to queens, workers in a queenright colony do not experience a change in the composition of their MC as they age (Figure I.1). However, when a colony becomes queenless, a false queen can occasionally arise. False queens have a MC intermediate between that of a queenright worker and a mature queen. These individuals must therefore gain the ability to oxidize 9-HDA and may also experience a shift in the specificity of β-oxidation similar to young queens. The observation that false queens only arise after prolonged queenlessness suggests that the biosynthetic pattern characteristic of workers is maintained by inhibition of queen-specific β-oxidation and/or 9-HDA oxidation in queenright colonies. When this unknown inhibitory factor disappears after loss of the queen and the brood, a few individuals become false queens. Numerous studies with queenless workers reveal that MC composition in workers does not correlate with their ovarian development (Plettner *et al.* 1993 and references therein), so mandibular acid biosynthesis and ovarian development may be under separate control. Furthermore, different subspecies of *Apis mellifera* and different strains within these subspecies vary in their ease of false queen formation, which suggests that there is a genetic predisposition for this phenomenon (Robinson *et al.* 1990).

IV.3 Concluding remarks

The present study outlines the biosynthetic pathway of the ω- and ω-1-functionalized 10-carbon acids found in the mandibular glands of workers and queens. The precursor to these compounds is octadecanoic acid which can be incorporated directly or synthesized *de novo* from acetate. Conversion of octadecanoic acid to the 10-carbon mandibular acids requires three steps. Octadecanoic acid is hydroxylated at the ω- or ω-1-position and the resulting 18-carbon hydroxy acids are chain shortened to the 10-carbon length. The 10-carbon ω- and ω-1-hydroxy acids are oxidized to diacids and keto acids, respectively.

The estimated rates of the three steps for both types of acid in both castes give insights into the total glandular production and the caste-specificity in the biosynthesis. Both castes hydroxylate octadecanoic acid to the same extent, but they differ in their ability to chain-shorten the 18-carbon hydroxy acids and to oxidize the ω- and ω-1-hydroxy acids. This two-point control over caste-specificity ensures that workers produce mainly 10-HDA, only trace levels of 9-HDA and no detectable ODA, and that mature queens produce more 9-HDA and ODA than 10-HDA. Both castes, however, can hydroxylate octadecanoic acid at the ω- and ω-1-position and therefore have the potential to produce the other caste's characteristic compounds.

This work has led to new insights into honey bee primer pheromone production and the establishment of distinct queen and worker chemical signals. However, many questions remain unanswered. The mechanisms and the stereoselectivities of the ω-1 hydroxylation and hydroxy group oxidation are not known. These stereoselectivities are of interest because the 9-HDA in mature queens is variable with a mean of 70 % R and 30 % S (Slessor *et al.* 1990) and the two enantiomers of 9-HDA have different activities in swarm stabilization (Winston *et al.* 1982). A more detailed picture of β-oxidation in queens and workers is also of interest because it is the first caste-specific step in the pathway. The data presented in this thesis indicate that caste-specific β-oxidation occurs from the 12-carbon length onward. However, each cycle of β-oxidation consists of four reactions which may not contribute equally to the caste-specific pattern.

Workers produce their characteristic mandibular acids at levels that are comparable to 9-HDA and ODA production in queens, yet little is known about the functions of the worker acids in the colony. Interactions between the queen and the workers that are mediated by queen mandibular pheromone are the most extensively studied form of chemical communication within the hive. However, many decisions, ranging from task distribution to foraging strategy, are made by the workers and require communication between them. Screening the worker mandibular acids for primer and releaser activities is likely to give new insights into communication among workers.

The mandibular gland ontogeny of workers and queens and the changes in some queenless workers present many questions for further research. The changes seen in queen ontogeny and false queens may correlate with changes in the caste-specific enzymes of the mandibular acid pathway. Furthermore, the maintenance of the caste-specific pattern of mandibular acid biosynthesis in queenright workers and in mated queens may require continuous pheromonal feedback from the colony.

Workers and queens have a characteristic pattern of mandibular acids at emergence, which suggests that the biosynthetic capabilities of the mandibular gland are fixed during larval development. With suitable probes, expression of the key enzymes in the pathway may be followed through larval and adult development. Such studies will greatly enhance knowledge about caste differentiation of physiological characters in eusocial insects and about the communication system that is at the heart of the social structure in a honey bee colony.

Literature cited

Abrams, S. R. 1982. Isomerization of acetylenic acids with sodium salt of 1,2-diaminoethane: a one step synthesis of megatomic acid. *Can. J. Chem.* **60**: 1238.

Agrawal, V. P., Kolattukudy, P. E. 1978a. Purification and Characterization of a Wound-Induced ω-Hydroxyfatty Acid:NADP Oxidoreductase from Potato Tuber Disks (*Solanum tuberosum* L.). *Arch. Biochem. Biophys.* **191**: 452-465.

Agrawal, V. P., Kolattukudy, P. E. 1978b. Mechanism of Action of a Wound-Induced ω-Hydroxyfatty Acid:NADP Oxidoreductase Isolated from Potato Tubers (*Solanum tuberosum* L.). *Arch. Biochem. Biophys.* **191**: 466-478.

Allsopp, M. H., Crewe, R. M. 1993. The Cape Honey Bee as a Trojan Horse Rather Than the Hordes of Genghis Khan. *American Bee Journal* **133**: 121-123.

Ando, T., Takema, H., Rika, A., Uchiyama, M. 1988. Biosynthetic Pathway of Bombykol, the Sex Pheromone of the Female Silkworm Moth. *Agric. Biol. Chem.* **52**: 473-478.

Arnold, G., Roger, B. 1979. Group Effect on the Content of 10-Hydroxydec-2-enoic acid in the Head of Worker Bees. *Apidologie* **10**: 35-42.

Arsequell, G., Fabrias, G., Camps, F. 1990. Sex Pheromone Biosynthesis in the Processionary Moth *Thaumetopoea pityocampa* by Delta-13 Desaturation. *Arch. Insect Biochem. Physiol.* **14**: 47-56.

Asencot, M., Lensky, Y. 1976. The effect of sugars and juvenile hormone on the differentiation of the female honeybee larvae (*Apis mellifera* L.) to queens. *Life Sciences* **18**: 693-700.

Attygalle, A. B., Siegel, B., Vostrowsky, O., Bestman, H. J., Maschwitz, U. 1989. Chemical Composition and Function of Metapleural Gland Secretion of the Ant *Crematogaster deformis* Smith (Hymenoptera Myrmicinae). *J. Chem. Ecol.* **15**: 317-328.

Babler, J. H., Coghlan, M. J. 1979. A facile Method for Monoacetylation of Symmetrical Diols: Application to the Total Synthesis of Z-8-Dodecenyl Acetate, the Sex Attractant of the Oriental Fruit Moth. *Tetrahedron Lett.* **# 22**:1971-1974.

Barker, S. A., Foster, A. B., Lamb, D. C. , Hodgson, N. 1959. Identification of 10-Hydroxy-Δ^2-decenoic Acid in Royal Jelly. *Nature* (Lond.) **183**: 996-997.

Beetsma, J. 1979. The Process of Queen-Worker Differentiation in the Honeybee. *Bee World* **60**: 24-39.

Beetsma, J. 1985. Feeding behavior of nurse bees, larval food composition and caste differentiation in the honey bee (*Apis mellifera* L.). In: Experimental Behavioral Ecology (Ed's: B. Hölldobler and M. Lindauer). Fischer Verlag, Stuttgart, pp 407-410.

Beilsteins Handbuch der Organischen Chemie. Springer-Verlag, Berlin.

Biemann, K. 1962. Mass Spectrometry. Mc Graw Hill, New York.

Bjorkhem, I., Hamberg, M. 1971. ω-Oxidation of Fatty Acids. *J. Biol. Chem.* **246**: 7417-7420.

Bjorkhem, I., Jornvall, H. Zeppezauer, E. 1973. Oxidation of ω-Hydroxylated fatty acids and steroids by alcohol dehydrogenase. *Biochem. Biophys Res. Comm.* **52**: 413-420.

Bjostad, L. B., Wolf, W. A., Roelofs, W. L. 1987. Pheromone Biosynthesis in Lepidopterans: Desaturation and Chain Shortening. In: Pheromone Biochemistry (Ed's: G. D. Prestwich and G. J. Blomquist). Academic Press Inc., London, pp 77-120.

Blomquist, G. J., Tillman-Wall, J. A., Guo, L. Quilici, D. R., Gu, P., Schal, G. 1993. Hydrocarbon and Hydrocarbon Derived Sex Pheromones in Insects: Biochemistry and Endocrine Regulation. In: Insect Lipids: Chemistry, Biochemistry and Biology (Ed's D. W. Stanley-Samuelson and D. R. Nelson), U. of Nebraska Press, Lincoln, pp 317-351.

Blum, M. S., Novak, A. F., Taber, S. 1959. 10-Hydroxy-Δ^2-decenoic acid, and antibiotic found in royal jelly. *Science* **130**: 452-453.

Boch, R., Shearer, D. A. 1967. 2-Heptanone and 10-Hydroxy-*trans*-dec-2-enoic Acid in the Mandibular Glands of Worker Honey Bees of Different Ages. *Z. vergl. Physiol.* **54**: 1-11.

Boddupalli, S. S., Estabrook, R. W., Peterson, J. A. 1990. Fatty Acid Monooxygenation by Cytochrome P-450_{BM-3}. *J. Bio. Chem.* **265**: 4233-4239.

van den Bosch, H., Schutgens, R. B. H., Wanders, R. J. A., Tager, J. M. 1992. Biochemistry of Peroxysomes. *Ann. Rev. Biochem.* **61**: 157-197.

Brown, H. C., Geoghegan, P. 1967. The Oxymercuration-Demercuration of Representative Olefins. A Convenient, Mild Procedure for the Markovinkov Hydration of the Carbon-Carbon Double Bond. *J. Am. Chem. Soc.* **89**: 1522-1524.

Callow, R. K., Johnston, N. C., Simpson, J. 1959. 10-Hydroxy-Δ^2-decenoic Acid in the Honeybee (*Apis mellifera*). *Experientia* **15**: 421-422.

Carey, F. A., Sundberg, R. J. 1983. Advanced Organic Chemistry, second Ed., Plenum, New York.

Charlton, R. E., Roelofs, W. L. 1991. Biosynthesis of a Volatile, Methyl-Branched Hydrocarbon Sex Pheromone From Leucine by Arctiid Moths (*Holomelina* Spp.) *Arch. Insect Biochem. Physiol.* **18**: 81-97.

Chiron, R. 1982. New Synthesis of Royal Jelly Acid. *J. Chem. Ecol.* **8**: 709-713.

Christensen, E., Gronn, M., Hagve, T.-A., Christophersen, B. O. 1991. Omega-oxidation of fatty acids studied in isolated liver cells. *Biochim. Biophys. Acta* **1081**: 167-173.

Christensen, E., Hagve, T.-A., Gron, M. Christophersen, B. O. 1989. β-Oxidation of medium chain (C8-C14) fatty acids studied in isolated liver cells. *Biochim. Biophys. Acta* **1004**: 187-195.

Clarke, S. E., Brealey, C. J., Gibson, G. G. 1989. Cytochrome P-450 in the housefly: induction, substrate specificity and comparison to three rat hepatic enzymes. *Xenobiotica* **19**: 1175-1180.

Cooke, M. J. 1987. Are we opening Pandora's box again? *Am. Bee J.* **127**: 15-17.

Corey, E. J., Schmidt, G. 1979. Useful procedures for the oxidation of alcohols involving pyridinium dichromate in aprotic media. *Tetrahedron Letters* **# 5**: 399-402.

Costa-Leonardo, A. M. 1980. Estudios morfologicos do ciclo secretor das glândulas mandibulares de *Apis mellifera* L. (Hymenoptera, Apidae). *Rev. Bras. Ent.* **24**: 143-151.

Crane, E. 1946. The World's Beekeeping Past and Present. In: The Hive and the Honey Bee (Ed. Dadant & Sons), Dadant & Sons, Illinois, Chapter 1.

Crewe, R. M. 1982. Compositional variability: the key to social signals produced by the honeybee mandibular glands. In: The Biology of Social Insects (Ed's M. D. Breed, G. D. Michener, H. E. Evans), Westview Press, London, pp 318.

Crewe, R. M., Velthius, H. H. W. 1980. False queens: a consequence of mandibular gland signals in worker honeybees. *Naturwissenshaften* **67**: 467-469.

Devore, J. L. 1991. Probability and Statistics for Engineering and the Sciences. Third Ed. Brooks/Cole Pub. Co., Pacific Grove, CA.

Ellin, A., Orrenius, S., Pilotti, A., Swahn, C.-G. 1973. Cytochrome $P450_K$ of Rat Cortex Microsomes: Further Studies on its Interaction with Fatty Acids. *Arch. Biochem. Biophys.* **158**: 597-604.

Fabrias, G., Arsequell, G. Camps, F. 1989. Sex Pheromone Precursors in the Processionary Moth *Thaumetopoea pityocampa* (Lepidoptera: Thaumetopoeae). *Insect Biochem.* **19**: 177-181.

Fendrich, G., Abeles, R. H. 1982. Mechanism of Action of Butyryl-CoA Dehydrogenase: Reactions with Acetylenic, Olefinic and Fluorinated Substrate Analogues. *Biochemistry* **21**: 6685-6695.

Foster, S. P., Roelofs, W. L. 1988. Pink Bollworm Sex Pheromone Biosynthesis from Oleic Acid. *Insect Biochem.* **18**: 281-286.

Free, J. B. 1982. Bees and Mankind. George Allen & Unwin, London.

Free, J. B. 1987. Pheromones of Social Bees. Chapman and Hall, London.

Gibson, G. G., Orton, T. C., Tamburini, P. P. 1982. Cytochrome P-450 induction by clofibrate. *Biochem. J.* **203**: 161-168.

Gotuda, H. 1991. Production of Hydroxy and Oxo Fatty Acids by Microorganisms as a Model of Adipocere Formation. *Hokkaido-Igaku-Zasshi* **66**:142-150.

Graham, S. H., Williams, A. J. S. 1966. Alkylidenecyclobutanes. Part II. The Oxidation of Benzylidenecyclobutane and of Bis-(p-methoxyphenyl) methylenecyclobutane. *J. Chem Soc.* (C) **5**:655-660.

Gries, G., Li, J., Gries, R., Bowers, W. W., West, R. J., Wimalaratne, P. D. C., Khaskin, G., King, G. G. S., Slessor, K. N. 1994. (E)-11,13-tetradecadienal: major sex pheromone component of the eastern blackheaded budworm *Acleris variana* (Fern.) (Lepidoptera: Tortricidae). *J. Chem. Ecol.* **20**: 1-7.

Guo, L., Latli, B., Prestwich, G. D., Blomquist, G. J. 1991. Metabolically Blocked Analogs of Housefly Sex Pheromone: II. Metabolism Studies. *J. Chem. Ecol.* **17**: 1769-1781.

Hamberg, M., Bjorkhem, I. 1971. ω-Oxidation of Fatty Acids. Mechanism of Microsomal ω1- and ω2-hydroxylation. *J. Biol. Chem.* **246**: 7411-7416.

Heinz, E., Tulloch, A. P., Spencer, J. F. T. 1969. Stereospecific Hydroxylation of Long Chain Compounds by a Species of *Torulopsis*. *J. Biol. Chem.* **244**: 882-888.

Hendry, L. B., Korzeniowski, S. H. Hindenlang, D. M., Kosaruch, Z., Mumma, R. O, Jugovich, J. 1975. An Economical Synthesis of the Major Sex Attractant of the Oak Leaf Roller- cis 10-Tetradecenyl Acetate. *J. Chem. Ecol.* **1**, 317-322.

Higo, H. A., Colley, S. J., Winston, M. L. Slessor, K. N. 1992. Effects of Honey Bee (*Apis Mellifera* L.) Queen Mandibular Gland Pheromone on Foraging and Brood Rearing. *Can. Ent.* **124**: 409-418.

Ho, P., Fulco, A. 1976. Involvement of a Single Hydroxylase Species in the Hydroxylation of Palmitate at the ω-1, ω-2 and ω-3 Positions by a Preparation from *Bacillus megaterium*. *Biochim. Biophys. Acta* **431**: 249-256.

Hölldobler, B., Wilson, E. O. 1990. The Ants. Harvard University Press. Cambridge, Mass.

Ichihara, K., Kusunose, E., Kusunose, M. 1969. Some Properties and Distribution of the ω-Hydroxylation System of Medium-Chain Fatty Acids. *Biochim. Biophys. Acta* **176**: 704-712.

Johnston, N. C., Law, J. H., Weaver, N. 1965. Metabolism of 9-ketodec-2-enoic Acid by Worker Honeybees (*Apis mellifera* L.). *Biochemistry* **4**: 1615-1621.

Jurenka, R. A., Haynes, K. F., Adolf, R. O., Bengtsson, M., Roelofs, W. L. 1994. Sex Pheromone Component Ratio in the Cabbage Looper Moth Altered by a Mutation Affecting the Fatty Acid Chain-Shortening Reactions in the Pheromone Biosynthetic Pathway. *Insect Biochem. Molec. Biol.* **24**: 373-381.

Jurenka, R. A., Roelofs, W. L. 1989. Characterization of the Acetyltransferase used in Pheromone Biosynthesis in Moths: Specificity for the Z isomer in Tortricidae. *Insect Biochem.* **19**: 639-644.

Jurenka, R. A., Roelofs, W. L. 1993. Biosynthesis and Endocrine Regulation of Fatty Acid Derived Sex Pheromones in Moths. In: Insect Lipids: Chemistry, Biochemistry and Biology (Ed's D. W. Stanley-Samuelson and D. R. Nelson), U. of Nebraska Press, Lincoln, pp 353-388.

Kaatz, H. H., Hildebrandt, H., Engels, W. 1992. Primer effect of queen pheromone on juvenile hormone biosynthesis in adult worker honey bees. *J. Comp. Physiol. B* **162**: 588-592.

Kaminski, L.-A., Slessor, K. N., Winston, M. L., Hay, N. W., Borden, J. H. 1990. Honeybee response to queen mandibular pheromone in laboratory bioassays. *J. Chem. Ecol.* **16**: 841-859.

Kandil, A. A. 1985. Ph. D. Thesis: Chiral Synthesis of Insect Pheromones. Simon Fraser University, Burnaby, Canada.

Kinoshita, G., Shuel, R. W. 1975. Mode of action of royal jelly in honeybee development. Some aspects of lipid nutrition. *Can. J. Zool.* **53**: 311-319.

Kumar, R., Weintraub, S. T., Hanahan, D. J. 1983. Differential susceptibility of mono- and di-O-alkyl ether phosphoglycerides to acetolysis. *J. Lipid Res.* **24**: 930-937.

Kuwahara, Y., Nakamura, S. 1985. (Z)5 and (E)5-Undecenoic Acid: Identification of the Sex Pheromone of the Varied Carpet Beetle, *Anthrenus verbasci* L. (Coleoptera: Dermestidae). *Appl. Ent. Zool.* **20**: 354-356.

Lazarow, P. B., de Duve, Ch. 1976. A fatty acyl-CoA oxidizing system in rat liver peroxisomes; enhancement by clofibrate, a hypolipidemic drug. *Proc. Nat. Acad. Sci.* (U. S. A.) **73**: 2043-2046.

Lu, A. Y. H., Junk, K., Coon, M. J. 1969. Resolution of the Cytochrome P-450-containing ω-Hydroxylation System of Liver Microsomes into Three Components. *J. Biol. Chem.* **244**: 3744-3721.

Lukoschus, F. S., Keularts, J. L. W. 1968. Eine weitere Funktion der Mandibeldrüse der Arbeiterin von *Apis mellifica* L.- Produktion eines Pollenkeimung hemmenden Stoffes. *Z. Bienenforschung* **9**: 333-343.

Martínez, T., Fabrias, G., Camps, F. 1990. Sex Pheromone Biosynthetic Pathway in *Spodoptera littoralis* and Its Activation by a Neurohormone. *J. Biol. Chem.* **265**: 1381-1387.

Mitchell, M. J., Smith, S. L. 1986. Characterization of ecdysone 20-monooxygenase activity in wandering stage larvae of *Drosophila melanogaster*. *Insect Biochem.* **16**: 525-537.

Moritz, A., Kirchner, H., Crewe, R. M. 1991. Chemical Camouflage of the Death's Head Hawkmoth (*Acherontia atropos* L.) in honeybee colonies. *Naturwissenschaften* **78**: 179-182.

Morse, D., Meighen, E. 1984. Aldehyde Pheromones in Lepidoptera: Evidence for an Acetate Ester Precursor in *Choristoneura fumiferana*. *Science* **226**: 1434-1436.

Morse, D., Meighen, E. 1987. Pheromone Biosynthesis: Enzymatic Studies in Lepidoptera. In: Pheromone Biochemistry (Ed's G. D. Prestwich, G. J. Blomquist). Acad. Press Inc., London, pp 121-158.

Naumann, K., Winston, M. L., Slessor, K. N., Prestwich, G. D., Webster, F. X. 1991. Production and transmission of honey bee queen (*Apis mellifera* L.) mandibular gland pheromone. *Behav. Ecol. Sociobiol.* **29**: 321-332.

Obin, M. S., Van der Meer, R. K. 1985. Gaster Flagging by Fire Ants (*Solenopsis* spp): Functional Significance of Venom Dispersal Behavior. *J. Chem. Ecol.* **11**: 1757-1768.

Oliver, J. E., Waters, R. M., Lusby, W. R. 1994. A Convenient Synthesis of α Fluoro Carboxylic Acids. *Synthesis* # **3**:273-275.

Pain, J., Barbier, M., Bogdanovsky, D., Lederer, E. 1962. Chemistry and biological activity of the secretions of queen and worker honeybees (*Apis mellifera* L.). *Comp. Biochem. Physiol.* **6**: 233-241.

Pankiw, T., Winston, M. L., Plettner, E., Slessor, K. N., Pettis, J. S., Taylor, O. R. In preparation. Mandibular Gland Components of European and Africanized Honey Bee Queens (*Apis mellifera* L.).

Pierce, A. E. 1968. Silylation of Organic Compounds. Pierce Chemical Co. Rockford, Illinois, pp 35.

Plettner, E., Slessor, K. N., Winston, M. L., Robinson, G. E., Page, R. E. 1993. Mandibular Gland Components and Ovarian Development as Measures of Caste Differentiation in the Honey Bee (*Apis mellifera* L.) *J. Insect Physiol.* **39**: 235-240.

Plettner, E., Sutherland, G. R. J., Slessor, K. N., Winston, M. L. 1995. Why not be a queen?- Regioselectivity in the mandibular secretions of honey bee castes. *J. Chem. Ecol.* **21**: 1017-1029.

Rachinsky, A. 1990. Juvenilhormon und Ekdysteroide in der Kastenentwicklung der Honigbiene (*Apis mellifera* L.) Ph. D. Thesis. Eberhard-Karls-Universitat, Tübingen, Germany.

Rembold, H., Lackner, B. Geistbeck, I. 1974. The chemical basis of honeybee, *Apis mellifera*, caste formation. Partial purification of queen bee determinator from royal jelly. *J. Insect Physiol.* **20**:307-314.

Robinson, G. E. 1987. Regulation of honey bee age polyethism by juvenile hormone. *Behav. Ecol. Sociobiol.* **20**: 329-338.

Robinson, G. E., Page, R. E., Fondrk, K. 1990. Intracolonial behavioral variation in worker oviposition, oophagy and larval care in queenless honey bee colonies. *Behav. Ecol. Sociobiol.* **26**: 315-323.

Robinson, G. E., Strambi, C., Strambi, A., Feldlaufer, M. F. 1991. Comparison of Juvenile Hormone and Ecdysteriod Haemolymph Titres in Adult Worker and Queen Honey Bees (*Apis mellifera*). *J. Insect Physiol.* **37**: 929-935.

Ronis, M. J. J., Hodgson, E. 1989. Cytochrome P-450 monooxygenases in insects. *Xenobiotica* **19**: 1077-1092.

Ronis, M. J. J., Hodgson, E., Dauterman, W. C. 1988. Characterization of Multiple Forms of Cytochrome P-450 from an Insecticide Resistant Strain of House Fly (Musca Domestica). *Pesticide Biochem. Physiol.* **32**: 74-90.

Rooney, T. A. 1981. Theory of Chromatography. In: High Resolution Gas Chromatorgaphy (Ed. R. R. Freeman) Second Ed. Hewlett-Packard Co. pp 7-21.

Rosell, G., Hospital, S., Camps, F., Guerrero, A. 1992. Inhibition of a chain shortening step in the biosynthesis of the sex pheromone of the Egyptian armyworm *Spodoptera littoralis*. *Insect Biochem. Mol. Biol.* **22**: 679-685.

Ruttner, F., Koeninger, N., Veith, H. J. 1976. Queen substance bei eierlegenden Arbeiterinnen der Honigbiene (*Apis mellifera* L.). *Naturwissenschaften* **63**: 434.

Ryan, R. O., de Renobales, M., Dillwith, J., Heisler, Ch. R., Blomquist, G. J. 1982. Biosynthesis of Myristate in an Aphid: Involvement of a Specific Acylthioseterase. *Arch. Biochem. Biophys.* **213**: 26-36.

Sakagami, S. F. 1958. The false-queen: fourth adjustive response in dequeened honeybee colonies. *Behaviour* **13**: 280-296.

Schroepfer, G. J. 1966. Stereospecific Conversion of Oleic Acid to 10-Hydroxystearic Acid. *J. Biol. Chem.* **241**: 5441-5447.

Schulz, S., Toft, S. 1993. Identification of a Sex Pheromone from a Spider. *Science* **260**: 1635-1637.

Silverstein, R. M., Rodin, O. J., Burkholder, W. E., Gorman, J. E. 1967. Sex Attractant of the Black Carpet Beetle. *Science* **157**: 85-86.

Slessor, K. N., Kaminski, L.-A., King, G. G. S., Borden , J. H., Winston, M. L. 1988. Semiochemical basis for the retinue response to queen honey bees. *Nature* **332**: 354-356.

Slessor, K. N., Kaminski, L.-A., King, G. G. S., Winston, M. L. 1990. Semiochemicals of the honey bee queen mandibular glands. *J. Chem. Ecol.* **16**: 851-860.

Soliday, Ch. L., Kolattukudy, P. E. 1977. Biosynthesis of Cutin. ω-Hydroxylation of Fatty Acids by a Microsomal Preparation from Germinating *Vicia faba*. *Plant Physiol.* **59**: 1116-1121.

Soliday, Ch. L, Kolattukudy, P. E. 1978. Midchain Hydroxylation of 16-Hydroxypalmitic Acid by the Endoplasmic Reticulum Fraction from Germinating *Vicia faba*. *Arch. Biochem Biophys.* **188**: 338-347.

Stanley-Samuelson, D. W., Jurenka, R. A., Cripps, C. Blomquist, G. J., de Renobales, M. 1988. Fatty Acids in Insects: Composition, Metabolism and Biological Significance. *Arch. Ins. Biochem. Physiol.* **9**: 1-33.

Tasayco, M. L., Prestwich, G. D. 1990. Aldehyde-Oxidizing Enzymes in an Adult Moth: In Vitro Study of Aldehyde Metabolism in *Heliothis virescens*. *Arch. Biochem. Biophys.* **278**: 444-451.

Vallet, A., Cassier, P., Lensky, Y. 1991. Ontogeny of the fine structure of the mandibular glands of the honeybee (*Apis mellifera* L.) workers and the pheromonal activity of 2-heptanone. *J. Insect Physiol.* **37**: 789-804.

Vanderwel, D., Johnston, B., Oehlschlager, A. C. 1992. Cucujolide Biosynthesis in the Merchant and Rusty Grain Beetles. *Insect Biochem. Mol. Biol.* **22**: 875-883.

Vanderwel, D., Oehlschlager, A. C. 1989. Pheromone Biosynthesis in Coleoptera. *Chemica Scripta* **29**: 401-406.

Vaz, A. H., Jurenka, R. A., Blomquist, G. J., Reitz, R. C. 1988. Tissue and Chain Length Specificity of the Fatty Acyl CoA Elongation System in the American Cockroach. *Arch. Biochem. Biophys.* **267**: 551-557.

Weatherston, J., Roelofs, W., Comeau, A. Sanders, C. J. 1971. Studies of physiologically active Arthropod secretions. X. Sex pheromone of the Eastern Spruce Budworm, *Choristoneura fumiferana*. *Can. Entomol.* **103**: 1741-1747.

Weaver, N., Johnston, N. C., Benjamin, R., Law, J. H. 1968. Novel Fatty Acids From the Royal Jelly of Honeybees (*Apis mellifera* L.) *Lipids* **3**: 535-538.

Willis, L. G., Winston, M. L., Slessor, K. N. 1990. Queen honey bee mandibular pheromone does not affect worker ovary development. *Can. Ent.* **122**: 1093-1099.

Wilson, E. O. 1971. The Insect Societies. Harvard University Press. Cambridge, Mass.

Winston, M. L. 1987. The Biology of the Honey Bee. Harvard University Press, Cambridge, Mass.

Winston, M. L., Fergusson, L. A. 1985. The effect of worker loss on temporal caste structure of the honeybee (*Apis mellifera* L.). *Can. J. Zool.* **63**: 777-780.

Winston, M. L., Higo, H. A., Colley, S. J., Pankiw, T., Slessor, K. N. 1991. The role of queen mandibular pheromone and colony congestion in the honey bee (*Apis mellifera*) reproductive swarming. *J. Insect Behav.* **4**: 649-660.

Winston, M. L., Higo, H. A., Slessor, K. N. 1990. Effect of various dosages of queen mandibular pheromone on the inhibition of queen rearing in the honey bee (Hymenoptera: Apidae). *Ann. Ent. Soc. Am.* **83**: 234-238.

Winston, M. L., Slessor, K. N. 1992. The essence of Royalty: Honey Bee Queen Pheromone. *American Scientist* **80**: 374-385.

Winston, M. L., Slessor, K. N., Smirle, M. J., Kandil, A. A. 1982. The influence of a queen-produced substance, 9-HDA, on swarm clustering behavior in the honeybee *Apis mellifera* L. *J. Chem. Ecol.* **8**: 1283-1288.

Winston, M. L., Slessor, K. N., Willis, L. G., Naumann, K., Higo, H. A., Wyborn, M. H., Kaminsky, L.-A. 1989. The influence of queen mandibular pheromones on worker attraction to swarm clusters and inhibition of queen rearing in the honey bee. *Insect Soc.* **36**: 15-27.

Wolf, W. A., Roelofs, W. L. 1986. Properties of the Δ11 desaturase enzyme used in cabbage looper moth sex pheromone biosynthesis. *Arch. Insect Biochem. Physiol.* **3**: 45-52.

Wolf, W. A., Roelofs, W. L. 1989. Enzymes Involved in the Biosynthesis of Sex Pheromones in Moths. In: Biocatalysis in Agricultural Technology (ACS Symposium Series 389, Ed's: J. R. Whitaker, P. E. Sonnet). Am. Chem. Soc. pp 323-331.

Wood, G. W., Cornwell, M., Williamson, L. S. 1989. High performance thin-layer chromatography and densitometry of synaptic plasma membrane lipids. *J. Lipid Res.* **30**: 775-779.