Unusual Degradation Pathway of Pheromone Odorants in Gypsy Moth

by

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ABSTRACT

Gypsy moth is a major pest in Europe and North America. The closely related species, nun moth, is also a destructive pest in Europe. Both species use the pheromone *cis* 7(*R*), 8(*S*)-2-methyl-7, 8-epoxyoctadecane, (+)-disparlure, for sexual communication. The alkene precursor of disparlure, 2-methyl-7(Z)-octadecene, and (-)-disparlure are also of biological importance in both species.

In this thesis I describe the metabolic fate of 2-methyl-7(Z)-octadecene, (+)-disparlure and (-)-disparlure in the gypsy moth. Unexpectedly, I found that the incubation these odorants with moth extracts stimulates the formation of methyl and ethyl esters of linoleic and oleic acid. I hypothesize that the incubated compounds are degraded into smaller units and these units are then incorporated into the biosynthesis of oleic and linoleic acids. These fatty acids are then esterified into their corresponding fatty esters. The formation of these fatty esters from the incubated substrates is NADPH and FAD dependent.

DEDICATION

I dedicate this document to my dear family for their unlimited and unconditional love and support.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ACP	Acyl carrier protein
AMP	Adenosine monophosphate
BSTFA	N,N-bis(trimethylsilyl)trifluoroacetamide
CoA	Co-enzyme A
Cys	Cysteine
DMDS	Dimethyl disulphide
FAD	Flavine adenine dinucleotide
FAS	Fatty acid synthase
HPLC	High Performance Liquid Chromatography
msec.	Milliseconds
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotidephosphate
SAM	S-adenosylmethionine
UV	Ultra Violet

CHAPTER 1

1.1 Introduction

1.1.1 Chemical communication

Communication is an essential element of life. At its basic level, communication is comprised of biological functions such as sender and receiver, messages and their recognition patterns. Every signal-receptor interaction is based on non-covalent molecular interactions. Signals emitted by the sender are carried through the environment *via* molecular diffusion and convection. The partners are then brought together through active searching behaviour, as in bacterial chemotaxis [1].

Organisms interact with each other in many different ways. These interactions may occur within one species, such as mating interactions, or, happen between two different species, as in predator-prey interactions. Law and Regnier (1971) defined all chemicals acting as messages between organisms as semiochemicals [2]. Depending on whether the interactions are intra or inter-specific, semiochemicals are subdivided into two major groups, pheromones and alleochemics [3]. Depending on whether chemical cues involved in interaction are of advantage to the producing species or the receiving species, they are respectively classified as allomones and kairomones [3].

Our knowledge of odorous signals in insects dates back to the late 1800s, when freshly emerged female wild silk moths were reported to attract their males even when hidden in a house, but not when they were tightly covered by a glass cup [4]. In 1959 the

first of such intra-specific communication mediums was chemically identified for the silk moth [5]. The class of these signalling substances was called *pheromones* [6]. The word "pheromone" first appeared in the 1950s, from the Greek words "pherein" and "horman", meaning carrier of excitement. Pheromones were defined as a chemical or a blend of chemicals that is produced by an organism and that elicits a specific behavioral or physiological response in another member of the same species" [6]. Base on their effect, pheromones are subdivided into two classes: releasers elicit an immediate, short-lived behavioural response, while primers elicit a delayed long-lived physiological response [3].

1.2 Insect pheromones

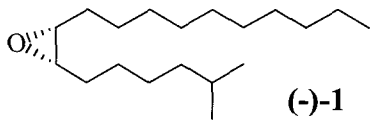
Pheromone systems of insects are among the most extensively studied over the past four decades. The pheromone components of over 1500 of the estimated 875,000 species of insects have been chemically and behaviourally elucidated [7]

Insect pheromones are multi-component blends of volatile compounds that can, in some systems, function synergistically. Often these components are attractive in a certain species-specific ratio where the relative amounts of a synergistic component can be less than 0.1%, whereas, the same components mixed in a different ratio might represent the pheromone of a different species [8]. In most cases studied to this date, one compound has shown the strongest effect (major component), this effect may further increase by other constituents (synergists). Insect pheromone structures represent a great number of chemical functional groups, however, several biological or behavioural responses such as sex, aggregation, dispersal, alarm and trail following are mediated through a multi-

component blend. For example, gypsy moth uses (+)-disparlure, +**1**, as a sex attractant, while, the nun moth uses +**1** (10%) and –**1** (90%) as its sex pheromone blend (Figure 1) [9]. Male Jeffrey pine beetles uses 1,5-dimethyl-6,8-dioxabicyclo[3.2.1]octane, **3** (Figure 1), as an aggregation pheromone [10]. The honey bee queen produces pheromones that function in both releaser and primer roles. The primer and aggregation pheromone blend in this insect contains (2E) 9-ketodec-2-enoic acid, (2E, 9R) 9-hydroxydec-2-enoic acid and (2E, 9S) 9-hydroxydec-2-enoic acid. In a pheromone blend the configuration and composition of each component can be critical for triggering the behavioural response. Insect sex and aggregation pheromones are the most widely researched classes of these categories and have had the richest contribution to the nascent science of chemical ecology [7].

Figure 1. Pheromone blend components.

(+)-disparlure, **(+)-1**, and 2-methyl-7(Z)-octadecene, **2**, in the gypsy moth; **(+)-1** and **(–)-1** in the nun moth and **3** is a component of pheromone blend in the Jeffrey pine beetle.



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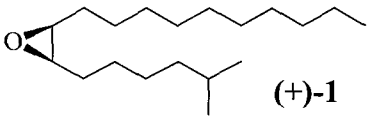

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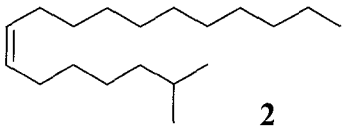

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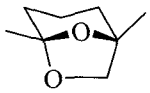

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1.3 Pheromone biosynthesis in insects

1.3.1 De novo biosynthesis or conversion of dietary host precursor

Studies show that many insects are able to biosynthesise their pheromone components *de novo.* However, there are several reported cases where a host precursor is utilized directly or converted to a pheromone component through a simple chemical transformation [7]. For instance, males of ornate moth convert monocrotaline, a pyrrolizidine alkaloid from their food plant, to (R)-(-)-hydroxydanaidal, a courtship pheromone in this insect [11]. Fatty acid derived pheromones are typical examples for *de*

novo pathway. This group of pheromones are described in section 1.3.3. However, due to the importance of *de novo* biosynthesis of fatty acids in this report, I will first discuss the mechanisms involved in this biosynthesis in more detail.

1.3.2 Fatty acid biosynthesis

Long chain fatty acids (16 and 18 carbons) are important substrates for the cell energy metabolism. In addition, they are essential constituents of membrane lipids. Fatty acids are supplied by the diet or synthesized *de novo* by cytosolic or microsomal fatty acid synthase enzymes.

In insects, fatty acids have special functions as precursors to some hydrocarbons and pheromone components [12]. Insect hydrocarbons are complex mixtures of straight-chain saturated, unsaturated and methyl branched compounds. The major role of these hydrocarbons is to protect insects against desiccation and participate in chemical communication processes [13]. To convert to hydrocarbons, fatty acids are first elongated to very long chain fatty acids (VLCFA). They are then reduced to aldehydes and furthermore to hydrocarbons with one carbon shorter [14]. In insects, the biosynthesis of straight-chain saturated and unsaturated fatty acids was reported in the 1960s [15, 16]. However, methyl-branched fatty acids were first described in the integument of *Blatella germanica* [17]. Fatty acid synthase activity has also been found in the microsomal and cytosolic fractions of the egg of *Triatoma infestans.* The incorporation of [^{14}C] malonyl-CoA and [^{3}H] acetyl-CoA into palmitic acid was observed in this insect [14]. In the housefly, *Musca domestica* L. eighteen methyl-branched fatty acids were identified. The

incorporation of methylmalonyl-CoA into methyl-branched fatty acids increased with increasing concentration of this compound [18].

Fatty acids are synthesized from acetyl-CoA, malonyl-CoA and NADPH [19]. The absence or presence of methylmalonyl-CoA determines whether the fatty acid formed is straight or branched chain. Fatty acid biosynthesis involves initial carboxylation of acetyl-CoA to malonyl-CoA. This reaction requires ATP, CO_2, and the coenzyme biotin [20], [21]. The acetyl group elongates by two carbon units from malonyl-CoA *via* Claisen condensation in a stepwise manner [19]. Radiotracer studies proved that the carboxyl group introduced into malonyl-CoA is simultaneously lost by a decarboxylation reaction during the Clasien condensation [20] The nature of this reaction became known from studies of fatty acid synthesis in cell-free extracts of *Escherichia coli* [22].

Fatty acid biosynthesis is catalyzed by a multifunctional enzyme known as fatty acid synthase (FAS). In animals, FAS contains all of the catalytic activities required for initiation, elongation and release of C16 and C18 fatty acids [19]. Fatty acid synthase protein contains an acyl carrier protein (ACP) binding site as well as an active site cysteine (Cys) residue [20], [19, 23].

Acetyl-CoA and malonyl-CoA are converted into enzyme-bound thioesters (Figure 2). The β-carbon of the malonyl group is then condensed to an acetyl group with a simultaneous release of CO_2 giving an acetoacetyl product. The latter is stereospecifically reduced to the corresponding β-hydroxyester. The presence of NADPH is essential for this reduction. The reduction is followed by elimination of water from β-

hydroxyester to generate α,β-unsaturated ester. The latter ester is further reduced to its saturated form, consuming NADPH again. The chain is transferred to the thiol of Cys. The process is repeated several more times, with each cycle the acyl group is elongated by a two-carbon unit to yield a even numbered fatty acyl thioester derivative. The fatty acyl derivative is either hydrolysed to its corresponding fatty acid or converts to its fatty acyl-CoA derivative. Fatty acid ethyl esters (FAEE), which are important non-oxidative metabolites of ethanol, can form from ethanol and fatty acyl-CoA or non-esterified fatty acid, catalysed by acyl-CoA : ethanol *O*-acyltransferase (AEAT) [24, 25] or FAEE synthase respectively [26]. In a fatty acid biosynthesis system, the acyl derivative intermediates remain tightly bound to the enzyme during the product formation, therefore, the shorter chain products are not released [19].

Figure 2 Fatty acid biosynthesis mechanism from acetyl and malonyl-CoA.
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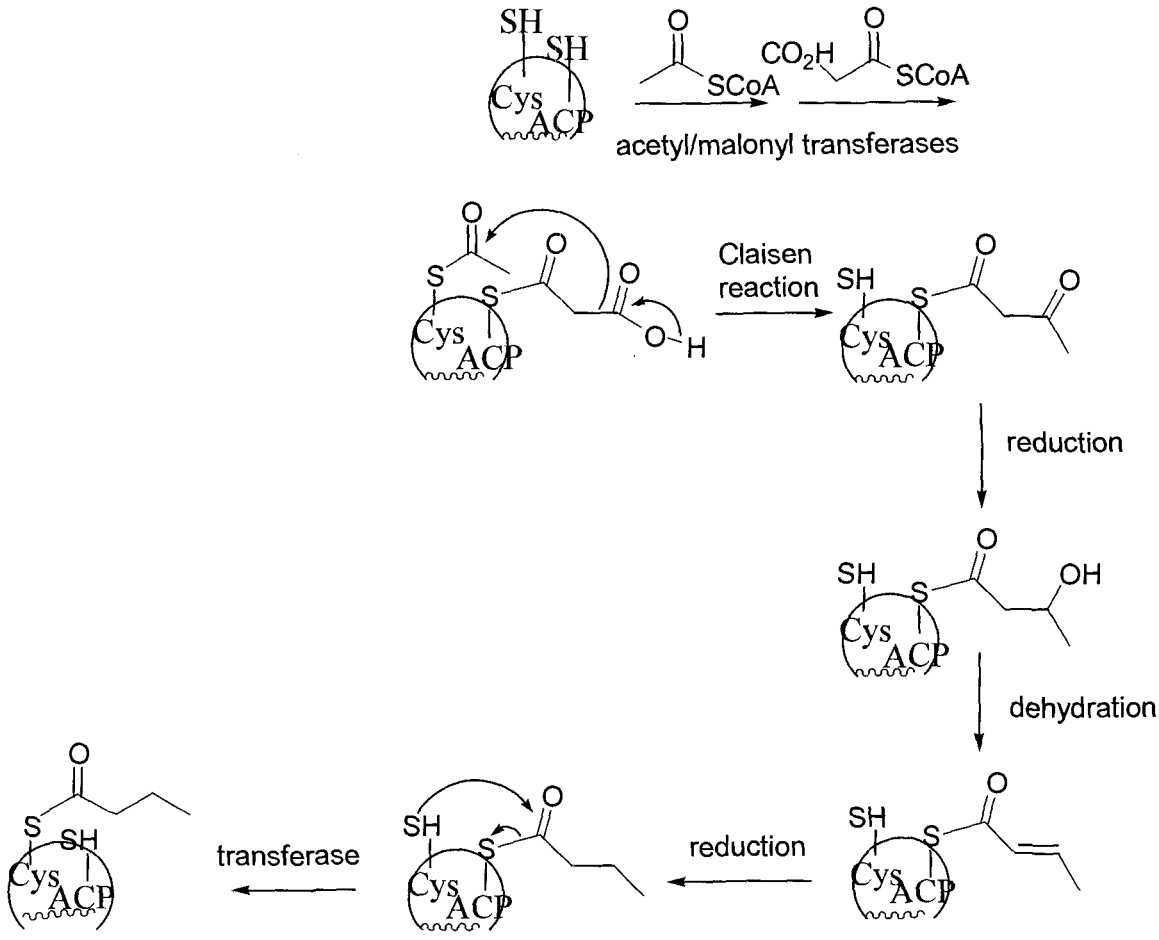

1.3.3 Fatty acid derived pheromones (*de novo* biosynthesised pheromones).

As mentioned before, fatty acid derived pheromones are a class of *de novo*–synthesized pheromones. In the gypsy moth, the biosynthesis of *cis* 7(*R*), 8(*S*)-2-methyl-7,8-epoxyoctadecane, the sex pheromone of this insect, begins with the biosynthesis of a hydrocarbon, 2-methyl-7(Z)-octadecene, in oenocyte cells located in the abdomen [27]. The biosynthesis of this even chain-length hydrocarbon is initiated using valine to supply

the carbon for the 2-methyl branch. The biosynthesis proceeds by chain elongation to 19 carbons and an unusual Δ12 desaturation followed by a decarboxylation. After being synthesized, the precursor hydrocarbon is transported to the sex pheromone gland and transformed to the corresponding epoxide by addition of oxygen across the double bond [27]. Another example is seen in females of *Tenebrio molitor,* which synthesize 4-methyl-1-nonanol *via* a fatty acid biosynthesis mechanism initiating with one unit of propionate followed by an incorporation of a second propionate unit to provide the methyl branch [28].

In *Carpophilus freemani* the biosynthesis of 2E,4E,6E-5-ethyl-3-methyl-2,4,6-nonatriene, an unusual ethyl branched component, was studied using ^{13}C and ^{2}H labeled precursors [29]. The biosynthesis of this hydrocarbon is initiated by one acetate unit and elongated with a propionate and a butyrate unit to provide the methyl and ethyl branch respectively. In *B. germanica* the biosynthesis of 3,11-dimethylnonacosan-2-one involves a fatty acid biosynthesis pathway from malonyl-CoA and methylmalonyl-CoA. The biosynthesis of the intermediate, 3,11-dimethyloctadecanoyl-CoA, involves the substitution of malonyl-CoA with methylmalony-CoA at specific points during chain elongation to provide the two methyl branches [12]. Radiotracer studies *M. domestica* and *B. germanica* showed that propionyl-CoA, derived from one of the amino acids valine, isoleucine, or methionine, is the source of the methyl branching unit, methymalonyl-CoA [30, 31].

1.3.4 Pheromones biosynthesised *via* modification of host compounds

Pheromone biosynthesis can be exclusively or partially *de novo*. Studies show that some insects utilize host precursors for their pheromone biosynthesis. For instance, females of the salt marsh caterpillar moth, and the ruby tiger moth convert host derived linolenic acid (Z9,Z12,Z15-octadecatrienoic acid) to the sex pheromone component of Z3,Z6-*cis*-9,10-epoxyheneicosadiene. L inolenic acid is first elongated by four carbons followed by decarboxylation and epoxidation to the C_{21} epoxide [32].

Terpene-derived pheromones are also common examples for this class of pheromones. In *A.grandis,* t ritium labeled geraniol and nerol, two geometric isomer terpenoids from the host plant were incorporated into the four cyclic monoterpenoid pheromone components after these substrates were injected to the insect [33].

It is believed that the biosynthesis of monoterpene alcohol or ketone pheromones from host monoterpenes involves an allylic oxidation followed by an additional oxidation, hydrogenation or rearrangement of the carbon skeleton [34]. The oxidative steps are most likely catalysed by oxidases. Studies show that oxidative reactions of the host terpenes can be highly stereo- and enantio-selective. In *Dendroctonus terebrans*, a microsomal P450 exhibits high specificity for the host monoterpene α-pinene [35]. Many species of leaf beetles secrete iridoids, a group of cyclopentanoid monoterpenoids, as a defense against predators. Using stable isotope techniques, it has been shown that some leaf beetle larvae can synthesize these iridoids *de novo* as well as sequester plant-produced molecules [36].

1.4 Insect pheromone olfaction

The importance of pheromones differs from species to species. For insects, pheromones are indispensable for reproduction and, in social insects for maintaining their social interactions. As mentioned before, pheromones are blends of volatile compounds produced by various glands in the insect body. A pheromone blend often includes more than two components. After being biosynthesised, pheromones are air transported to the vicinity of recipient insect. Each component in the pheromone blend is perceived by a specific type of receptor cell, called an olfactory specialist. Olfactory receptors in insects are located in the antennae, which are sometimes branched into sensilla. The olfactory neurons and receptors are located inside each of these sensilla surrounded by aqueous lymph. Unlike vertebrates, in insects, these receptor cells are morphologically and physiologically well-defined units [37]. The degree of sensitivity of pheromone receptor cells are such that male moths are attracted to female moths over distances of one kilometre or more [38, 39]. This extreme sensitivity of moths to pheromone is related to the optimal geometry and arrangement of sensilla on the insect antenna for trapping molecules from the air space. A protective cuticle covers the surface of each antennae sensillum. Inside the sensillum a cascade of well-organized and coordinated events takes place and these events constitute the olfactory process. Subtle differences in this process can differentiate species. Thus, the antennae serve as a kind of olfactory lens which concentrates the molecules on the sensillar hairs with the receptor cell dendrites. Olfactory transduction begins with the adsorption of pheromone molecules on the waxy surface of sensory hairs on the antenna. These molecules will then diffuse to the inner face of the cuticule through pores. From the surface, they are bound to pheromone-

binding proteins (PBPs) and transported through the aqueous lymph to the receptor-cell membrane. This is an essential step for olfaction due to the insolubility of the pheromone molecules in the lymph [40]. Originally, it was hypothesized that the proteins, (odorant binding proteins (OBP) and pheromone binding proteins (PBP)), acted principally as carries or solubilizers of the odorant molecule [41]. It has also been speculated that the binding proteins protect the odorant molecule from degradation enzymes [42], [43]. Once transported to the vicinity of the receptor, the odorant molecule-binding protein complex stimulates the receptor [42, 44, 45]. Stimulation of the olfactory receptor triggers a secondary messenger leading to the opening of sodium ion channels (Steinbrecht 1999) and thus a subsequent neural signal down the olfactory nerve to the brain. Tritium-labelled pheromones are important tools to measure the percentage of the stimulus molecules adsorbed by the moth antennae from the odour source. They were also used to determine the velocity of pheromone transport to the receptor cells.

During exposure to pheromone, the amount of the uptake of pheromone by the antennae increases. However, the electrophysiological response does not increase indefinitely during constant stimulation, yet, it starts to decline immediately after stimulus decline. Thus, the pheromone molecules need to be constantly removed from cuticular surfaces and be prevented from accumulation in order to maintain high sensitivity and rapid electrophysiological response [46]. If they readily desorb, they would provide false sensory information. Therefore, the reasonable sequence is that the stimulant molecule is inactivated by either binding to binding proteins [47] or by enzymatic degradation [48], or both.

1.5 Pheromone degradation

Degradation of the resident pheromone components from antennal and body surfaces is essential in olfaction to maintain efficacious sensitivity to new incoming pheromone components. Insects possess substrate-specific catabolic enzymes in order to clear the pheromone from their sensory tissues. The biochemistry of this phenomenon has been studied using ^{3}H-labeled pheromones and pheromone analogues. The application of radioactive probes has led to discovery and elucidation of esterase [41], epoxide hydrolase [49] and aldehyde dehydrogenase activities [50] in moths. For example, in the gypsy moth both enantiomers of disparlure, the sex pheromone of this moth, are metabolised by epoxide hydrolase(s) located in the antennae of adult males [51]. Epoxide hydrolases add water to strained 3 membered ring epoxides. Evidence suggests that the hydration of the epoxide occurs by an attack on one epoxide carbon by activated water resulting in inversion with retention of configuration of the other carbon [52]. However, a two-step mechanism involving an attack by a nucleophilic acid instead of direct attack of active water on the epoxide was suspected [53]. Body scales of the wild silk moth, *Antheraea polyphemus*, were also found to have a potent esterase which metabolised 25% of the air-adsorbed pheromone to the corresponding alcohol in 5 min [54]. The pheromone esterase showed tissue and sex specificity in this insect. In the presence of physiological concentrations, the pheromone has a maximum estimated half-life of 15 msec, suggesting that the enzyme acts as a rapid inactivator to maintain a low stimulus noise level in the sensillum antennae [55]. The pheromone mixture of *Antheraea polyphemus* also contains an aldehyde the degradation of which is associated with an antenna-specific aldehyde oxidase found in this moth [56]. In *Choristoneura fumiferana,*

in vivo incubation of the major pheromone component, (E)-11-tetradecenal, resulted in conversion of this compound to (E)-11-tetradecenoic acid as the only metabolite observed. The domestic silk moth, *Bombyx mori*, utilizes an alcohol (bombykol) and an aldehyde (bombykal) as pheromones. An aldehyde oxidase from *Bombyx mori* which is also unique to the antennae has been reported [56]. In general, in odorant metabolism, substrates are chemically modified to more water-soluble intermediates. In these pathways, oxygen may be added to a specific substrate and/or electrons removed from the substrate resulting in substantially more oxidised product than the parent substrate. For example, the addition of a hydroxyl group by cytochrome P450-dependent monooxygenases is a common initial step in metabolising a variety of compounds [57]. The hydroxylated intermediates will then undergo subsequent oxidation(s) by various oxidoreductases such as alcohols and aldehyde dehydrogenases. Evidence suggests that for each class of molecule represented in a pheromone blend, there exists an antennal (sensillar) enzyme capable of metabolising it. This in turn, supports the suggestion that rapid pheromone degradation plays a critical role in pheromone perception. In addition, antenna-specific enzymes may be found in antennae of both sexes, however, they are usually enriched in antennae of the male [58]. These enzymes may serve a dual function, optimising the perception of both pheromones and general odorants. The latter becomes important in those species that feed or find their habitats via their general sense of smell [58].

While first product metabolites of pheromones have been the main focus in these studies, it is not valid to assume that the first product of pheromone degradation is behaviourally inert. In other words, pheromone-clearance requirement to maintain

optimal olfactory sensitivity is not necessarily a one-step mechanism. For instance, when antennae of male European corn borer, *Ostrinia nubilalis*, were treated with 11-tetradecenyl acetate (the insect uses geometric isomer mixtures of 11-tetradecenyl acetate as pheromone), the pheromone component was effectively hydrolyzed by antennal esterase and the resulting alcohol also disappeared [59]. However, coevaporation of (Z)-11-tetradecenol with the pheromone component inhibited the upwind flight response of the male insect [60]. Therefore, it is essential that the alcohol, the first degradation metabolite, be degraded further, in order to not interfere with the pheromone perception. Further studies by Klun et al. (1992) revealed that in male European Corn Borer when the labelled pheromone was applied to male antennae, it was converted to the alcohol, and the only other major radiolabeled metabolite observed was tritiated water [61]. This observation along with the detection of a trace radiolabeled catabolic pool of the 11-tetradecanoic acid suggested that clearing of the pheromone from male European corn borer antennae involves hydrolysis and oxidation of the alcohol to fatty acid and consequently the degradation of the fatty acid, via β-oxidation, to acetate units [61]. β-Oxidation is a well-known degradation pathway of fatty acids (Figure 3) [62]. In this pathway, carbon 3 of the acid undergoes a twofold oxidation to yield a β-ketoacyl that cleaves to form acetic acid and a fatty acid with two carbons shorter than the starting acid. The shortening process continues until the starting acid is degraded completely to acetyl-CoA. The acetyl-CoA can be further oxidized to CO_2 and water by entering the tricarboxylic acid cycle.

Figure 3. β-Oxidation mechanism of fatty acids.

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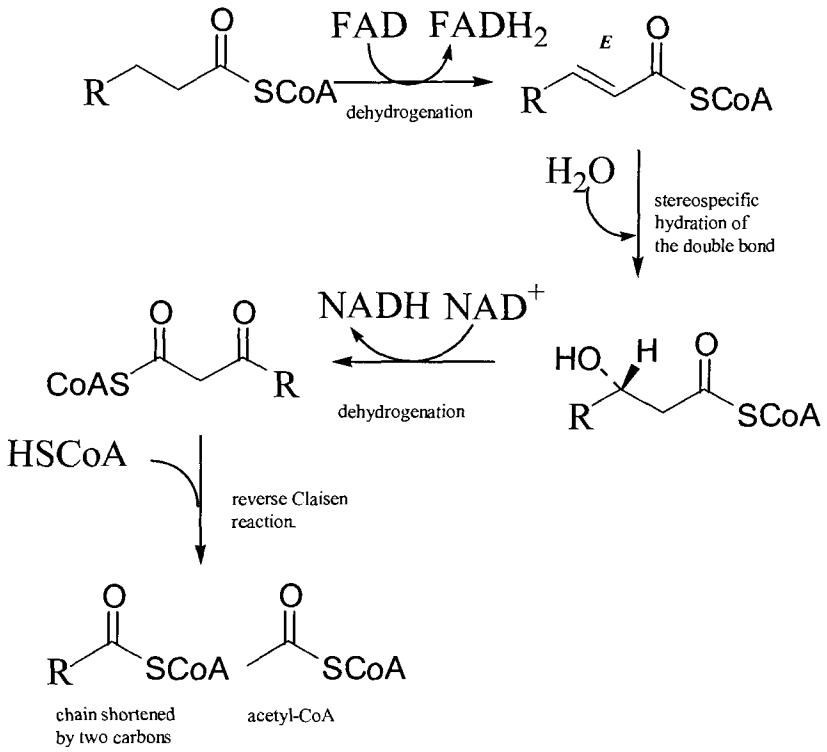


1.6 Gypsy and Nun Moth; Sexual communication, sex pheromone blends in the two species.

Gypsy moth, *Lymantria dispar*, is a major pest of decidious forests in Europe and North America [63]. The closely related species, nun moth, *Lymantria monacha,* is a destructive pest of coniferous forests in Europe [64]. Both gypsy and nun moths use the pheromone disparlure (Fig. 1) for sexual communication [65], [66], [67]. Electroantennographic studies showed that gypsy moths produce almost 100% (+)-disparlure while nun moths produce both enantiomers with about 90% being (-)-disparlure [68]. Males of both species were attracted by *cis* 7(*R*), 8(*S*)-2-methyl-7, 8-epoxyoctadecane, (+)-disparlure [66, 69]. However, the addition of *cis* 7(*S*), 8(*R*)-2-

methyl-7, 8-epoxyoctadecane, (-)-disparlure, to the (+)-enantiomer suppressed the attraction of the gypsy moth males, while it did not alter the attraction of males of the nun moth [68]. Gyspy moth has two distinct populations of receptors for the disparlure enantiomers (high sensitivity for the (+), low sensitivity for the (-) enantiomer) [70], whereas nun moth has two receptors both of which sensitive to (+)-disparlure and no receptor for (-)-enantiomer was detected in the nun moth [68]. In other words, (-)-disparlure is a behavioural antagonist in gypsy moth while in nun moth this compound is behaviourally inactive [9, 71]. Therefore, it is thought that (-)-disparlure contributes to reproductive isolation between the two species [68]. The alkene precursor of disparlure, 2-methyl-7 (Z)-octadecene, is present in gland extracts of both species [67, 72]. The presence of this alkene reduces the attractiveness of (+)-disparlure for the gypsy moth while increases the attractiveness of the (+)-disparlure for the nun moth [9]. Thus, the hydrocarbon has been proven to be a behavioural antagonist in the gypsy moth while a synergist in the closely related nun moth [73]. Therefore, in the gypsy moth, the catabolism of 2-methyl-7 (Z)-octadecene is essential for efficacious attractiveness of (+)-disparlure and prevention of cross attraction between the two closely related species.

1.7 The objective of this thesis

In this thesis, I describe a new pheromone degradation pathway in the gypsy moth. Unexpectedly, I found that **(+)-1**, **(-)-1** and alkene **2** stimulate the formation of linoleic and oleic acid methyl and ethyl esters. I describe the work that has led to the discovery and identification of these insect produced methyl and ethyl esters.

CHAPTER 2

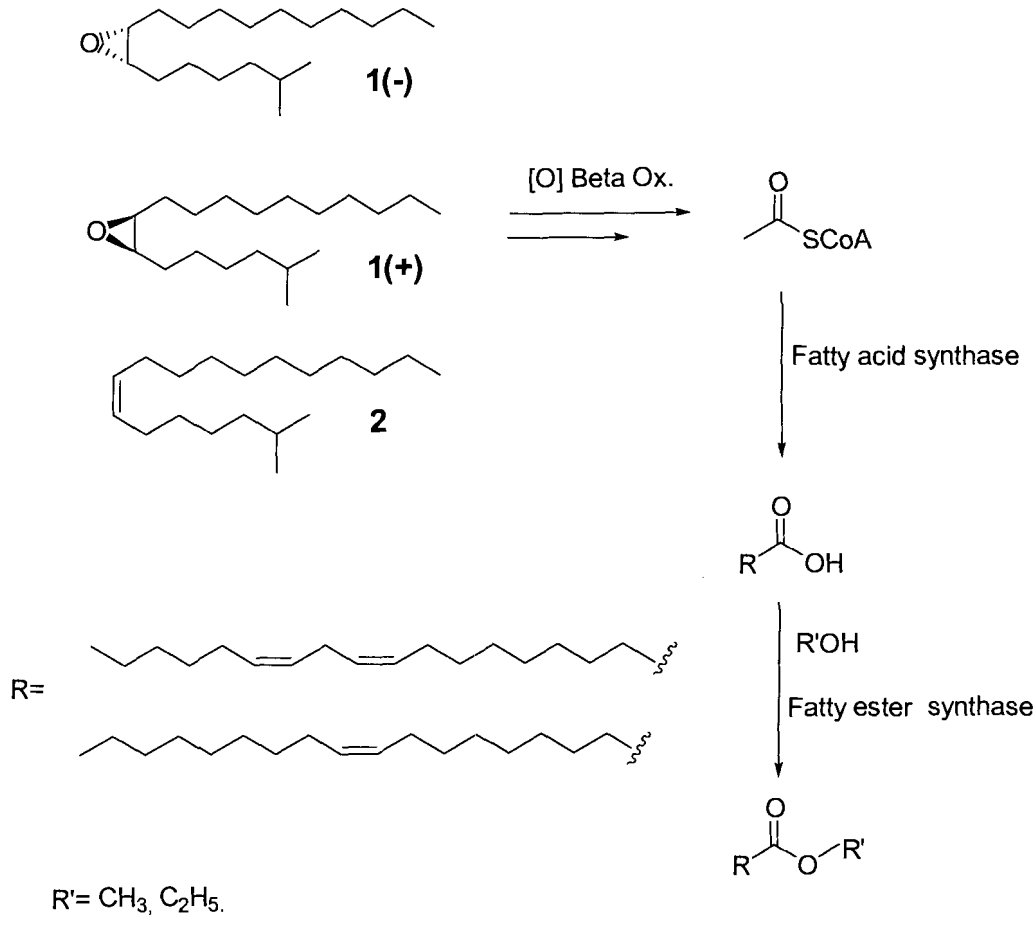
2.1 Introduction

As we discussed in the previous chapter, the clearance of (+) and (-)-disparlure and the alkene precursor of disparlure, 2-methyl-7 (Z)-octadecene, from gypsy moth's body is essential in order to maintain efficacious pheromone perception in this moth.

This chapter represents the experiments that led me to first identify the two sets of homologous compounds formed upon *in vitro* incubation of gypsy moth extracts with these biologically important substrates in this moth. Due to the fact that only a few nanograms of each product obtained in every treatment, I had no choice but carry out functional group tests to confirm the final product identity. Similar to other bioconversions in the nature, the observed bioconversion was dependent on the presence of some cofactors. Therefore, a series of other experiments was carried out to screen the cofactors necessary in this bioconversion. Further investigation, utilizing 1-^{13}C glucose, revealed the involvement of a fatty acid synthase in the product formation. Ultimately, the results we obtained from these series of experiments, led me to hypothesize that the incubated compounds **(+)-1**, **(-)-1** and alkene **2** are degraded into acetate units and these units are then incorporated in the biosynthesis of the observed products as shown in figure 1. This hypothesis will be discussed in detail in the next chapter.

Figure 4 Degradation of (+)disparlure, (-)- disparlure and alkene, **2.**

These odorants are degraded into acetate units *via* β-oxidation. The acetate units are then incorporated into methyl and ethyl esters of linoleic and oleic acids.



2.2 Experimental

2.2.1 General material and methods:

Male gypsy moth pupae were reared by Agriculture Canada. They were emerged individually in tall petri dishes in an incubator at 19 °C. The newly emerged moths were frozen at -35°C.

Solvents were distilled in glass prior to use, alkene **2** was synthesized by E.Plettner Dept. Chem. S.F.U., disparlure was a gift from G.D Prestwich U.Utah and from Dr. G.Gries Dept. Bio. S.F.U., D-Glucose-1-^{13}C was from *ISOTEC,* purchased through *Sigma*, oleic and linoleic methyl and ethyl esters were purchased from *Sigma*, and methyl disulfide was purchased from *Aldrich*.

GC/MS spectra were obtained on a Varian CP 3800 gas chromatograph, equipped with a DB5 column and interfaced with a Saturn 2000 ion trap mass spectrometer. The chromatograph was operated in splitless mode on the following programs; **a)** Injector 220°C, column oven (140 °C (1.00 min), 20°C/min to 205°C (11.00 min)), 40°C/min to 260°C (4.37 min). Mass spectra were recorded from 5-14 min. **b)** Injector 220°C, column oven (140 °C (1.00 min), 20°C/min to 205°C (11.00 min)), 40°C/min to 260°C (9.37 min). Mass spectra were recorded from 5-24 min. **c)** Injector 220°C, column oven (100 °C (1.00 min), 10°C/min to 220°C (11.00 min)), 40°C/min to 260°C (4.37 min). Mass spectra were recorded from 5-16 min.

2.2.2 Experiments

2.2.2.1 Incubation of (+)-1, (-)-1, alkene 2 and 1-^{13}C glucose with gypsy moth extracts

a. Moth extract preparation.

Antennae, legs and wings were cut and extracted with Tris buffer (80 mM with 400 mM KCl, pH 6.6) containing phenylmethanesulfonylfluoride (PMSF 10µl of mg/25µl stock in ethanol for every 1.5ml of buffer) and 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF 1mg in 1ml of water). Approximately 10 moth equivalents were used for each experiment. Antennae, legs and wings were removed and extracted in Ependrof tubes with ~ 220 µl of buffer. The tubes were then centrifuged at 10,000 ×g for 10 min. The supernatant ~70 µl was collected and used immediately in an assay.

b. Incubation of moth extracts with substrates, +1, -1, 2 and 1-^{13}C glucose.

Moth extracts were aliquoted for individual treatments (15 µl/ treatment). Each aliquot was treated with necessary cofactors such as NADPH (7.5×10^{-4} µmol), NADH (1.0×10^{-3} µmol), FAD (2.5×10^{-4} µmol.), glucose (0.03 µmol) and glucose dehydrogenase (0.02 unit/treatment). The mixtures were then treated with 0.013 mmol of the substrate and incubated for 8 hours at 4°C. The treatments were then quenched with saturated NaCl (5 µl) and the products were extracted with freshly distilled ethyl acetate (2×50 µl two times). The extracts were dried over Na_2SO_4 and subjected to GC and GC-MS analysis.

2.2.2.2 Functional group tests

a. Transesterification of products obtained in 2.2.2.1, b.

Organic products obtained from incubation of moth extracts with substrates were transesterified as in [74], by taking 20 µl of each treatment (< 8 ng. of each product) and treating it with 20 µl of a) 0.01 mmol KOH in MeOH, b) 0.01 mmol KOH in EtOH.

b. Hydrogenation of the products obtained in 2.2.2.1, b.

Hydrogenation of the products was performed a) in the presence of palladium, 5 wt.% on calcium carbonate, poisoned with lead, (Lindlar catalyst)[1], b) in the presence of palladium on charcoal. In each case a treatment containing < 20 ng of each product was diluted with distilled hexane and treated with catalytic amount of the catalyst and flushed with hydrogen for 3 hours. The mixture was then filtered and examined using the GC/MS.

c. Separation of the first set of products obtained in 2.2.2.1,b.

As mentioned earlier in this chapter, two sets of homologous compounds formed upon *in vitro* incubation of gypsy moth extracts with **+1**, **-1**, and alkene **2**. Here, I separated the first two products, of lower molecular weight and therefore lower boiling point than their homologous compounds, from each other.

Several treatments were pooled together to provide us with a higher concentration of the products. The concentrated sample (50 µl) was injected into a HPLC, coupled to a UV detector, with a reverse phase column and eluted with H_2O (1% vol.)/ CH_3CN

[1] The efficiency of the catalyst was tested by hydrogenation of few milligrams of an alkyne standard under similar conditions.

(99%vol). Compounds A and B (Figure 5) were separated, and, >250 ng of each product was obtained.

d. Dimethyldisulfide derivatization of the products.

Each purified compound (200-400 ng) was individually dissolved in 70 µl of dimethyldisulfide in the presence of catalytic amount of iodine. The reaction mixture was heated at 60° C for 48 hours. The reaction was quenched with aqueous $Na_2S_2O_3$ as in [75].The organic phase was extracted with ethyl acetate and examined on GC/MS. Standards of DMDS adducts were synthesized from commercial methyl linoleate and oleate by similar procedure.

2.2.2.3 Cofactor requirement examination for product formation.

a. Is FAD an essential requirement in this bioconversion?

Moth extracts were prepared as in part 2.2.1, a. Alkene **2** was then incubated with moth extracts as in 2.2.1, b, however, coenzyme FAD was not added in this experiment.

b. Are CoASH and ATP essential requirements in this bioconversion?

Moth extracts were prepared as in part 2.2.1, a. Alkene **2** was then incubated with moth extracts as in 2.2.1, b. In addition, extracts were treated with 0.5µl of CoASH (4 mg/ml) and 0.5µl of ATP (8 mg/ml) solution.

c. Is S-adenosylmethionine (SAM) the source of methyl in the methyl esters?

The methyl building unit can be supplied from L-methionine and is introduced by a nucleophilic substitution reaction. In nature, the leaving group is enhanced by converting L-methionine into S-adenosylmethionine [76].

Moth extracts were prepared as in part 2.2.1, a. Alkene **2** was then incubated with moth extracts as in 2.2.1, b. In addition, extracts were treated with 0.5µl of 0.4 mg/ml of SAM solution.

2.2.2.4 Incorporation of labelled alcohols, methanol and ethanol, in the final products.

Moth extracts were prepared as in part 2.2.1, a. Alkene **2** was delivered in a) C_2D_5OH (8% *vol.*) / C_2H_5OH (92% *vol.*), b) CD_3OD (8% *vol.*) / CH_3OH (92% vol.) and incubated with moth extracts as in 2.2.1, b.

2.2.2.5 Incubation of moth extracts with alkene 2, optimal mixture of cofactor and excessive aeration.

Moth extracts were prepared as in part 2.2.1, a. Alkene **2** was then incubated with moth extracts as in 2.2.1, b. The treatments were aerated for the first two hours of incubation.

CHAPTER 3.

3.1. Introduction

The formation of four products, A, B, C, D, was observed in the organic phase of the incubated moth extracts with substrates as shown in Figure 5 (see 2.2.2.1, chapter 2). Solvent extracted products were examined on a non-polar GC column using GC programs **a** and **c** mentioned in experimental section of chapter two. The fragmentation patterns of these products by EI GC-MS were consistent with those of methyl and ethyl esters: loss of m/z 32, $[CH_3OH]^{\cdot+}$, for A and B and observing the homologous pattern for C and D followed by loss of m/z 74 fragment resulting from the familiar McLafferty rearrangement, a cleavage one bond removed from carbonyl group. The retention times and the fragmentation patterns by EI GC-MS of products A to D were compared to standards of methyl and ethyl esters of usual fatty acids found in insects such as palmitic, stearic, linoleic and oleic acids. In all cases the retention times and the fragmentation patterns of products A to D were consistent with those of methyl and ethyl esters of linoleic and oleic acids (Fig. 6). In addition, products A and B were examined on polar DB17, DB23 and DB210. In all cases, the retention times of these products were consistent with those of methyl linoleate and oleate (Table 1). The CI spectrum of each product also matched the CI spectrum of the corresponding commercial standards (Figure 7).

To further confirm the identity of these products I carried out three sets of functional group test experiments (see chapter 2).

In this chapter I present the results obtained upon performing the functional group test sets of experiments. Once the identity of the products was established, I studied the cofactors required to support the pathway.

Table 1 Retention time of standards of methyl linoleate and oleate examined on different GC columns[2].

GC columns	Program [3]	Methyl linoleate (min)	A (min)	Methyl oleate (min)	B (min)
DB 17	c	15.0	15.0	14.8	14.8
DB 23	c	12.13	12.13	11.74	11.74
DB 210	c	10.5	10.5	10.4	10.4
DB 5	a	11.3	11.3	11.5	11.5

In all cases the retention times matched those of compounds A and B.

[2] I am grateful to R. Gries for allowing me to use their several GC columns.
[3] Please refer to chapter 2, 2.2.1.

Figure 5 GC spectra of solvent extracted products from incubation of alkene **2** with moth extracts.

a. Substrate blank, alkene **2** (delivered in EtOH) incubated with buffer and cofactors, NADPH and FAD. **b**. Moth extract blank, moth extract and cofactors, NADPH and FAD. **c.** Solvent extracted products, moth extract, cofactors, NADPH and FAD and alkene **2**.

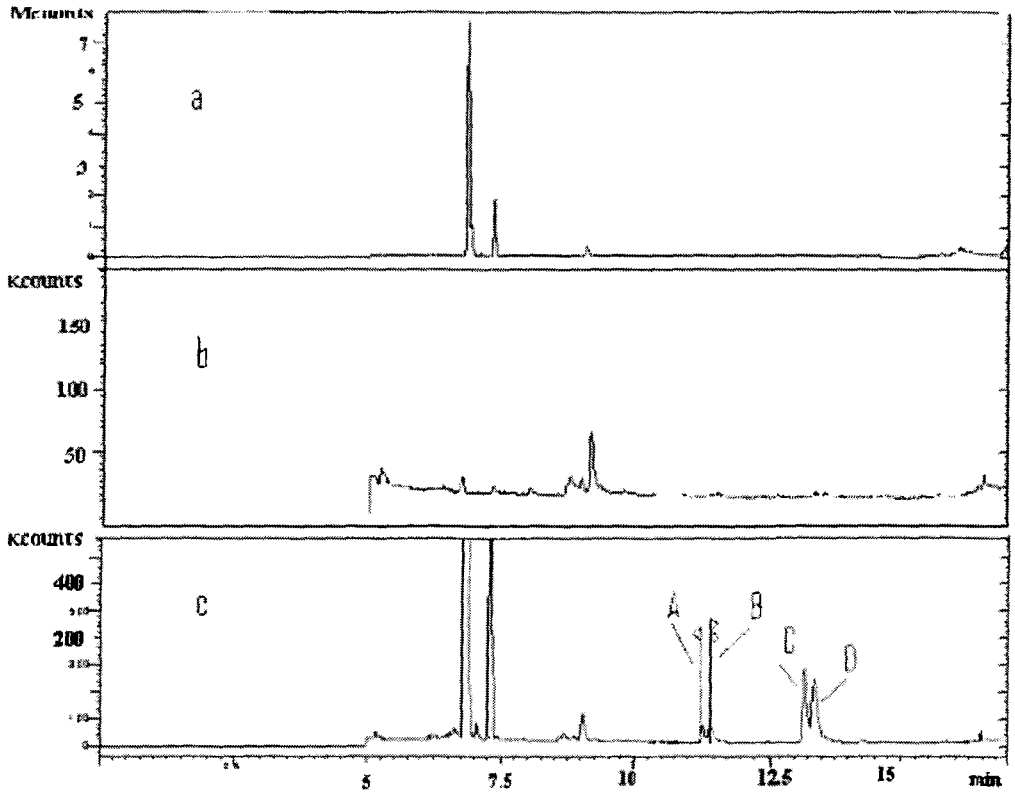


Figure 6. Mass spectra of compound A, B, C, and D.

The compounds fragment analogous to methyl and ethyl esters of linoleic and oleic acids respectively. Loss of the alcohol portion from M^{+} or M+1 is observed (m/z=264 for oleates m/z=262 for linoleates).

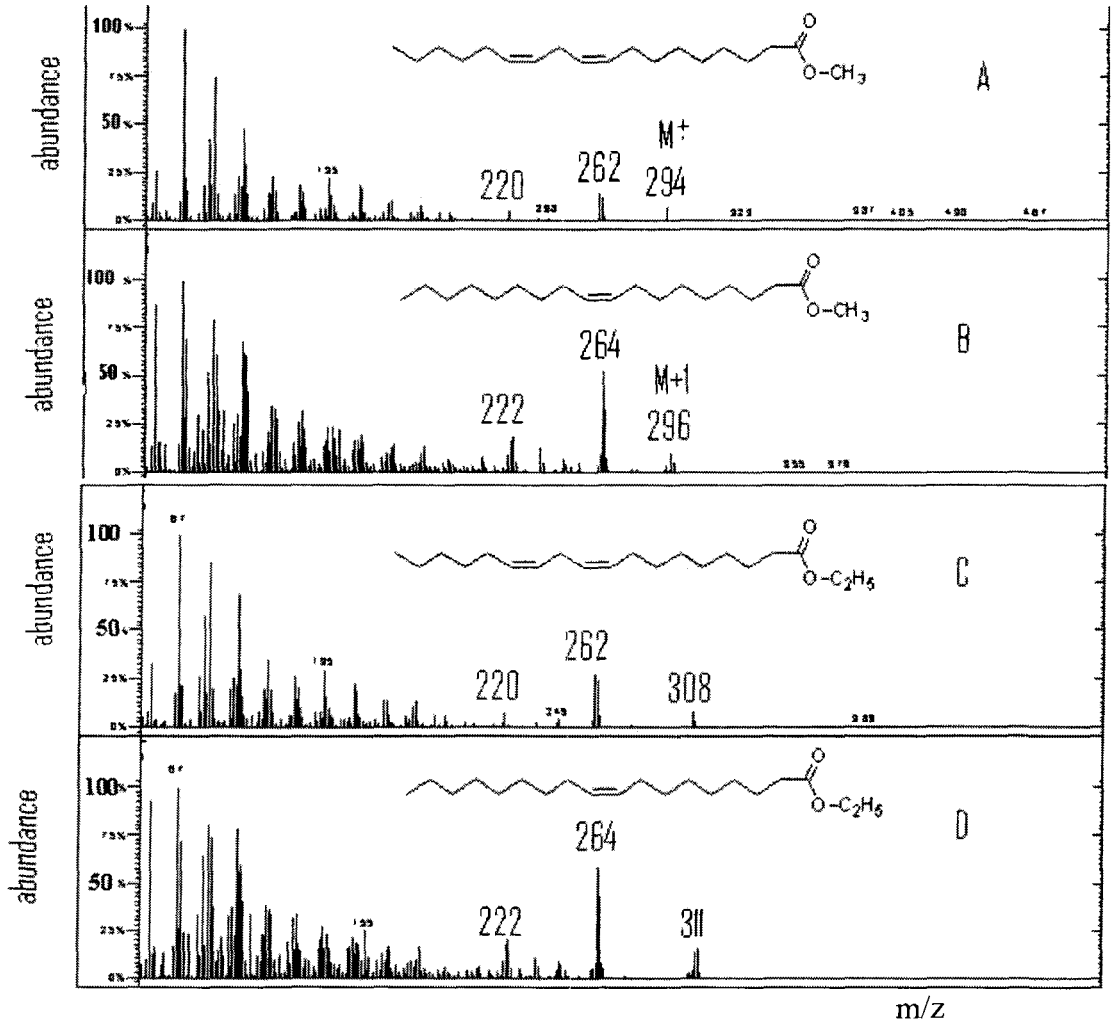
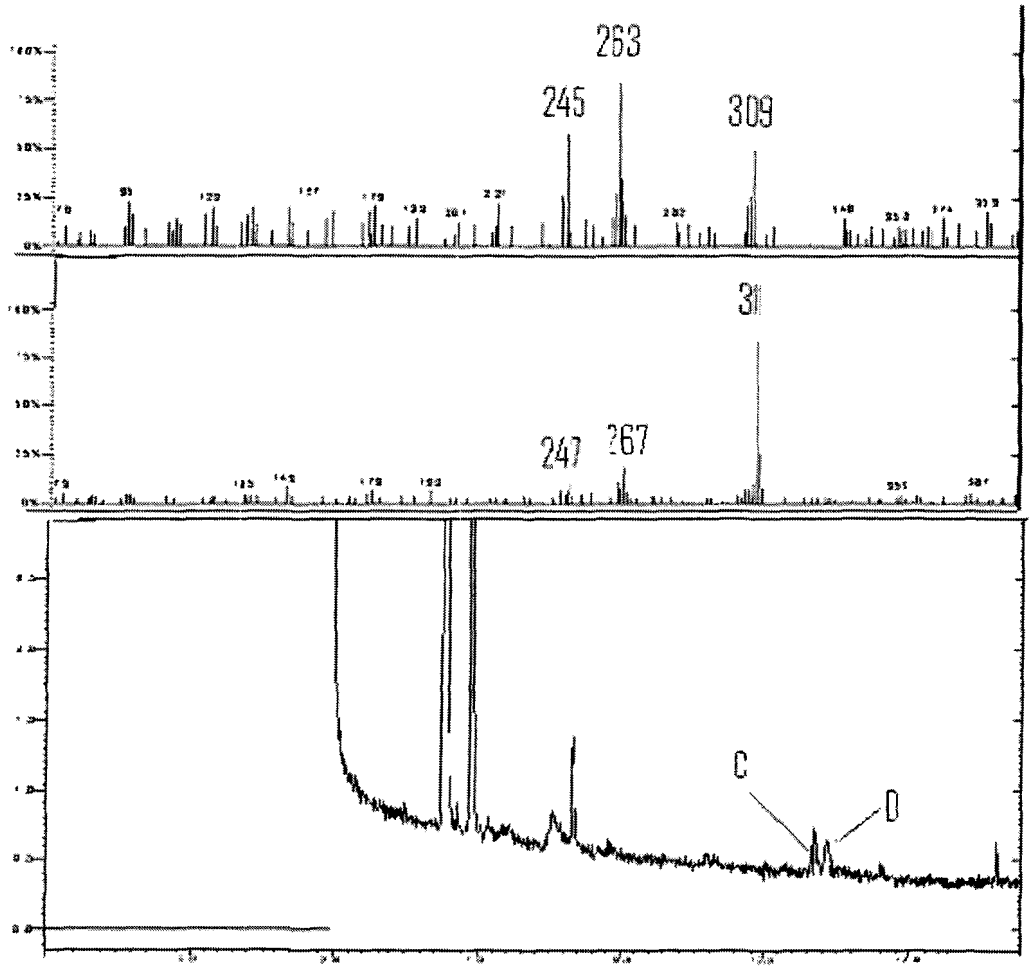


Figure 7. CI spectra of solvent extracted products.

The products obtained from incubation of moth extracts with alkene **2** (in EtOH) in the presence of NADPH and FAD.



3.2. Functional group tests results.

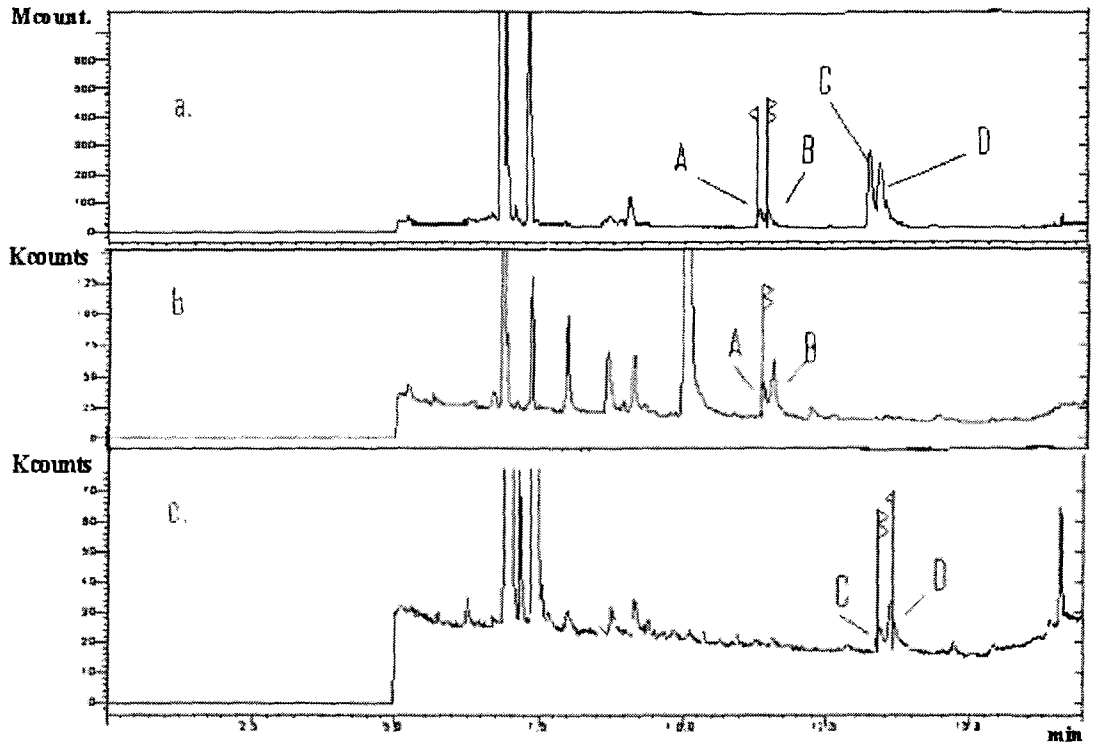
3.2.1 Transesterification of products A to D.

Products were transesterifed using **a.** KOH/MeOH **b.** KOH/EtOH solution, as mentioned in chapter two. In case **a**, compounds C and D were converted to A and B, while, in case **b**, compounds A and B were converted to C and D. This experiment

showed that C and D are ethyl esters while A and B are methyl esters of the same carboxylic acids.

Figure 8 Transesterification of products A-D.

Original trace: GC spectrum of solvent extracted products obtained from incubation of alkene **2** (in EtOH) with moth extract and cofactors, NADPH and FAD, **a**, transesterification of the original trace with methanol resulted in disappearance of peaks C and D, **b**. Upon transesterification of the original trace with ethanol, peaks A and B disappeared, **c**.

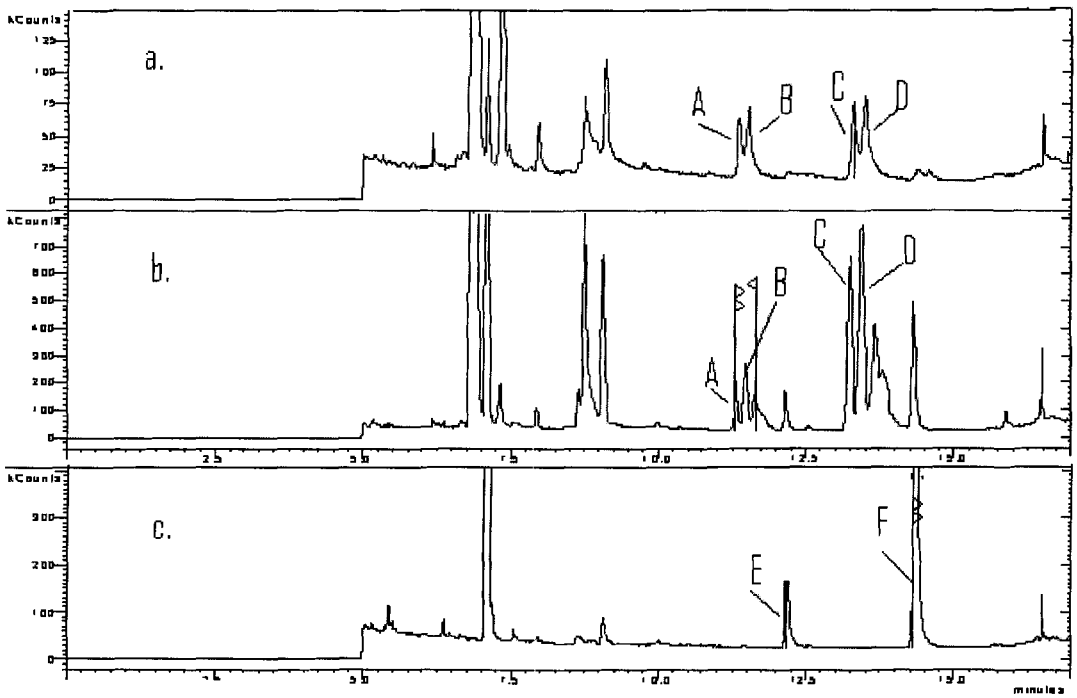


3.2.2 Hydrogenation of products A to D.

To prove that the products contain double bond(s) and not triple bond(s), I hydrogenated the products in the presence of Lindlar catalyst (chapter 2). No major products of higher molecular weight were formed upon this hydrogenation which

indicates that there is no triple bond present in compounds A, B, C and D. The hydrogenation was also tried over Pd/charcoal which resulted in formation of two homologous products which were consistent with standards of methyl and ethyl esters of stearic acid.

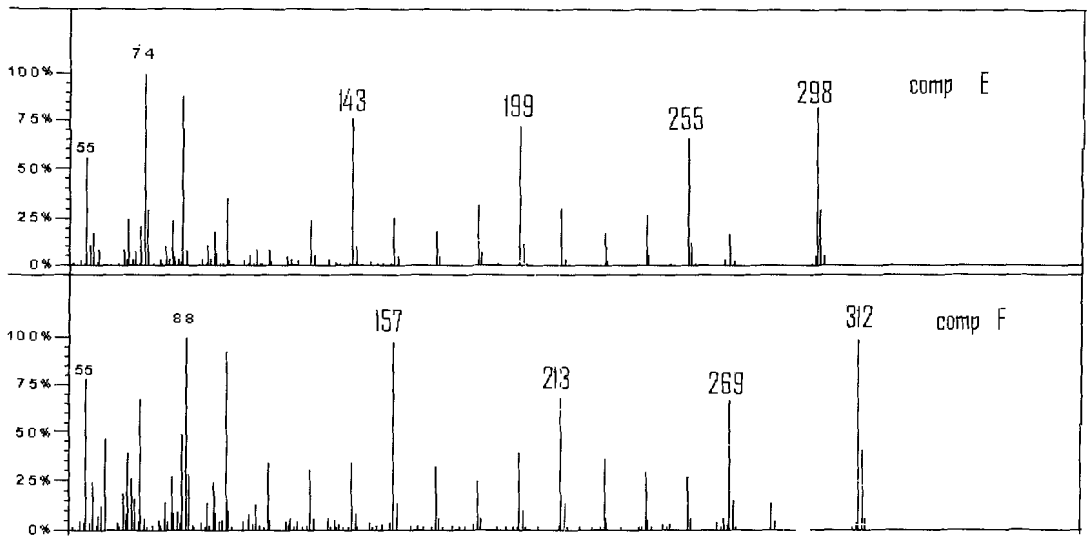
Figure 9 Hydrogenation of products A-D.

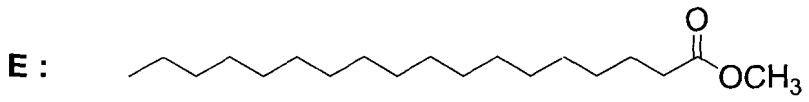


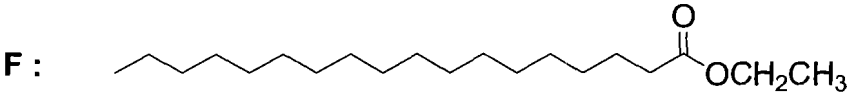
a Original trace: GC spectrum of solvent extracted products obtained from incubation of alkene **2** with moth extract and cofactors, NADPH and FAD. **b.** Hydrogenation in the presence of Lindlar catalyst. The appearance of new peaks right after product peaks is related to double-bond isomerization of the products. The formation of some saturated products (E and F in **c**) can be related to small (ng) scale of the reaction. **c.** Hydrogenation over Pd/C, formation of saturated products E and F, methyl and ethyl stearate was observed.

Figure 10 Mass spectra of compounds E and F.

The fragmentation of these compounds matched with methyl and ethyl stearate.





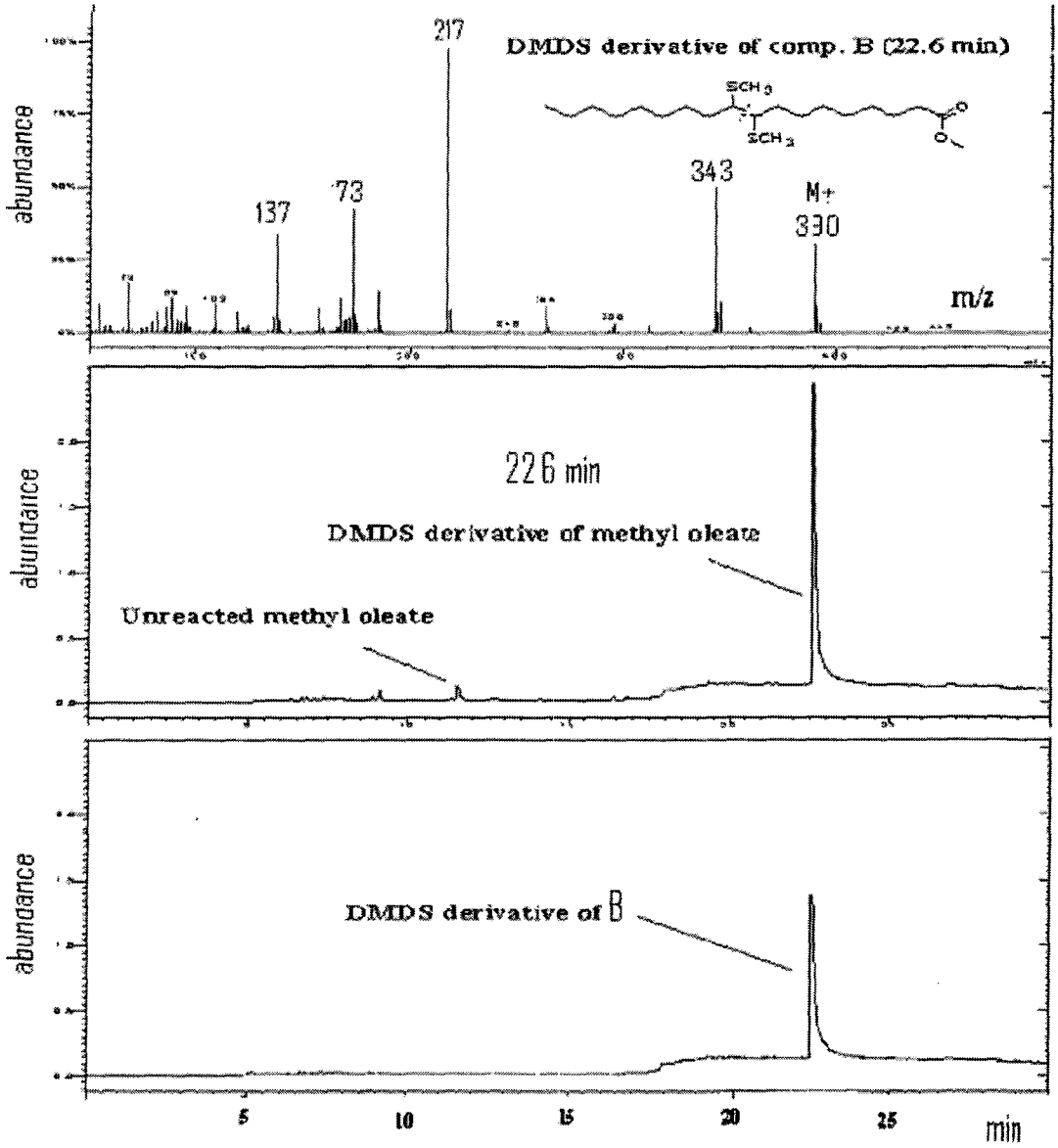


3.3.3 Dimethyldisulfide derivatization of the products.

The double bond positions in the products A and B were determined by dimethyldisulfide derivatization of A and B individually. The single-step iodine-catalyzed procedure described in chapter 2, leads to formation of α, β-bis(methylthio) adducts which are stable to gas chromatographic conditions (Figure 10). The mass spectra under electron impact mode of this class of derivatives present two characteristic ions arising from the fragmentation of the RCH(SMe)-RCH(SMe) bond [77]. Dimethyl disulfide derivative of B, had a molecular ion of m/z 390, the

base ion at m/z 217 $[H_3CSCH(CH_2)_2CO_2CH_3]^+$ and m/z 173 for $[H_3CSCH(CH_2)_7CH_3]^+$. The ions are consistent with a synthetic standard of dimethyl disulfide derivative of methyl oleate. The standard was prepared from commercial methyl oleate by the same procedure, and the product analysed by 500 MHz.^{1}H NMR. ^{1}H NMR ($CDCl_3$): δ (ppm) 0.8 (t, 3H, J=6.8, CH_3), 1.3 (m, 20H, CH_2's), 1.6 (m, 4H, 2×SCH**CH_2**), 1.8 (m, 2H, **CH_2**CH_2CO), 2.1 (s, 6H, 2× **CH_3**-S), 2.3 (t, 2H, J=7.6, **CH_2**CO), 2.7 (dt, 2H, J=9.1, J=3.1, 2×**CH**-S), 3.7 (s, 3H, O**CH_3**).

Figure 11 DMDS derivative of compound B.



For compound A, several compounds formed upon treatment with DMDS. Two peaks among the observed peaks were more significant for analysis. These two peaks in the isolated material had the same retention times as the two products obtained from a methyl linoleate standard. I attempted to purify the product mixture over a mini column of silica eluted by hexane : ethyl acetate (8 : 2) . This, however, resulted in decomposition of products and therefore no recovery of the sulphide adducts.

It should be mentioned that the later peak, X, in the original sample shown in figure 11, had meaningful ion fragments according to the reported DMDS adduct for this diene [75]. A well detectable molecular ion at m/z 420, and a fragment ion m/z 373, corresponding to loss of CH_3S from the molecular ion, were observed. The characteristic cleavage of bonds between the two carbon atoms linked to sulphur generates ions at m/z 217 and m/z 74 (a four- member ring [$(CH_2)_3S$]) which might be indicative of the presence of one CH_2 group between the double bonds (Fig.12). However, I could not interpret the fragmentation pattern of compound Y.

Figure 12 Matched standard & DMDS derivative of A chromatograms.

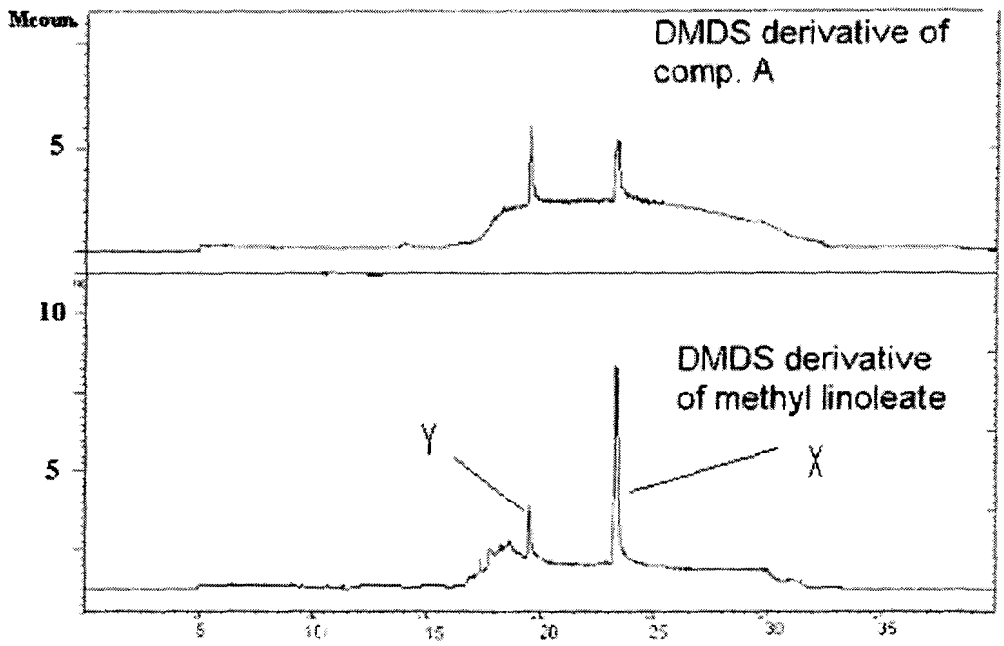
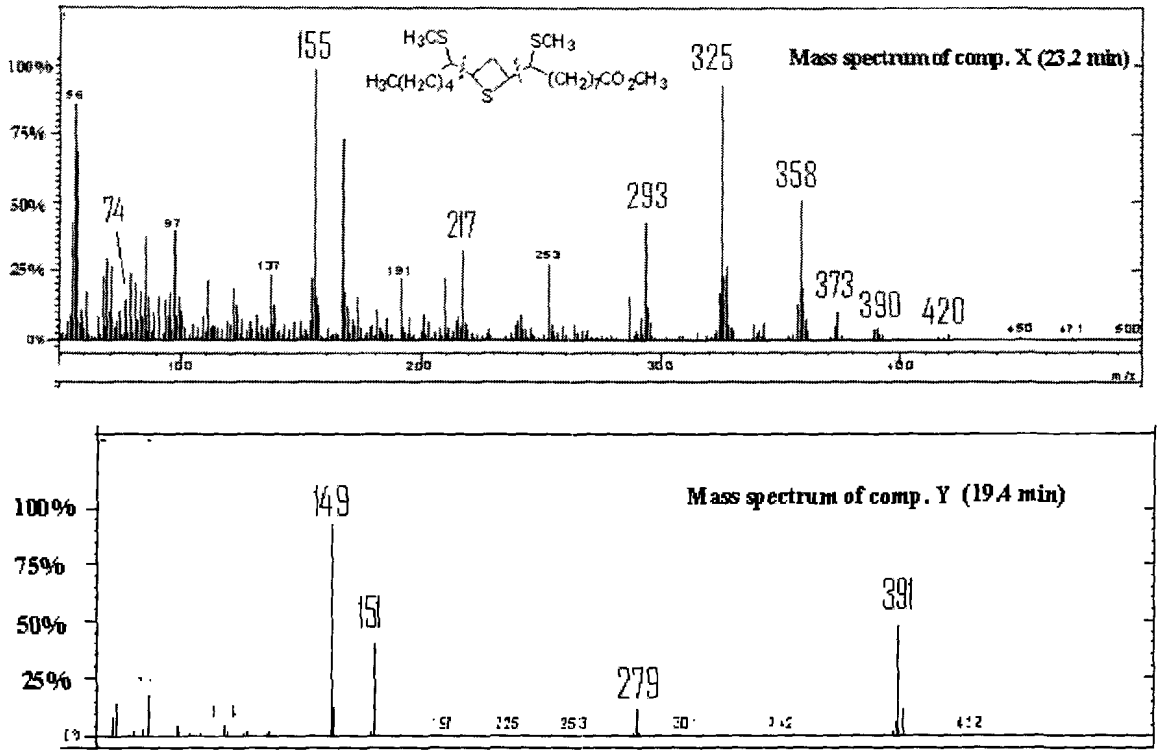


Figure 13 Mass spectrum of compound X.

The fragmentation pattern of this product is meaningful according to previously reported adduct in the literature. The mass spectra I obtained are shown below.

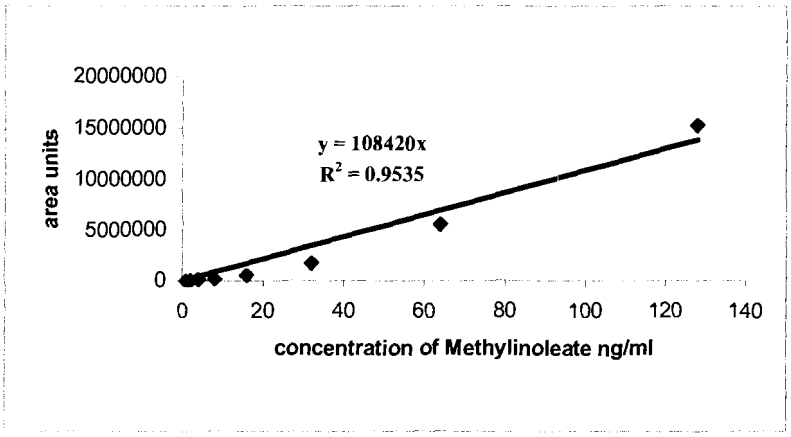


3.3 Results obtained from incubation of +1, -1 and alkene 2 with gypsy moth extracts.

Esters A, B, C and D were formed in statistically significant quantities upon treatment of moth extracts with alkene **2**, **+1** and **-1** (Table 2). No methyl or ethyl esters were detected in the substrate free moth extracts (Table 2). Moth extracts were incubated with various substrates and cofactors, and the total quantities of methyl

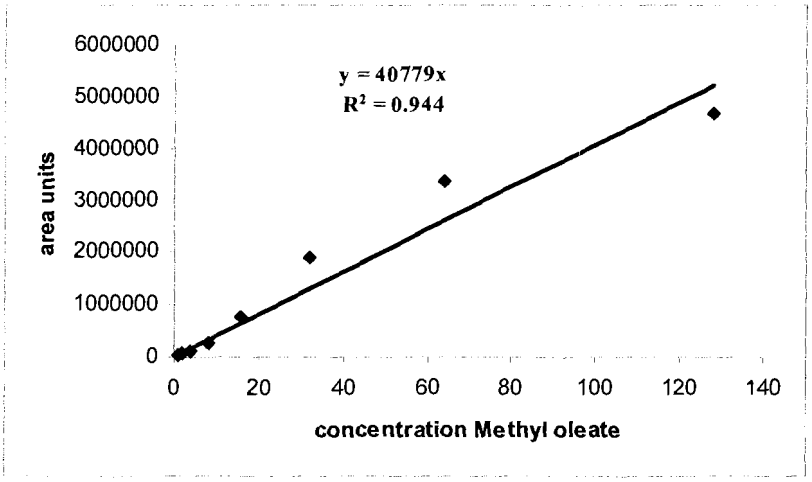
and ethyl linoleate and oleate formed were detected from standard calibration lines shown in figures 13 and 14[4].

Figure 14. The Standard graph of methyl linoleate.



The volume injected on GC/MS was 1 µl.

Figure 15 The Standard graph of methyl oleate.



The volume injected on GC/MS was 1 µl.

[4] I am grateful to Mr. Cris Barzan for making the standards and assisting with quantifications of ester products.

Table 2. Formation of A, B, C and D from incubation of gypsy moth extracts with various substrates[5].

Compounds / Treatment	Rep.	Me linoleate A (ng/100µl)	Me oleate B (ng/100µl)	Et linoleate C (ng/100µl)	Et oleate D (ng/100µl)
Extract blanks[6] NADPH +FAD	11	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Alkene 2 (in ethanol) + NADPH +FAD	14	5.4 ± 2.0 P = 0.01	2.8 ± 0.9 P = 0.00	13.9 ± 3.6 P < 0.01	81.2 ± 52.2 P = 0.06
1-^{13}C Glucose (in ethanol)+ NADPH +FAD	7	2.3 ± 0.9 P = 0.02	6.6 ± 2.6 P = 0.02	4.5 ± 2.7 P = 0.06	17.3 ± 9.9 P = 0.05
(+)Disparlure (in ethanol) + NADPH +FAD	4	15.5 ± 9.1 P = 0.02	11.5 ± 6.2 P = 0.008	253±132 P = 0.06	590 ± 311 P = 0.06
(-)Disparlure (in ethanol) + NADPH +FAD	4	0.0 ± 0.0	0.0 ± 0.0	131 ± 84.7 P = 0.08	352 ± 220 P = 0.08
Alkene 2 (in C_2D_5OH) + NADPH +FAD	4	1.3 ± 0.2 P = 0.00	3.1 ± 0.8 P = 0.01	87.1 ± 30.5 P = 0.02	159.2 ± 56.7 P = 0.02
Alkene 2[7] (in CD_3OD) + NADPH +FAD	4	321 ± 77.9 P = 0.0	627 ± 164 P = 0.0	11.3 ± 1.9 P = 0.0	22.6 ± 2.9 P = 0.0
Alkene 2 (in ethanol) + NADPH +FAD+ CoA+ATP	4	0.6 ± 0.2 P = 0.05	0.6 ± 0.3 P = 0.05	11.8 ± 6.9 P = 0.07	39.8 ± 17.9 P = 0.04
Alkene 2 (in ethanol) +NADPH with no FAD	6	0.0 ± 0.0	0.0 ± 0.0	1.0 ± 0.4 P = 0.03	4.1 ± 1.1 P = 0.00
Alkene 2 (in ethanol) + NADPH +FAD SAM	4	0.2±0.1 P=0.09	0.1±0.0 P=0.3	1.9±0.7 P=0.03	9.9±1.9 P=0.00
Alkene 2 (in ethanol) + NADPH +FAD+ Excess air	4	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.0 P=0.0	0.5 ± 0.1 P=0.0

[5] I am thankful to C.Barzan for helping me with the quantifications of the products.
[6] Insect extract with cofactors and buffer, but no odorant.
[7] In this experiment, no ethanol was applied in preparation of PMSF stock solution (see 2.2.2.1 chapter 2).

Values shown in this table are averages ± standard error. The P values shown are for pair comparisons between treatments and moth extract blanks.

There was no detectable presence of these compounds in extract blanks (entry 1), which contained cofactors but no odorants. Entries 2, 8 & 9 show the cofactor requirements. For entries 3-5 treatments all contained the optimal cofactor mixture. (See chapter 2). Product formation is NADPH and FAD dependent, however, addition of CoASH + ATP or SAM did not increase product formation (entries 8 & 10). Upon excessive aeration (entry 11) less amount of product was formed.

3.3.1. Incubation of 1-^{13}C- glucose with moth extracts.

The results obtained from incubation of **(+)-1**, **(-)-1** and alkene **2** with gypsy moth extracts suggested that products A-D are forming from the applied odorants. To test this is a general metabolic pathway in the moth, we treated extracts with 1-^{13}C glucose, a known source of 2-^{13}C acetate (see 1.2 of chapter 2). Treatment with 1-^{13}C glucose for 8 hours led to formation of A-D (Table 2). The results of the stable isotope labelling experiment demonstrated slight incorporation of ^{13}C into oleate esters (Table 3 and 4). The products from this experiment were analyzed by conventional GC-MS. It was hoped that this method would provide information about the incorporation of ^{13}C. However, mass spectrometric analyses showed almost no ^{13}C enrichment in the final products.

Table 3 Incorporation of ^{13}C in formation of linoleate esters upon overnight incubation of 1-^{13}C glucose was with moth extracts.

Compound / Ion Int.	Standard 263/262 M+1	Standard 264/262 M+2	1-^{13}C-glu. treatments 263/262 M+1	1-^{13}C-glu. treatments 264/262 M+2	% Inc. M+1	% Inc. M+2
Methyl linoleate	2.01±0.0	0.39±0.0	1.16±0.2	0.21±0.0	N/A	N/A
Ethyl linoleate	2.01±0.0	0.39±0.0	0.84±0.0	0.22±0.0	N/A	N/A

Table 4. Incorporation of ^{13}C in formation of oleate esters upon overnight incubation of 1-^{13}C glucose was with moth extracts.

Compound / Ion Int.	Standard 265/264 M+1	Standard 266/264 M+2	1-^{13}C-glu. treatments 265/264 M+1	1-^{13}C-glu. treatments 266/264 M+2	% Inc. M+1	% Inc. M+2
Methyl oleate	0.75±0.0	0.14±0.0	0.71±0.0	0.21±0.0	N/A	17.6
Ethyl oleate	0.75±0.0	0.14±0.0	0.79±0.0	0.15±0.0	5.1	6.7

3.3.2. Cofactors required for formation of methyl esters from +1, -1 and 2.

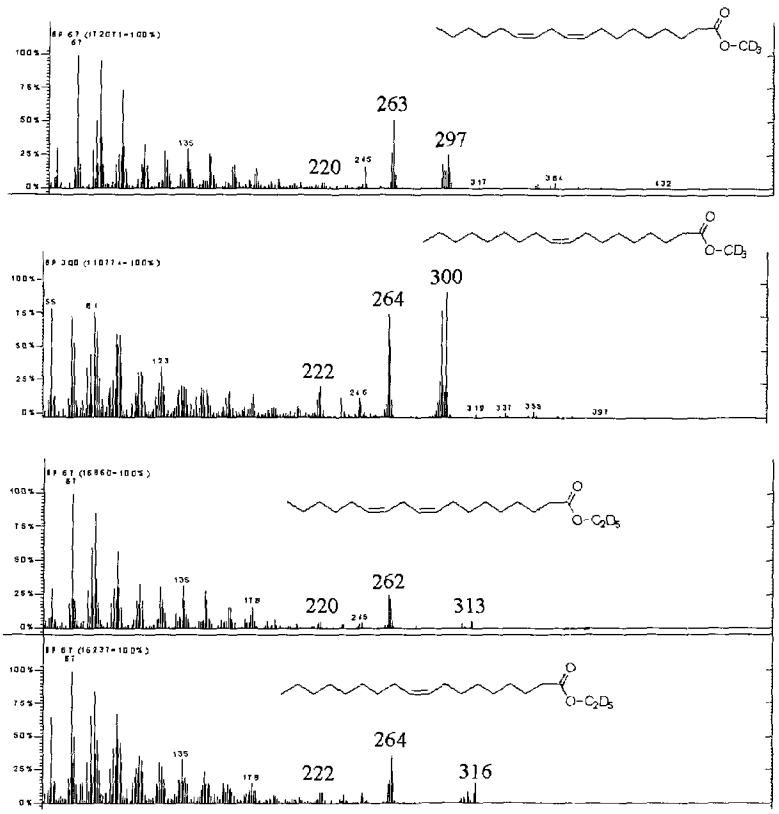
The formation of A, B, C and D from **(+)-1, (-)-1** or **2** is dependent on NADPH and FAD (Table 2). Comparing formation of esters from **2** with NADPH and FAD versus CoASH and ATP, significantly ($P<0.05$, t test) more products formed from **2** in the presence of NADPH and FAD than with CoASH and ATP (Table 2). Furthermore, no product formed in the absence of FAD (Table 2).

3.3.3 Results from incubation of labelled alcohols, methanol and ethanol, in the final products.

To determine whether the alcohol portion of the observed esters came directly from methanol or ethanol, we delivered the substrate in different alcoholic solutions such as a) C_2D_5OH (8% *vol.*) / C_2H_5OH (92% *vol.*), b) CD_3OD (8% *vol.*) / CH_3OH (92% vol.)[8]. In case a, ethyl oleate and linoleate were major products formed ($P<0.05$, t-test) Table 2. However, in case b, the methyl esters of these fatty acids were dominantly formed ($P<0.05$, t- test) Table 2. In both cases the incorporation of the deuterium label was observed in the products. (Fig. 17), suggesting that exogenous alcohols are readily incorporated, see table 5 for quantifications.

[8] In this experiment no ethanol was applied in preparation of PMSF stock solution (see 2.2.2.1 chapter 2).

Figure 16 Mass spectra of products formed upon delivery of alkene **2** in CD_3OD and C_2D_5OH.



In methyl linoliate (M^+ = 294 unlabeled) and in the M+1 peak for methyl oleate (M+1= 297 unlabeled). For ethyl linoliate (M^+ = 308 unlabeled) and for ethyl oleate (M+1= 311 unlabeled).

Table 5. Quantification of deuterium labelled products.

Compound	Replicates	RCO_2CD_3 (ng/100µl)	$RCO_2C_2D_5$ (ng/100µl)	% Labelled product
Methyl linoleate	4	22.4±5.3	N/A	5.8
Methyl oleate	4	169.8±10.0	N/A	20.8
Ethyl linoleate	4	N/A	6.5 ± 0.4	7.5
Ethyl oleate	4	N/A	30.0±4.0	18.8

Labelled products were quantified from intensities of characteristic fragments in mass spectra. Fragments used for calculations were m/z 220, 262, 297 for [$^2H_3$]-methyl linoleate, m/z 222, 264, 300 for [$^2H_3$]-methyl oleate, m/z 220, 262, 313 for [$^2H_5$]-ethyl linoleate and m/z 222, 264, 316 for [$^2H_3$]-methyl oleate. % of labelled products indicates the ratio of the labelled products to those reported in table 2, entries 6 and 7.

CHAPTER 4

4.1 Discussion

This study shows that gypsy moth can completely metabolize 2-methyl-7(R),8(S)-7,8-epoxyoctadecane (known as (+)-disparlure) (+**1**), 2-methyl-7(S),8(R)-7,8-epoxyoctadecane (known as (-)-disparlure) (-**1**) and 2-methyl-7(Z)-octadecene (**2**). The process likely requires an initial hydrocarbon chain oxidation followed by β-oxidation. This clears the moth's body surface of the pheromone +**1**, and of -**1** and alkene **2**, two behavioural antagonists in this insect [9]. In this thesis I describe some steps of the degradation pathway of these compounds in the gypsy moth. I found that the incubation of alkene **2**, (+) and (-)-disparlure with moth extracts stimulates the formation of two sets of homologous compounds. Unexpectedly, these compounds were identified as methyl and ethyl esters of linoleic and oleic acids.

Further investigations revealed that the biosynthesis of these esters likely occurs from smaller building blocks, obtained from degradation of the odorants. The biosynthesis of these fatty esters is likely catalysed by a fatty acid synthase (FAS). To test this hypothesis, I treated moth extracts with 1-^{13}C glucose, a known source of 2-^{13}C acetate [78]. Treatment with 1-^{13}C glucose for 8 hours led to formation of methyl and ethyl esters of linoleic and oleic acids (Table 2 chapter 3), with slight incorporation of ^{13}C label in oleic esters (Table 4 chapter 3). Interestingly, with glucose much less methyl and ethyl ester formed than with odorants ($p<0.05$), suggesting that the odorants might provide a starter unit for the FAS.

The fatty acid intermediates are then converted to the methyl and ethyl esters. The incorporation of acetate units into fatty acids and involvement of fatty acid synthase as a key enzyme in biosynthesis of fatty acid precursors of cuticular hydrocarbons has been previously shown in other insects [14, 18]. Fatty esters can either form from carboxylic acid released from FAS, or by the nucleophilic attack of methanol or ethanol to fatty acyl units still bound to FAS. Released fatty acids would need to be activated by ATP and CoASH (Figure 16). I have noticed that no significant ester biosynthesis occurs when extracts are supplemented with CoASH and ATP ($P>0.05$). Therefore, the first pathway, attack to a fatty acyl thioester still bound to FAS, is probably occurring. Consistent with this proposal is the observation that almost no ethyl or methyl stearate is formed. If esterification occurred from free acids, one would expect a greater variety of esters being formed. The formation of the methyl esters is favoured when alkene **2** is delivered in a solution of methanol and CD_3OD. Similarly, the ethyl esters are major products when the substrate is delivered in ethanol and C_2D_5OH solution. In both cases the incorporation of the deuterium label was observed in the products. (Table 5 chapter 3), suggesting that exogenous alcohols are readily incorporated. In addition, oleate esters were formed more abundantly than linoleates with very similar ratios in both methyl and ethyl esters (Table 5 chapter 3). This might suggest that linoleate esters are likely formed from oleates by a $\Delta 12$ desaturase. Esterification of ethanol to ethyl oleate has been observed in other organisms. For example, honey bees use ethyl oleate as a "social pheromone", and worker bees synthesize the ester *de novo* from glucose and ethanol [79]. In vertebrates, ethyl ester synthases have been detected in the liver where they may be involved in detoxification of ethanol [80].

I also investigated the cofactors necessary for these bioconversions. The formation of the ester products is dependent on NADPH and FAD (Table 2 chapter 3). This observation is consistent with the proposed pathway (Figure 17): a. FAD, NADPH and air are required for multiple–step oxidation of the odorants (V.Hung, E.Plettner, T.Lajevardi, unpublished), b. a fatty acid synthase which catalyzes the synthesis of fatty acids from acetyl-CoA and malonyl-CoA, likely uses NADPH as the reductant [19]. This process likely begins with monooxygenases that functionalise the hydrocarbon odorants. The functionalised odorants yield building blocks that are channelled into fatty acid biosynthesis. In insects, FASes that build 16 and 18-carbon chain thioesters are common. We then propose that the saturated 18-carbon chain thioester is desaturated. If desaturation occurred after release from FAS, then one would expect to see free stearic acid. However, BSTFA (N,N-bis(trimethylsilyl)trifluoroacetamide) analysis of the extracts has revealed mostly oleic acid. Fatty acyl desaturases which operate on the ACP thioesters, are also known [81], [82]. Furthermore, the labelling percentages with D_5 ethanol or D_4 methanol (Table 5, chapter 3) suggest that the linoleates could be derived from the oleates by desaturation. If the linoleates were formed from oleyl-ACP followed by esterification with ethanol or methanol, then one would expect the same percentage of deuterated alcohol to be incorporated into oleates and linoleates.

4.2 Future work

The objective of the future work in this project could be to delineate the pathway, proposed in Figure 18, more clearly. This may be performed by synthesis and incubation of isotope labelled odorants with moth extracts and follow up the percentage of the label incorporation into the accumulated products. The effect of a β-oxidation inhibitor, usually β-difluoro acetates, can be studied in earlier parts of this pathway which would further confirm the involvement of β-oxidation pathway in degradation of the applied odorants. The involvement of Δ9 and Δ12 desaturases in later stages of this pathway may be studied by incubation of deuterium labelled stearate and oleate esters with moth extracts. Ultimately, the involvement of some key enzymes such as mono-oxygenases in degradation of the odorants, a fatty acid and a fatty acid ester synthase may be proven by necessary experiments.

4.3 Conclusion

I have mapped the late stage of an odorants degradation pathway in the gypsy moth. Surprisingly, the pheromone and related odorants stimulate formation of methyl and ethyl oleate and linoleate. I identified the esters formed and determined the cofactors required for their formation.

Figure 17 Two possible routes for esterification of fatty acid units.

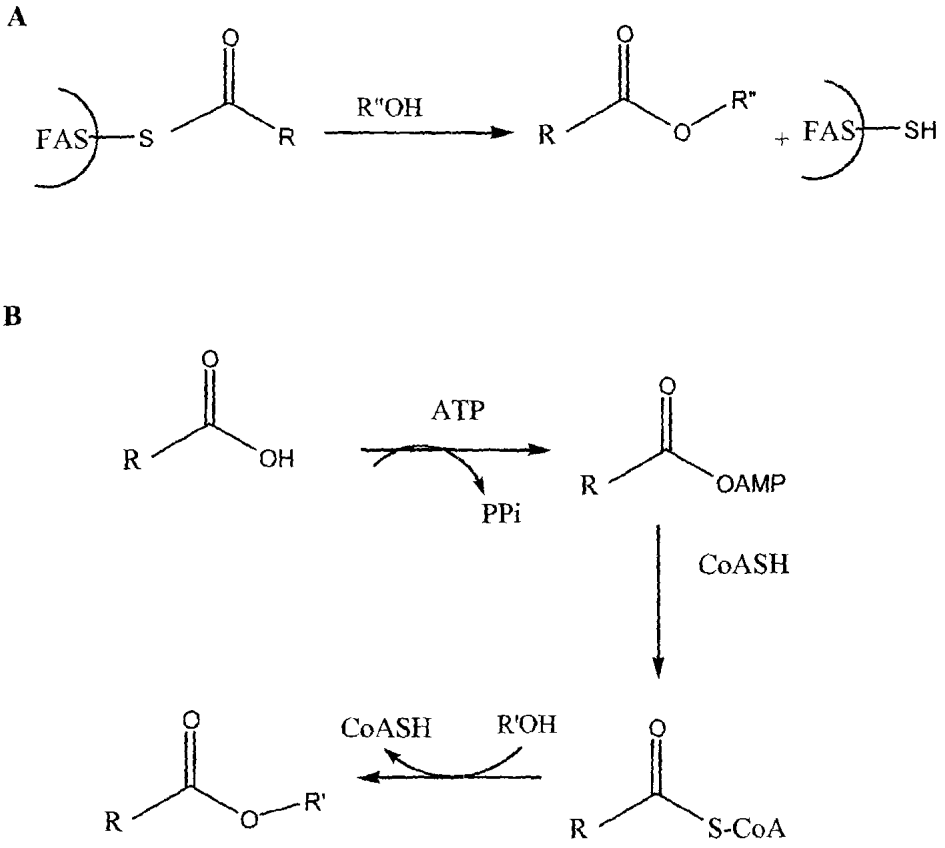
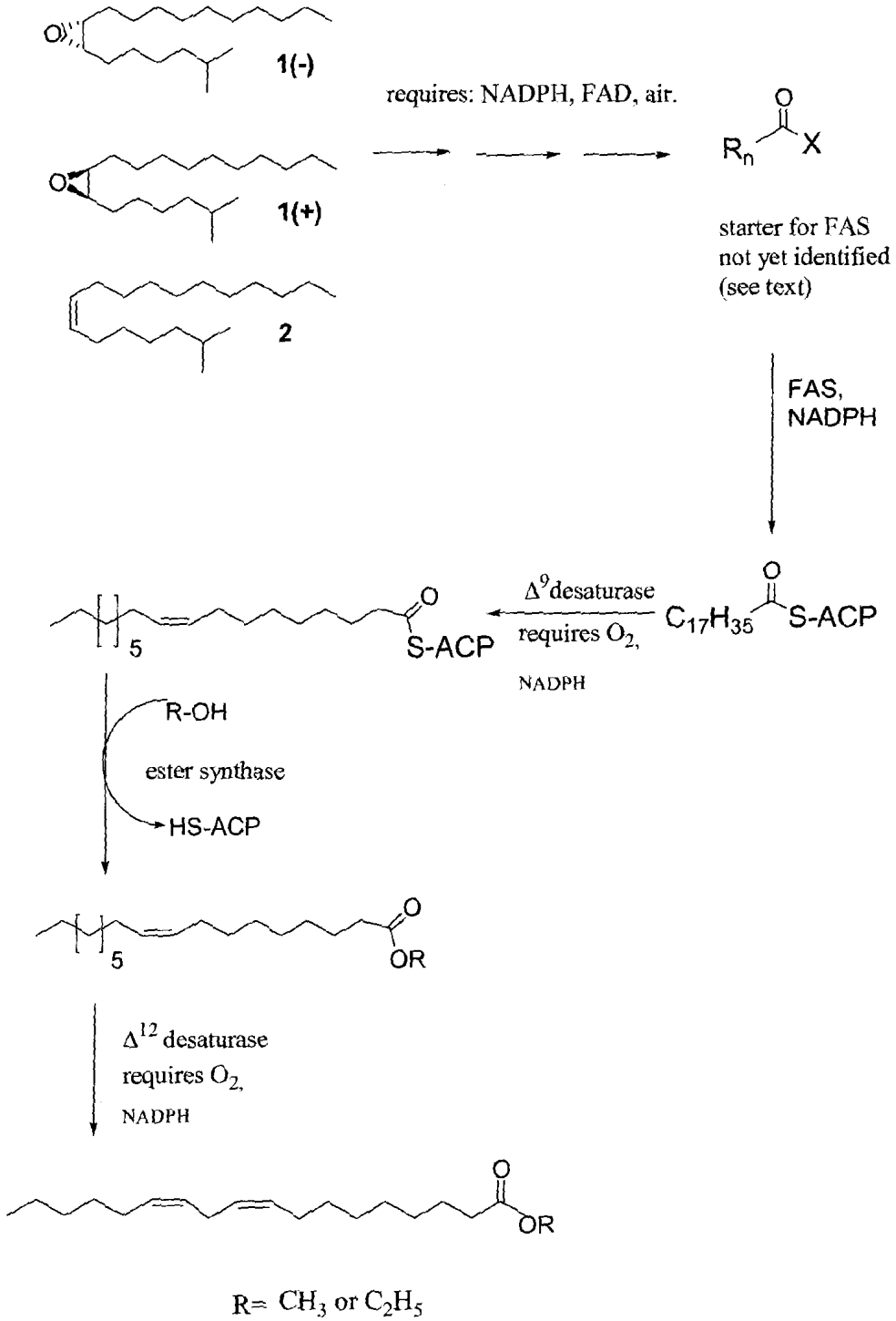


Figure 18 Proposed pathway from odorant to methyl and ethyl linoleate.



R= CH_3 or C_2H_5

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