PHEROMONE BIOSYNTHESIS AND REGULATION IN THE YELLOW MEALWORM BEETLE, *TENEBRIO MOLITOR*

BY

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PHEROMONE BIOSYNTHESIS AND REGULATION IN THE YELLOW MEALWORM BEETLE, *Tenebrio Molitor*

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A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

ABSTRACT

The overall goal of this thesis was to investigate the biosynthetic pathway and regulation of 4-methylnonanol, the female-produced sex pheromone of the yellow mealworm beetle, *Tenebrio molitor*. The first objective was to elucidate the biochemical pathway. Adult female beetles were fed defatted bran coated with putative stable isotope-labelled (carbon-13 or deuterium) precursors (acetate, propionate, pentanoic acid, 2-methylheptanoic acid or 4-methylnonanoic acid). Volatiles from feeding females were collected on Porapak Q columns attached to an aeration apparatus, and were eluted from the traps with diethyl ether after 6 days. Incorporation of the stable isotope into 4-methylnonanol was determined by gas chromatography/selected ion monitoring-mass spectroscopy (GC/SIM-MS). The biosynthetic pathway of the pheromone 4-methylnonanol was apparently a modification of normal fatty acid metabolism. The uneven number of carbons in the backbone resulted from initiation of chain biosynthesis with a propionyl derivative instead of the normal acetyl derivative. The second objective was to determine if 4-methylnonanol production is regulated by Juvenile Hormone (JH) and, if so, at what biochemical step this occurred. Behavioral bioassays indicated that methoprene (which is more stable and economical to use than JH) could be used as a JH analog. Pre-treatment of immature and mature decapitated female mealworm beetles with methoprene increased the biological activity of the extracts. Finally, methoprene was used to determine if the last step in the biosynthesis of 4-methylnonanol, the reduction of 4-methylnonanoic acid to pheromone, is hormonally regulated. Beetles were injected with [^{3}H]4-methylnonanoic acid and extracted with isopropanol:pentane (2:3) after 3 h. The 4-

methylnonanol and 4-methylnonanoic acid present in the extracts were separated on mini-columns of Florisel and quantified by scintillation counting. In immature females conversion was blocked in the absence of methoprene but occurred in the presence of methoprene, implicating the reduction of 4-methylnonanoic acid to 4-methylnonanol as one of the regulated steps in pheromone biosynthesis. This study is the first of its kind towards developing an understanding of the regulation of pheromone biosynthesis in beetles.

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LIST OF ABBREVIATIONS

20-HE- 20-hydroxyecdysone

ANOVA- Analysis of Variance

$CDCl_3$- Deuterated Chloroform

CoA- Coenzyme A

DMF- Dimethyl Formamide

DMSO- Dimethy Sulfoxide

FAS- Fatty Acid Synthase

GC/MS- Gas Chromatography/Mass Spectroscopy

HPLC- High Pressure Liquid Chromatography

JH- Juvenile Hormone

KOH- Potassium Hydroxide

$MgSO_4$- Magnesium Sulfate

$NaHCO_3$- Sodium Bicarbonate

PBAN- Pheromone Biosynthesis Activating Neuropeptide

SIM- Selected Ion Monitoring

Z9-23:Hy- (Z)-9-tricosene

Z9-27:Hy- (Z)-9-heptacosene

INTRODUCTION

I. GENERAL INTRODUCTION

Pheromones are semiochemicals or chemical signals used for communication between different individuals of the same species. Insects use a wide range of pheromones for intra-species communication. These may include sex, anti-aphrodisiac, aggregation, anti-aggregation, alarm, epidietic ("spacing") and "switching" pheromones (Happ, 1969; Borden, 1985). The first sex pheromone identified was that of the domesticated silkworm, *Bombyx mori,* after extraction of a half a million female silkworm pheromone glands and 30 years of classical chemical analyses (Butenandt et al. , 1959).

Pheromones have sparked a great deal of academic interest due to their potential for use in the detection and manipulation of insect pest populations. Direct control of moth pests has been achieved in some cases by the manipulation of insect sex pheromones to disrupt mating. So far, only pheromones of moths have been registered as mating disruption pheromones (reviewed by Cardé and Minks, 1995). These include pheromones of the artichoke plume moth in the United States (Ridgway, 1992) and pheromones of the beet army worm, peach tree moth and small tree tortrix moth in Japan (Wakamura, 1992). Pheromones can also be used in trapping insects for the purpose of monitoring and surveying pest populations (reviewed by Silverstein, 1981).

Earlier research focused mainly on the identification, synthesis and commercial use of

pheromones. More recently, researchers have begun to investigate the regulation and biosynthetic routes of pheromone production. A detailed knowledge of pheromone biosynthesis could be used to interrupt pheromone production in insect pest populations (Prestwich et al., 1984). Factors involved in the regulation of pheromone biosynthesis, such as hormones and dietary precursors, could be used to increase pheromone levels in insects and hence facilitate the identification of pheromones. For example, Francke et al. (1977) used juvenile hormone analogues to increase pheromone production in the bark beetle *Pityogenes chalcographus*. This eventually led to the discovery of the aggregation pheromone, 2-ethyl-1,6-dioxaspiro[4.4]nonane. Bjostad and Roelofs (1984a) predicted the existence of pheromones which had been previously overlooked by analyzing the biochemical pathway of the red-banded leafroller moth, *Argyrotaenia velutinana*.

The objective of this research was to study the biosynthetic pathway and regulation of pheromone production in the yellow mealworm beetle, *Tenebrio molitor*. The order "Coleoptera" includes over 333,000 species of beetles and as such is the largest order in the animal kingdom (Booth et al., 1990). Of these approximately 600 species are associated with stored food products (Hinton and Corbet, 1972). The yellow mealworm beetle is often found in stored grain. As early as the 1960's researchers had found that, although damage done by the mealworms compared to other grain beetles is minor, their presence in stored products is an indication of the poor quality of the grain (Munro, 1966). We chose the yellow mealworm as a model to study pheromone biosynthesis in beetles. Not only are they easy to rear and maintain in cultures in the laboratory, but the large size of the adult beetles (14-18

mm long) facilitates the injection and surgical manipulation of individual animals. A significant amount of research has been done on the physiology and the life cycle of the yellow mealworm beetle, but the biochemical aspects have been largely neglected.

Before focusing on the current research project, a brief literature review covering important aspects of pheromone biosynthesis and regulation in insects will be presented.

II. PHEROMONE BIOSYNTHESIS IN INSECTS

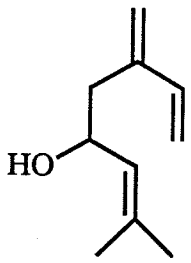
Insects use a great diversity of chemicals as pheromones. Beetle pheromones that have been isolated and identified range from fatty acid derivatives to aromatic and terpenoid compounds (FIGURE 1). Despite the fact that the structures are diverse and often unique, they appear to be formed through simple modifications of normal metabolism. Since the yellow mealworm beetle produces a fatty acid-derived pheromone, I will briefly summarize the biosynthesis of fatty acid-derived pheromones.

(A) FATTY ACID DERIVED PHEROMONES

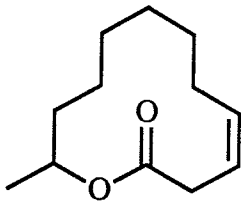
1. Unbranched pheromones:

Fatty acid derived pheromones are usually derived through simple modifications of normal fatty acid metabolism. A double bond may be inserted, the carbon chain may be shortened/elongated to the appropriate chain length, and/or the carboxylic acid group may be reduced (to an aldehyde or alcohol) or derivatized (usually to an ester) to produce the pheromone. This process has been best studied in moths but has also been demonstrated in

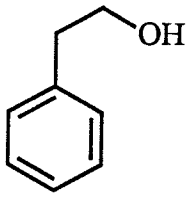
FIGURE 1: Structures of some different pheromones found in beetles



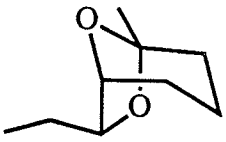
terpenoid
(eg. ipsdienol)



macrolide
(eg. cucujolide II)



aromatic
(eg. phenethyl alcohol)



bicyclic acetal
(eg. exo-brevicomin)

cockroaches, bees, houseflies and beetles. A brief outline of pheromone biosynthesis in a few insects will be presented here.

(a) *Moths*:

Pheromones of more than 300 insect species have been identified, and about 70% of these are sex pheromones of moths (Ridgway et al., 1986). Many sex pheromones found in moths are straight-chain alcohols, acetates and aldehydes derived from fatty acids. As mentioned above, in moths fatty acids are desaturated and subsequently the chain is shortened (β-oxidized) or elongated. A classic example is found in the red-banded leafroller moth. In this moth, hexadecanoate (C16) in the sex pheromone gland is chain-shortened to tetradecanoate (C14), which is then desaturated to form both (Z)-11 and (E)-11 tetradecenoyl moieties that are further reduced and acetylated to form the major sex pheromone components (Bjostad & Roelofs, 1981). This is summarized in FIGURE 2.

A chain elongation biosynthetic pathway has been detected in arctiid moth, *Estigmene acrea*. As shown in FIGURE 3, linolenic acid (C18:3) is chain elongated to docosatrienoic acid (C22:3) with subsequent reductive decarboxylation to eventually produce the pheromone (Z)-9,10-epoxyheneicosadiene (Rule and Roelofs, 1989). Investigation of sex pheromone biosynthetic pathways in a number of lepidopteran species have shown that intermediate saturated and unsaturated fatty acids can be synthesized *de novo* in the pheromone gland from acetate (Bjostad and Roelofs, 1984). Stearic acid and palmitic acid are synthesized from acetate and subsequently the chain is shortened and/or desaturated to produce the

FIGURE 2: Pheromone biosynthesis in the red banded leafroller moth, *Argyrotaenia velutinana*

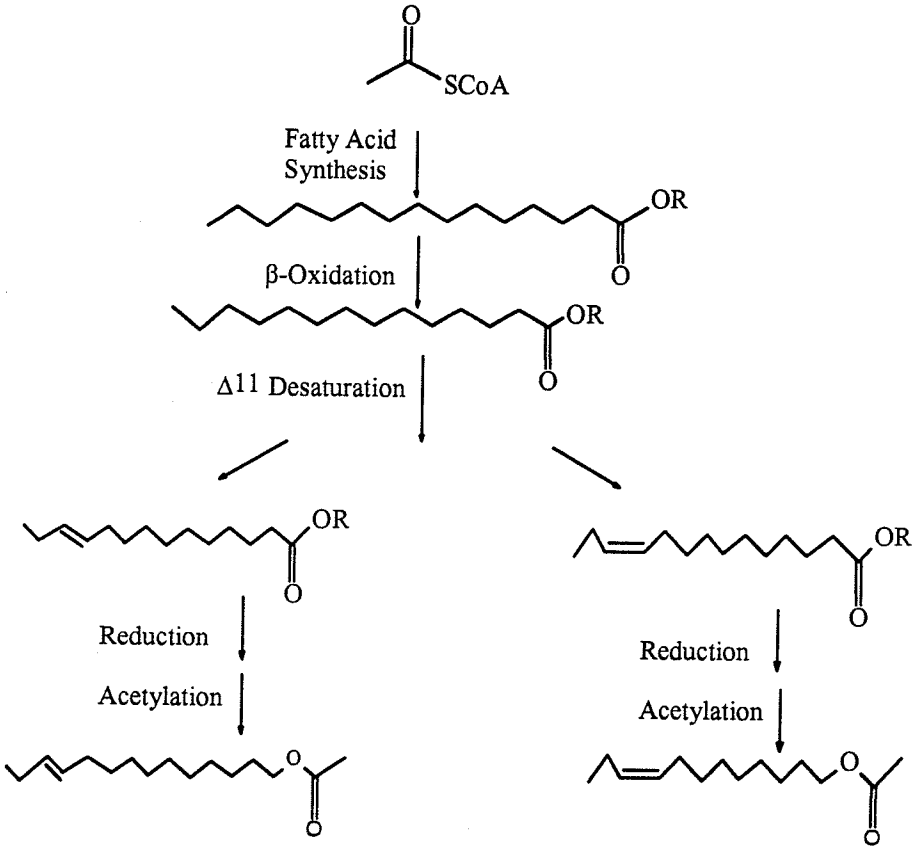
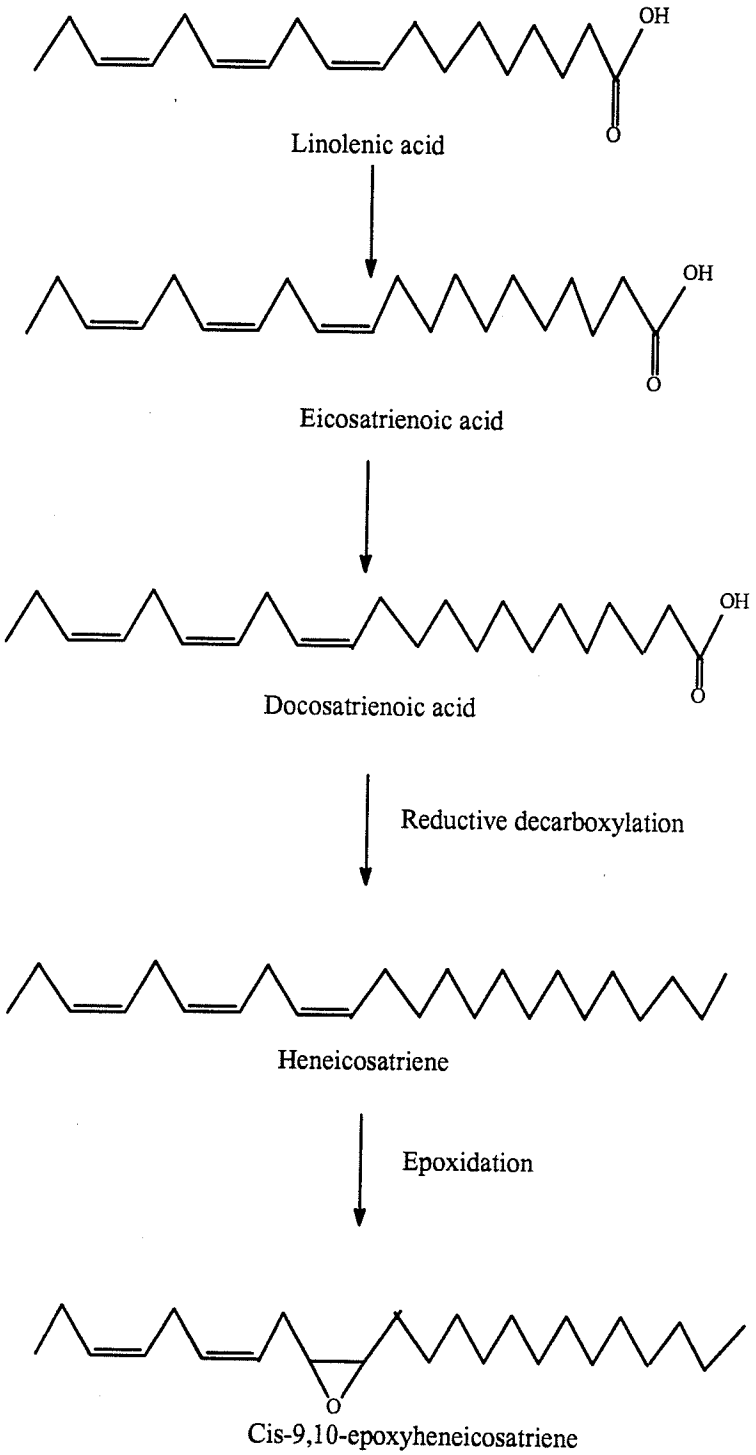


FIGURE 3: Pheromone biosynthesis in the arctiid moth, *Estigmene acrea*



intermediate fatty acid precursor to the pheromone components.

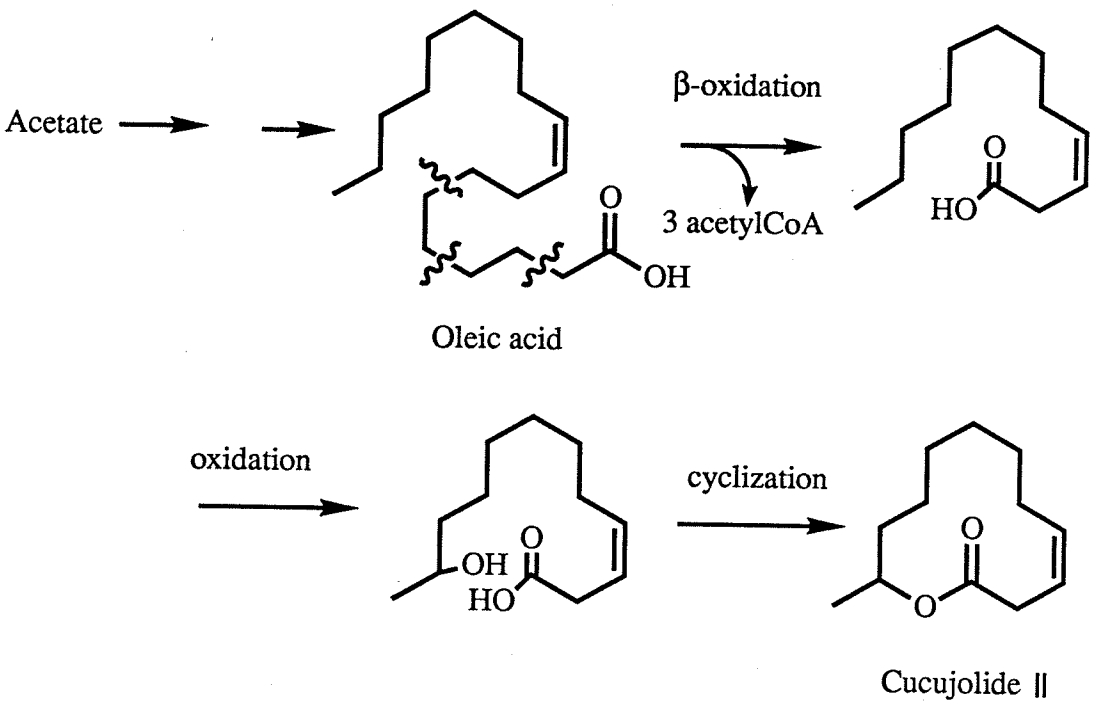
(b) *Beetles*:

The cucujid grain beetles produce several structurally similar macrocyclic lactones given the trivial name "cucujolides" (Oehschlager et al., 1988). Various combinations of these cucujolides act as species-specific aggregation pheromones for these cucujid beetles. The rusty grain beetle, *Cryptolestes ferrugineus*, can produce the pheromone cucujolide II [3(Z)-dodecen-11-olide] from a variety of fatty acids, including the common dietary fatty acid, oleic acid (C18:1). The fatty acid is first shortened to a 3(Z)-dodecenoyl derivative (C12) through β-oxidation, which is then oxidized at the penultimate carbon to form an 11-hydroxy-3-(Z)-dodecenoyl derivative. Cyclization of this intermediate (likely through a CoA derivative) results in the macrocyclic lactone, cucujolide II (Vanderwel et al., 1990; Vanderwel et al., 1992) (FIGURE 4). Cucujolide II can also be produced *de novo* in the rusty grain beetle (Vanderwel et al., 1990) (Figure 6). The other cucujolides are produced by similar pathways (Vanderwel et al., 1990).

2. Methyl-branched pheromones:

Methyl-branched hydrocarbons are found in the outer waxy cuticular surface of insects at all life stages (reviewed by Nelson, 1993). In addition to their role in protecting the insect from dessication, abrasion, and penetration of toxic compounds, some hydrocarbons are involved in chemical communication in some insect species (reviewed by Nelson, 1993). Since methyl-branched compounds are made as a matter of course (as part of the insect's cuticular

FIGURE 4: Biosynthesis of cucujolide II



hydrocarbon repertoire), methyl-branched pheromones are likely formed through normal metabolic pathways.

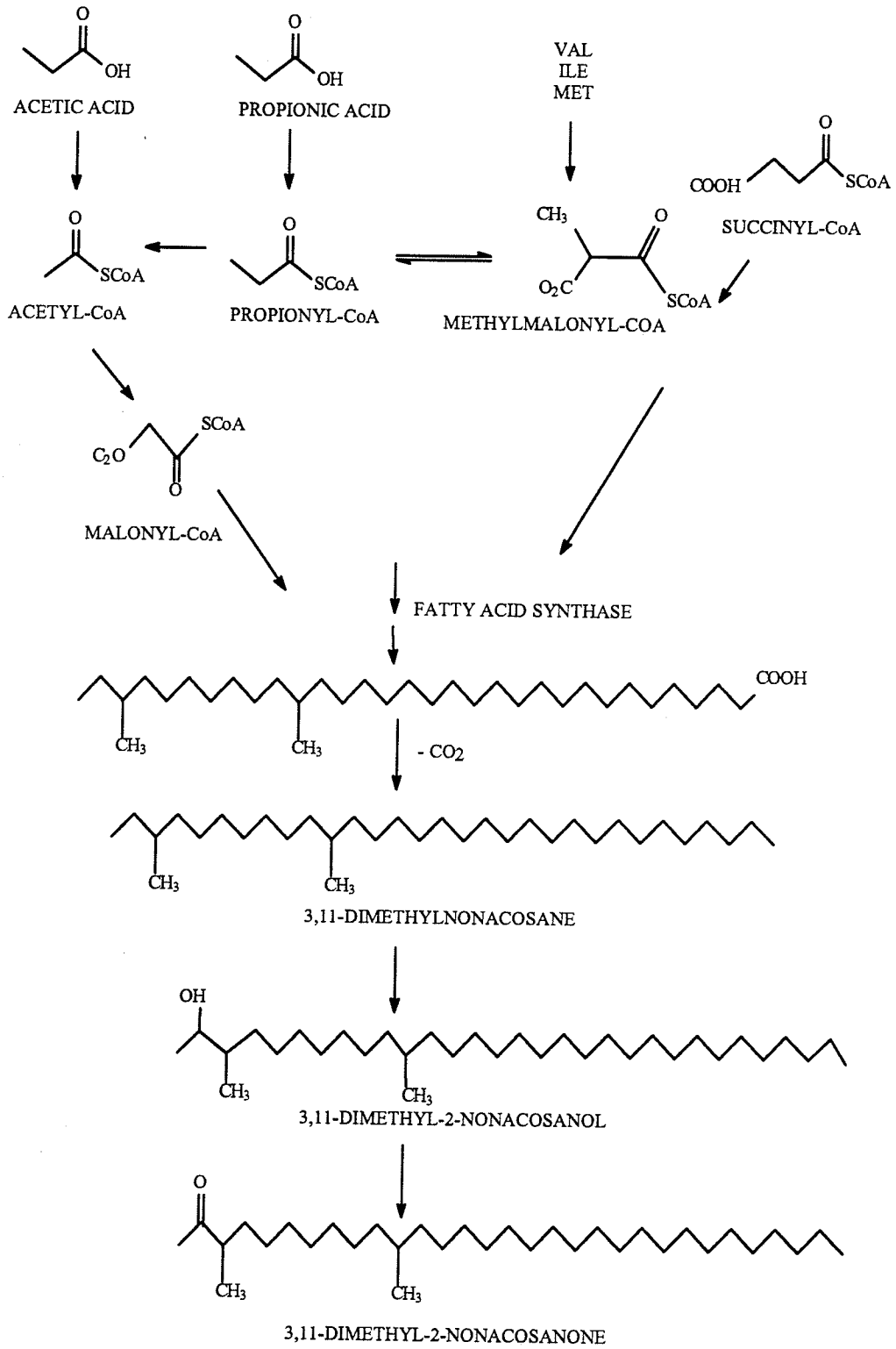
(a) *Cockroaches*

The female-produced contact sex pheromone of the German cockroach, *Blatella germanica*, is a multicomponent system of long chain, methyl branched methyl ketones (Chase et al., 1992). The source of the methyl branches of these pheromones have been particularly well studied, using stable-isotope labelled precursors (Blomquist et al., 1980) and NMR techniques (Dwyer et al., 1981). The methyl branches are produced when methyl malonyl CoA is inserted in place of malonyl CoA. The methyl malonyl CoA can be produced from propionate, succinate, isoleucine, valine and methionine (FIGURE 5). Vitamin B_{12}, which is a required cofactor for the mutase reaction that converts succinate to methylmalonyl-CoA, is present in appreciable quantities in the cockroach. The long chain methyl-branched fatty acids then give rise to long chain methyl-branched hydrocarbons in the German cockroach, which are eventually oxidized at the 2-position to form methyl ketone pheromones (see FIGURE 5).

(b) *Termites*

The use of succinate as the precursor to methylmalonyl-CoA in methyl-branched hydrocarbon biosynthesis was first demonstrated in the termite (Chu & Blomquist, 1980). In the two species of termites, *Zootermopsis nevadensis* and *Z. angusticollus,* both propionate and succinate can be metabolized to methylmalonyl-CoA, for insertion into methyl-branched

FIGURE 5: Pheromone biosynthesis in the German cockroach, *Blatella germanica*



pheromones (Chu & Blomquist, 1980). As in the cockroach the conversion of succinate to methylmalonyl-CoA is facilitated by the high levels of vitamin B_{12} present in the insect (Chu & Blomquist, 1980).

(c) *Houseflies*

Methylalkanes can act as sex attractants for the housefly, *Musca domestica*. The biosynthesis of methylalkanes involves the synthesis of a long chain methyl-branched fatty acid which gives rise to an alkane one carbon unit shorter. Propionate or the amino acids valine and isoleucine are converted to propionyl-CoA, which is subsequently converted to methylmalonyl-CoA (as shown before) and is incorporated into the methyl branches in the final product. In contrast to termites and cockroaches, houseflies lack vitamin B_{12} (Wakayama et al., 1986). Thus succinate cannot act as a source of methyl-branching carbons in houseflies (Blomquist et al., 1987).

To summarize, in every insect examined to date the methyl-branched pheromones are made through modifications of normal fatty acid metabolism. The methyl-branch is generally derived from a methylmalonyl-CoA intermediate. Propionate and succinate can serve as precursors of the methylmalonyl-CoA intermediate. However, when valine or leucine act as carbon chain initiators, they are not necessarily converted to methyl malonyl-CoA. Blailock and Blomquist (1976) showed that valine is converted to isobutyric acid which is then incorporated into the even-numbered carbon chain length methylalkanes, while leucine is converted to isovaleric acid and incorporated into odd-numbered carbon chain length

methylalkanes.

III. REGULATION OF PHEROMONE BIOSYNTHESIS IN INSECTS

Insects do not produce their entire repertoire of pheromones at all times. Environmental and physiological factors such as light intensity, temperature, host habitat, air velocity, time of the day, age, previous experience, population density, diet, hormones and seasonal rhythms can all affect pheromone production (reviewed by Raina and Menn, 1987). Obviously, the process must be tightly regulated.

Hormonal regulation is the most important mechanism for regulating pheromone production in insects. In some insects the hormones involved are associated with reproductive maturity. For example, JH III and ecdysone are often used to regulate sex pheromone production. These hormones play a role in the sexual maturation of the insect, therefore the sex pheromone is released when there is a higher probability of mating. However, in moths JH III and ecdysone apparently play a secondary role. A moth calls (rhythmically extends the ovipositor to expose the pheromone gland) at specific times everyday. In some moths it has been shown that pheromone production is maximized when calling occurs (Hendrikse, 1979). A special mechanism of hormonal control is necessary to maintain a circadian rhythm such as this (reviewed by Raina and Menn, 1987). Pheromone Biosynthesis Activating Neuropeptide (PBAN), plays a role in the regulation of pheromone biosynthesis in moths.

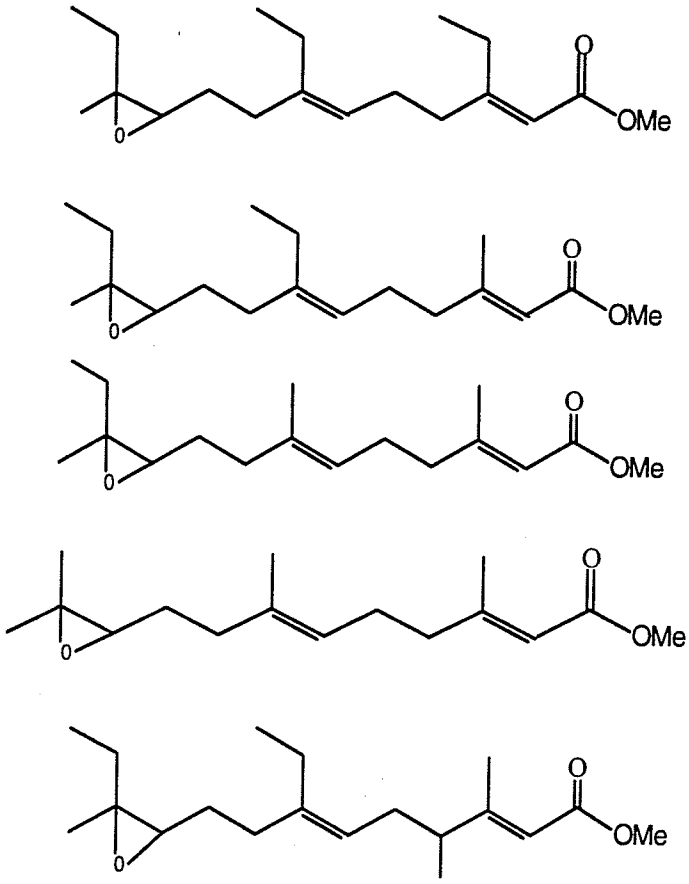
(A) JUVENILE HORMONE:

JH, the first insect hormone discovered, is an important hormone that is involved in the regulation of pheromone biosynthesis in many beetle species (Vanderwel & Oehschlager, 1987; Chen et al., 1988; Dickens et al., 1988). The hormone is produced by the corpora allata, stored in the corpora cardiaca, and secreted into the hemolymph through which it is transported to target cells (reviewed by King, 1983). The structure of JH III, the type of JH most commonly found in beetles, is shown in FIGURE 6. Other groups of insects, most notably the moths, produce JH's of related structure (also shown in FIGURE 6).

JH regulates pheromone production in several insects. JH analogues stimulate sex pheromone production in the German cockroach (Schal et al., 1991). JH has been found to play a role in control of sex pheromone production in only one species of moth, the armyworm moth (Cusson et al., 1994). JH or JH analogues also stimulate the production of aggregation pheromones by several species of beetles (including more than 10 species of grain beetles and bark beetles) (Pierce et al., 1986). To date three modes of JH action have been proposed.

Dickens et al. (1988) proposed that JH might regulate pheromone production by regulating the antennal response of the male boll weevil, *Anthonomus grandis*. When the antennae of the male boll weevil are removed, pheromone production is stimulated. When the boll weevils are treated topically with the JH analogue methoprene, two things occur simultaneously: pheromone production is increased and the sensitivity of the antennal olfactory receptors is decreased. This indicates that JH may influence pheromone production

FIGURE 6: Five different types of Juvenile Hormones



JH 0

JH I

JH II

JH III

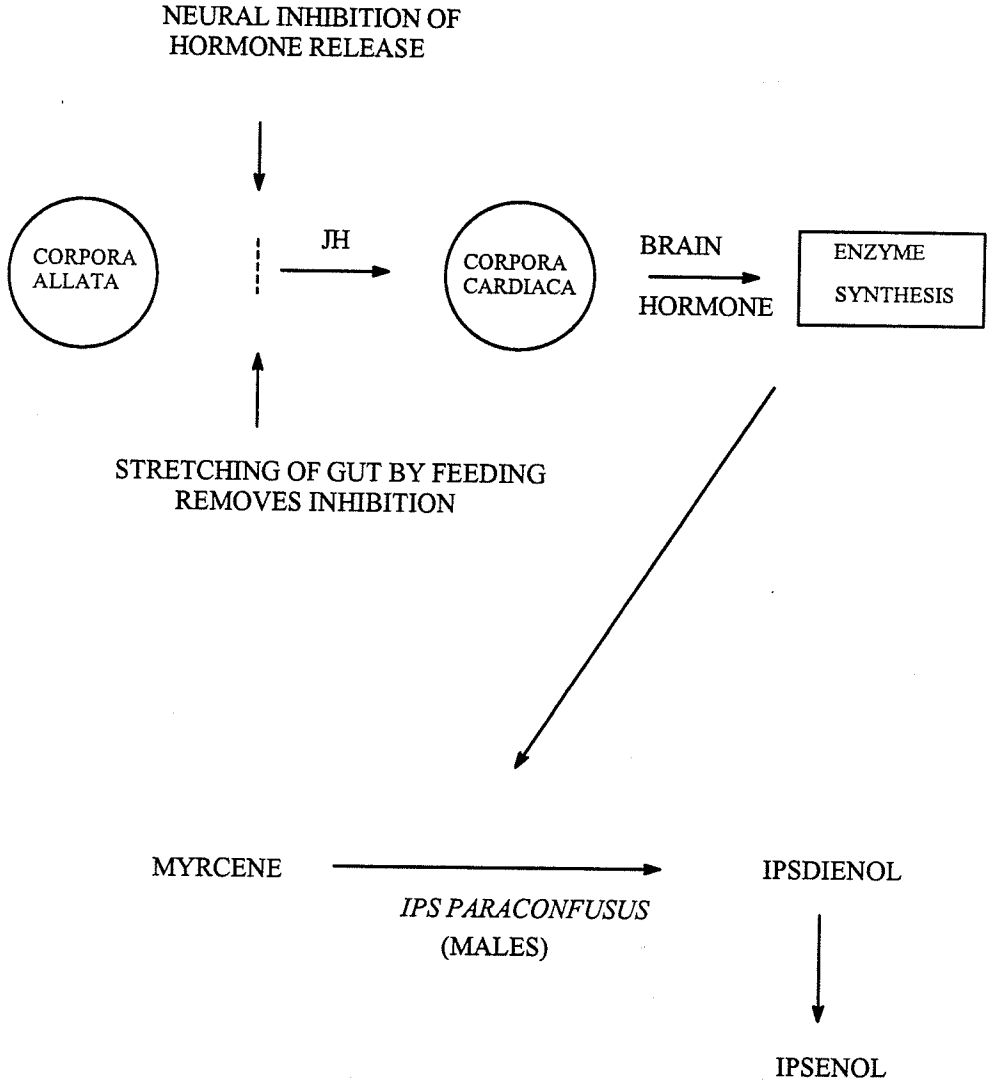
4-METHYL JH I

by decreasing antennal sensory responses, which in turn might stimulate pheromone production (Dickens et al., 1988).

A second mechanism by which JH might regulate pheromone production was proposed by Hughes and Renwick (1977). They confirmed earlier reports that pheromone production in the bark beetle, *Ips paraconfusus*, is stimulated by JH and that this stimulation is inhibited by decapitation. This led them to propose that JH might stimulate pheromone production by stimulating the corpora cardiaca to release a "brain hormone" which, in turn, might stimulate pheromone biosynthesis. There is some evidence that in *I. paraconfusus* neural inhibition is removed by stretching of the gut during feeding. This action might stimulate the corpora allata to release JH, which in turn could act on the corpora cardiaca to release the brain hormone (Hughes & Renwick, 1977) (see FIGURE 7). This putative "brain hormone" may be analogous to the PBAN found in moths (Vanderwel, 1994).

A third mechanism by which JH might stimulate pheromone biosynthesis is by acting directly at the site of pheromone production. When immature (3 day old) yellow mealworm females, which do not produce pheromone, are treated with JH analogues, a dramatic increase in pheromone production is observed (Menon, 1976). Decapitation or the removal of the corpora allata results in the decrease of pheromone production in female yellow mealworms, but treatment of decapitated insects with JH analogues restores pheromone production (Menon, 1970, 1976; Menon and Nair, 1976). Since JH could restore pheromone production in the absence of a "brain hormone" or other factors from the head such as nerve impulses,

FIGURE 7: Proposed scheme for neuroendocrine control of ipsdienol and ipsenol production (according to Hughes & Renwick, 1977)



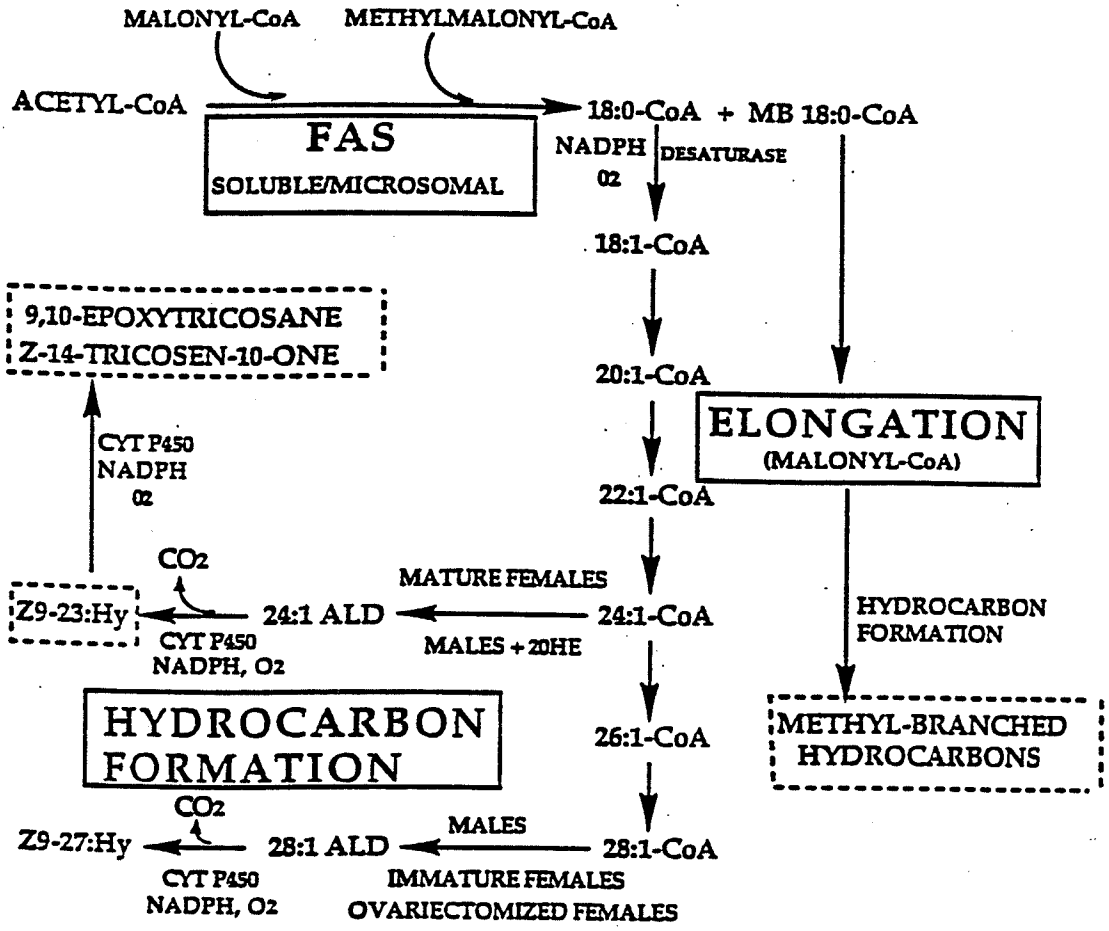
it could be acting directly at the site of pheromone production.

(B) ECDYSTEROIDS:

Sex pheromone production in the housefly is correlated with ovarian maturation, since pheromone production is first detected when females start producing vitellogenin (Dillwith et al., 1983). Vitellogenin is synthesized by the fat body, released into the hemolymph and cycled through the body during oogenesis (Adams and Filipi, 1983). Adams et al. (1984) showed that the removal of the corpora allata-corpora cardiaca complex has no effect on sex pheromone production, indicating that JH and possibly PBAN is not involved. In contrast, removal of the ovaries blocks sex pheromone synthesis. Pheromone production is restored by the reimplantation of ovaries into ovariectomized females, and this effect can be mimicked by treatment with 20-hydroxyecdysone (20-HE) (Adams et al., 1984; Blomquist et al. 1984, 1987). Dillwith and Blomquist (1982) concluded that the maturing ovary produces an ecdysteroid that is metabolized to 20-HE which, in turn, induces pheromone production in epidermal tissue.

In the housefly a novel microsomal fatty acid synthetase (FAS) is present in the epidermal tissue and plays a role in producing the methyl-branched fatty acyl-CoA elongation system (see FIGURE 8). In immature female and male houseflies of all ages the predominant alkene is (Z)-9-heptacosene (Z9-27:Hy). As the female houseflies begin to mature the level of Z9-27:Hy decreases and (Z)-9-tricosene (Z9-23:Hy) increases. Male houseflies do not produce Z9-23:Hy under normal circumstances but can be induced to do so by implanting ovaries or

FIGURE 8: Pheromone biosynthesis in the housefly, *Musca domestica* (reproduced from Blomquist et al., 1994)



treatment with 20-HE. Sex pheromone production is regulated by a 20-HE-induced change in the chain length specificity of the alkenes (reviewed by Blomquist et al., 1984) (see FIGURE 8).

(C) PBAN:

Riddiford and Williams (1971) were the first to propose that neuroendocrine control may be involved in the regulation of sex pheromone production in moths. Raina and Klun (1984) demonstrated that PBAN is critically involved in the regulation of pheromone production in female corn earworm, *Heliocoverpa zea*. Since then PBAN has been found to induce pheromone production not only in many other species of moths, but even in other orders of insects such as locusts, cockroaches and crickets. PBAN is produced in the suboesophagial ganglion and is stored in the corpora cardiaca. In response to environmental cues it is then released into the hemolymph and acts directly on the sex pheromone glands via a calcium/calmodulin-dependent adenylate cyclase (Raina, 1988).

Several theories have been presented regarding PBAN action on the enzymes involved in sex pheromone biosynthesis in moths. In some moths regulation of pheromone production by PBAN has been indicated very early in the biosynthetic process (i.e., at a step prior to fatty acid synthesis). Tang et al. (1989) suggested that PBAN regulates pheromone production in the red-banded leafroller moth, by activating the substrate supply for fatty acid synthesis. In other moths such as *Mamestra brassicae*, PBAN seems to affect a Δ-11-desaturation step (Bestmann et al., 1989). However, in *Spodoptera littoralis* (Martinez et al.,1990) and

Bombyx mori (Arima et al., 1991), later steps such as the reduction of fatty acyl moieties are regulated.

In summary, PBAN, ecdysteroids and JH are the major hormones that play a role in the regulation of pheromone biosynthesis in many insects (TABLE I summarizes endocrine regulation in selected species of insects). These hormones may work independently or in conjunction with other hormones. In beetles JH is the major hormone involved in pheromone biosynthesis. We examined the role of JH in pheromone biosynthesis of the yellow mealworm beetle.

TABLE I: ENDOCRINE CONTROL IN SELECTED SPECIES OF INSECTS

SPECIES	SEX	GLANDS & ORGANS INVOLVED	HORMONE INVOLVED	PROCESS REGULATED	REFERENCE
Leucophaea maderae	Female	Corpora allata			Luscher & Engelmann, 1966
Pycnoscelus indicus	Female	Corpora allata			Barth, 1965
Byrotria fumigata	Female	Corpora allata Brain	Juvenile Hormone	Production*	Bell & Barth, 1970
Periplaneta americana	Female	Corpora allata		Production*	Barth, 1965
Blaberus discoidalis	Female	Corpora allata		Production*	Barth & Lester, 1973
Anthonomous grandis	Male		Juvenile Hormone III	Production*	Hardee, 1970
Antheraea polyphemus	Female	Corpora cardiaca		Calling-release of pheromone	Riddiford & Williams, 1971
Hylaphora cecropia	Female	Corpora cardiaca		Calling-release of pheromone	Riddiford & Williams, 1971
Tenebrio molitor	Female	Corpora allata	Juvenile Hormone III	Production*	Menon, 1976

Ips paraconfusus	Male	Corpora allata Corpora cardiaca	Juvenile Hormone III "Brain hormone"	Biosynthesis	Hughes & Renwick, 1977
Pityokteins curvidens	Male	Corpora allata Brain	Juvenile Hormone	Biosynthesis	Harring, 1978
Pityokteins spinidens	Male	Corpora allata Brain	Juvenile Hormone	Biosynthesis	Harring, 1978
Pityokteins vorontzovi	Male	Corpora allata	Juvenile Hormone	Biosynthesis	Harring, 1978
Musca domestica	Female	Ovary	Ecdysone	Biosynthesis	Dillwith et al., 1980, 1981
Heliothis zea	Female	Corpora allata	PBAN	Production*	Raina, 1988

*Production= Biosynthesis + Release of pheromone

IV. RESEARCH PROPOSAL: PHEROMONE BIOSYNTHESIS IN THE GRAIN BEETLE, *TENEBRIO MOLITOR*

The overall goal of this research project was to study the pathway and regulation of pheromone biosynthesis in the yellow mealworm beetle, *T. molitor*. As mentioned previously, the yellow mealworm is an excellent beetle for the study of pheromone biosynthesis. Mature female yellow mealworms produce a sex pheromone, 4-methylnonanol (Tanaka et al., 1986) (see FIGURE 9). Much like the pheromones discussed earlier, 4-methylnonanol is methyl-branched and likely of fatty acid origin. The structure is interesting since it has an odd number of carbons in its carbon chain, the carbon chain is much shorter than normal (most methyl-branched pheromones studied to date are 24-30 carbons long), and the methyl branch is in an unusual ω-6 position.

The first step of the research project was to confirm that the female beetles in our lab colonies do indeed produce 4-methylnonanol. Pheromone extracts from female beetle were analyzed by Gas Chromatography (GC)/Mass spectroscopy (MS) and were compared to an authentic sample of 4-methylnonanol. Authentic 4-methylnonanol is not commercially available, but a synthetic route to this compound was developed using commercially available starting materials.

One of the major objectives of this project was to elucidate the biosynthetic pathway of 4-methylnonanol. As mentioned previously, methyl-branched fatty alcohols are generally formed through modification of normal fatty acid metabolism. The methyl-branch is generally

contributed by methyl-malonyl CoA, which may ultimately be derived from propionate. When the chain is of the appropriate length, it is converted to the alcohol. There are two possibilities: the fatty acid may be reduced to an alcohol directly (FIGURE 9 A); or the fatty acid may first be decarboxylated to form an alkane, dehydrogenated to an alkene, and finally converted to an alcohol with the addition of water (FIGURE 9 B). The conversion of alkane to alcohol via an alkene intermediate occurs in biological reactions, examples of which are β-oxidation and the TCA cycle in cells (Lehninger et al.,1993). However, we predicted that the fatty acid would be directly reduced to the alcohol since it is a simpler process and is known to occur in the production of pheromone in other insects (reviewed by Riendeau and Meighen, 1985).

The proposed biosynthetic route to 4-methylnonanol is shown in FIGURE 10. In this route the odd number of carbons in the backbone are shown to result from the initiation of chain biosynthesis with a propionyl derivative instead of the normal acetyl derivative. The unusual ω-6 methyl branch is proposed to be derived through elongation with a methylmalonyl derivative rather than a malonyl derivative.

The viability of this pathway was explored using stable isotope-labelled precursors. Hendry et al. (1980) were the first to use stable isotopes to study pheromone biosynthesis in insects. Since then stable isotopes have been successfully used to study pheromone biosynthesis in other beetles (eg. Vanderwel et al.,1992; Petroski et al., 1994), moths (eg. Bjostad et al., 1986), houseflies (eg. Blomquist et al., 1987) and cockroaches (eg. Chase et al.,1990).

FIGURE 9: Fatty acid may form alcohol directly (A) or via intermediates (B)

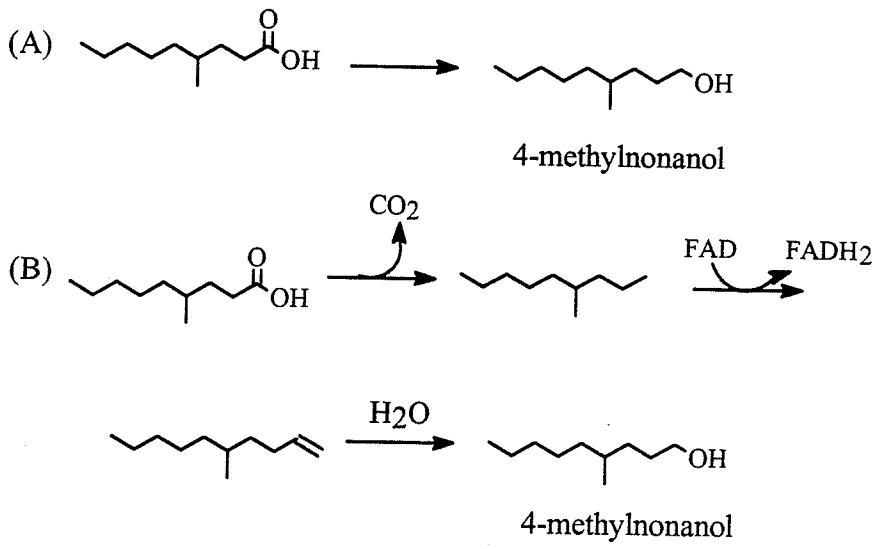
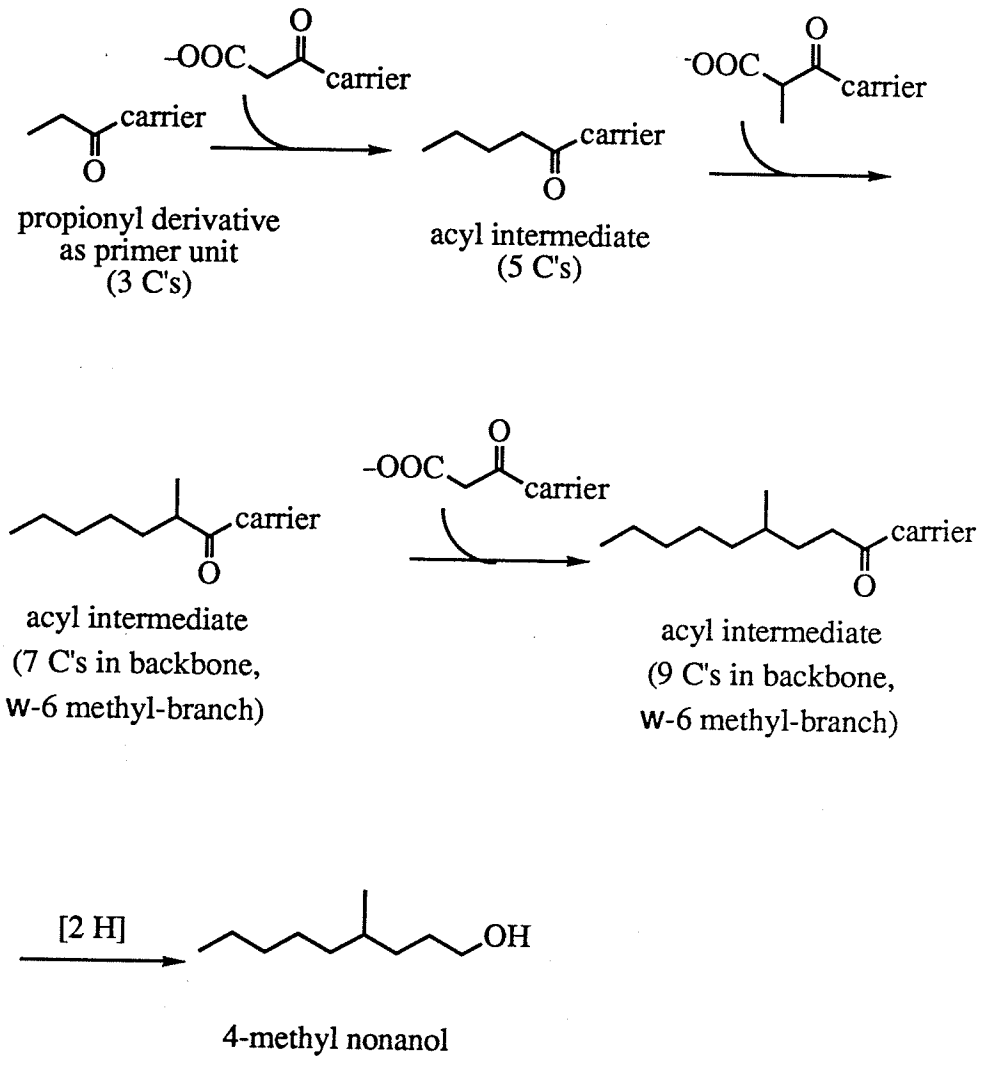


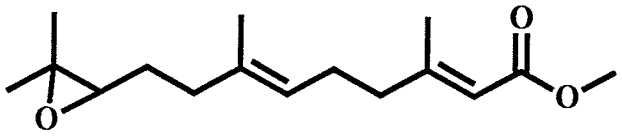
FIGURE 10: Putative biosynthetic route of 4-methylnonanol



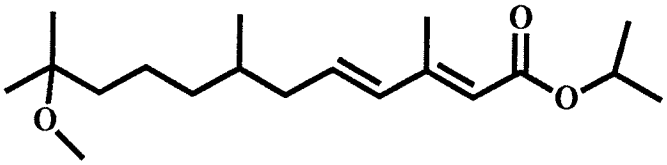
Incorporation of precursors labelled with carbon-thirteen (^{13}C) or deuterium (D) into 4-methylnonanol will increase the molecular weight of the pheromone, and should be readily discernible by GC/MS. The beetles were fed a diet containing organic acids labelled with stable isotopes. Mass spectroscopy was used to locate the label in the pheromone. Acetate and propionate labelled with ^{13}C are commercially available, while the other labelled precursors of interest were synthesized in our laboratory.

Menon (1970, 1976) showed that pre-treatment of immature female beetles with a JH analogue increased the biological activity of the extracts of other females. However, since the sex pheromone was not identified until 1986, she was unable to correlate the increase in biological activity with an increase in 4-methylnonanol production. Thus, one of our objectives was to determine if JH was indeed involved in the regulation of 4-methylnonanol biosynthesis. Since JH is very unstable and expensive we used the JH analogue methoprene in our experiments (see FIGURE 11). The first step was to see if methoprene could mimic the affects of JH by bioassay. Immature female beetles were treated with methoprene and extracted in solvent. The activity of the extracts was assessed by bioassay. An increase in the biological activity of the extracts of methoprene treated females would indicate that methoprene could mimick the effects of JH. If so, the next step was to show that enhanced biological activity was due to an increase in 4-methylnonanol production. Our approach was to compare pheromone production by immature females to that by mature females and immature females pre-treated with methoprene, using a radiolabelled precursor.

FIGURE 11: Juvenile Hormone III and its analogue methoprene



Juvenile Hormone III



Methoprene

This type of research can be used to extend the study of pheromone biochemistry in many beetles. Beetles are the largest group of animals, and yet the biochemistry of these insects has been largely neglected. An increased understanding of the biochemistry of beetles may lead to new strategies for pest control.

MATERIALS AND METHODS

I. CHEMICALS

Sodium[1-^{13}C]-propionate (99%) was purchased from Cambridge Isotope Laboratory. Sodium[1-^{13}C]-acetate (99.8%) was purchased from Merck Frost Canada, Inc. [1-^{13}C]-2-methylheptanoic acid and [1-^{13}C]-pentanoic acid were synthesized by Albert Moore, a summer student in our lab. 4-Methylnonanol, [3,4-D_2]-4-methylnonanoic acid and [^{3}H]-4-methylnonanoic acid were synthesized by Dr. Désirée Vanderwel. Methoprene was kindly donated by Dr. Paul Fields, Agriculture and Agri-Food Canada.

II. SYNTHESIS OF 4-METHYLNONANOL

All reactions were performed under an inert atmosphere of nitrogen gas. Structures were confirmed by ^{1}H NMR on a Varian Gemini 200 spectrometer at 200 MHz, using $CDCl_3$ as the solvent.

(A) 5-Bromo-2 pentanone

Acetyl butyrolactone (0.5 mol) was dissolved in 25 ml of water. Hydrogen bromide (0.7 mol) was added dropwise. The mixture was heated and the distillate was collected. The distillate was dissolved in water (100 mL) and extracted with diethyl ether (4 x 50 mL). The combined diethyl ether layers were extracted sequentially with 5% $NaHCO_3$ (2 x 10 mL) and brine (2 x 20 mL). The extract was dried over anhydrous $MgSO_4$, filtered, and the solvent removed by rotary evaporation under reduced pressure. The product was purified by bulb-to-bulb

distillation at 40-42 °C under vacuum. A colourless oil was obtained in 49.7 % yield.[1] H NMR: 2.12 (2H, m), 2.19 (3H, s), 2.66 (2H, t).

(B) 1-Bromo-4-methylnonane

1. Phosphonium salt: bromopentane (10 g) was added to triphenylphosphine (19 g) in xylene (25 mL) and refluxed overnight. Xylene was removed by decantation and the salt was dried by suction filtration. The white crystalline product was obtained in 80% yield and used directly in the next step.

2. (E) and (Z) 1-bromo-4-methyl-4-nonene: Butyllithium (2.5 M in hexane, 8.93 mL) was added to the phosphonium salt (9.22 g) in anhydrous tetrahydrofuran (20 mL) with stirring at R.T. The bright red colour of the ylide was obtained immediately. After 1 h the 5-bromo-2-pentanone (4.05 g) dissolved in anhydrous tetrahydrofuran was added to the ylide. The red colour disappeared, and the reaction mixture was left to stir overnight. The product was dissolved in water (100 mL) and extracted with hexane (3 x 50 mL). The combined hexane layers were extracted separately with brine (1 x 25 mL). The organic layer was dried over anhydrous $MgSO_4$, filtered, and concentrated by rotary evaporation under reduced pressure. The product was purified by bulb-to-bulb distillation. A colourless oil was obtained in 10% yield. ^{1}H NMR: 1.7 (3H, m), 3.4 (2H, t), 5.2 (1H, t) 0.9 (3H, t).

3. 4-methylnonyl bromide: The alkenes (E) and (Z) 1-bromo-4 methyl-4-nonene were dissolved in ethanol and added to a suspension of palladium on charcoal (5%) in ethanol (5

mL). The reaction mixture was stirred at R.T. under an atmosphere of hydrogen gas. Progress of the reaction was monitored by gas chromatography. The reaction was stopped after two days and product was gravity filtered and transferred with pentane. Water (50 mL) was added and extracted with pentane (3 x 50 mL). The organic extract was dried over anhydrous $MgSO_4$ and concentrated by rotary evaporation under reduced pressure. A colourless oil was obtained in 56% yield. ^{1}H NMR: 0.85 (6H, d), 1.1 (2H, m), 1.85 (1H, m), 1.3 (10H, s), 3.4 (2H, t).

(C) 4-methylnonanol

4-Methylnonyl bromide (0.05 g) was added to a 10% solution of KOH in methanol and stirred for 3 days. The product was dissolved in water (50 mL) and extracted with pentane (3 x 50 mL), dried over anhydrous $MgSO_4$, and concentrated by rotary evaporation under reduced pressure. A colourless oil was obtained in 10% yield. ^{1}H NMR: 0.85 (6H, d), 1.1 (2H,m), 1.3 (10H, s), 1.65 (1H, m),3.5 (2H, t).

III. PREPARATION OF EXPERIMENTAL CULTURES.

T. molitor was reared on a mixture comprised of 19% bran, 57% whole wheat, 14% rolled oats, 5% wheat germ and 5% brewers yeast (by weight), in the dark at 28-30 ° C. Paper towels spread over the colonies were moistened every day to provide ample moisture to the beetles. Pupae were gathered from the cultures and separated by sex using the method of Bhattacharya et al. (1970). Males and females were maintained separately for use in experiments.

IV. COATING THE LABELLED PRECURSORS ON THE BRAN

The yellow mealworms were exposed to stable isotope-labelled precursors by allowing the mature female beetles (5-6 days old) to feed on bran coated with precursors of interest. To reduce the dilution of the labelled compounds with unlabelled lipids, the bran was first defatted by Soxhlet extraction with diethyl ether for 1 h. The labelled compounds (100 mg) were dissolved in 75-80 mL of 95% ethanol or pentane in a 500 mL round-bottomed flask. Defatted oats (15 g) were added and the solvent was removed *in vacuo*. Remaining traces of solvent were removed by passing air over the bran (Vanderwel et al., 1992).

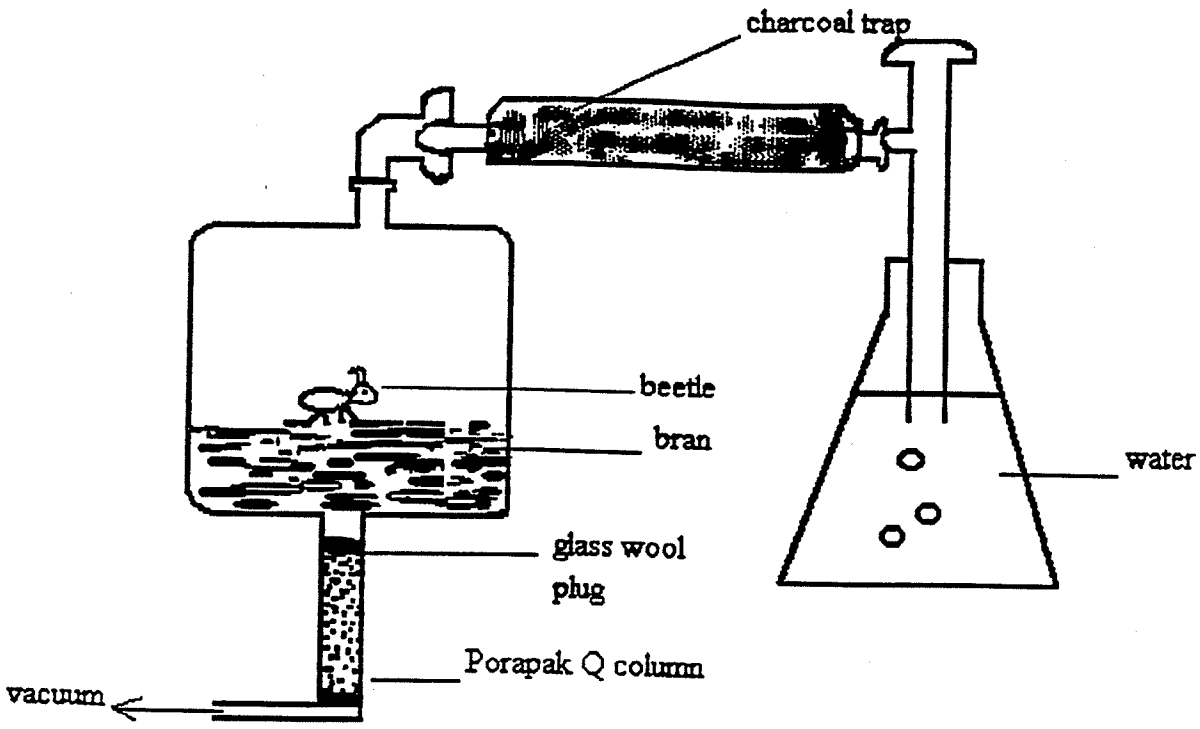
V. AERATIONS

The precursor-impregnated bran and untreated female beetles (30) were introduced into an aeration chamber (see FIGURE 12). Culture volatiles were captured on a Porapak Q trap for 6 days at 25-30 °C using the technique described by Pierce et al. (1984) and Vanderwel et al. (1990). The apparatus is diagrammed in FIGURE 12. Beetles were usually starved for 48 h before starting the experiment to reduce the endogenous fatty acid pools, thereby enhancing the incorporation of the stable-isotope labelled substrate (Vanderwel et al., 1990).

VI. PREPARATION OF PORAPAK Q EXTRACT.

After 6 days the Porapak Q columns were eluted with diethyl ether (20 mL). Eluates were concentrated to 40 μL with a gentle stream of nitrogen gas. The sides of the vials were washed down with pentane since some of the compounds tend to stick to the glass. After the diethyl ether extract was concentrated, hexane (50 μL) was added to facilitate GC analyses.

FIGURE 12: The aeration apparatus



(Hexane has a higher boiling point than diethyl ether and tends to give better peak shapes on the GC.)

VII. GC-MS ANALYSES OF 4-METHYLNONANOL.

Gas chromatographic analyses were performed on a Hewlett Packard 5890 gas chromatograph equipped with a capillary inlet system and a flame ionization detector (FID). The sample was injected using splitless injection into an open tubular column (25 mm x 0.55 mm I.D., 35 μm film thickness). The oven was programmed to provide an initial temperature of 50 ° C for 0.5 min followed by an increase of 4 °C/min to 180 ° C, with a total run time of 33 min. GC-MS analyses were recorded on a Hewlett Packard MS 5970 mass selective detector (EI, 70 EV). The same column and oven program as for GC analyses were used. 4-Methylnonanol in the Porapak Q extracts was identified through comparison of its fragmentation pattern and/or retention time with those of an authentic sample of 4-methylnonanol.

Selected Ion Monitoring (SIM) was used to determine the percentage incorporation of stable isotope-labelled precursors.

VIII. BIOASSAYS

Beetles were subjected to different treatments in order to assess the effect of methoprene on pheromone production. Immature female beetles (1-3 days old) were either treated topically between the thorax and the abdomen with 12 μg methoprene dissolved in 1 μL acetone, or

were left untreated. After 24 h the beetles were soaked in pentane (1 beetle/2.5 mL) overnight. Mature female beetles (6-7 days old) were either untreated, decapitated, or both decapitated and treated with methoprene. Mature female beetles that were decapitated had their wound sealed with wax. After 1 h some of the decapitated beetles were treated with methoprene in acetone (12 μg/μL). After 24 h they were extracted in pentane as described for the immature females.

The activity of the pentane extracts of the female beetles subjected to each treatment was determined by the response of male beetles by bioassays based on the method developed by Tschinckel et al. (1967). The activity of each extract was tested against 10 randomly selected virgin male beetles (7-10 days old). The entire procedure (preparation of new extracts and determination of activity by bioassay) was replicated 3 times. Each beetle was placed on the surface of a piece of brown paper under a glass petri dish (9 cm diameter), in a room illuminated by red light. The beetles were allowed to become accustomed to their surroundings for 20 min, before the experiment was begun. Glass rods (prepared from 3 mm glass tubing cut into rods 2.5 cm long and fire polished at both ends) were dipped into the extract and gently shaken before being placed under the petri dishes with the beetles (one rod/beetle). A positive result consisted of the male beetle climbing onto the rod and everting its phagus before 20 min elapsed. Observations were recorded by two other people that were not aware of the identity of the extracts, in order to ensure the results were unbiased.

Experiments where $p < 0.005$ after analyses by the ANOVA test, were taken to be statistically

significant.

IX. ANALYSES OF THE INCORPORATION OF RADIOACTIVE PRECURSOR IN THE BEETLES

The precursor [^{3}H]-4-methylnonanoic acid was dissolved in ethanol. Beetles were injected between the thorax and the abdomen with 0.5 µL of the ethanol solution, using a Hamilton 10 µL syringe (needle gauge 33.5, point type 4). The beetles were placed on food in an incubator, in the dark at 28°C. After 3 h the beetles were extracted by an adaptation of the procedure of Hara and Radin (1978). The beetles were crushed in pentane:isopropyl alcohol (3:2, 2 mL) and the extract was removed. This procedure was repeated three times. The extracts were combined and water (8 mL) was added. The mixture was vortexed and centrifuged (5 min, 2000 rpm) on a IEC HN-SII centrifuge to separate the layers. The upper pentane layer was removed. The lower layer (containing water and isopropanol) was then extracted with pentane (3 x 2 mL), to remove any organics still present. The organic layers were combined and extracted with water (3 x 2 mL), to remove all traces of remaining isopropanol. The extract was dried over anhydrous sodium sulfate and loaded onto a mini-column. These columns were prepared by inserting glass wool plugs into 2 mL Pasteur pipettes and filling them with florisel gel (100-200 mesh) up to a height of 6 cm. The beetle extracts loaded on the Florisel columns were sequentially eluted with pentane (10 mL), pentane:diethyl ether (80:20, 10 mL) and ethyl ether:acetic acid (100:1, 10 mL). Each sample was concentrated to 0.5 mL by blowing nitrogen over it. Ten millilitres of the scintillation fluid, ScintiLene^{TM}, was added to the samples with thorough mixing. The samples were

counted for 10 min on a Beckman LS 7500 microprocessor controlled scintillation counter.

The standards 4-methylnonanol and 4-methylnonanoic acid were analyzed by High Pressure Liquid Chromatography on a Beckman gradient liquid chromatograph, model 334, equipped with a Phenomenex Spherisorb 10 C18 (250 x 2.6 mm) column. Elution was started at 75% methanol:25% water and the methanol concentration was increased 1%/min over 25 min up to 100% methanol. The flow rate was 1 mL/min. Fractions (0.5 mL) were collected every 0.5 min. 4-Methylnonanol eluted with pentane:diethyl ether (80:20) and 4-methylnonanoic acid eluted with ethyl ether:acetic acid (100:1).

RESULTS

I. PREPARATION OF 4-METHYLNONANOL STANDARD

We chose a standard chemical approach to synthesize 4-methylnonanol as seen in FIGURE 13. The methyl branch was inserted in the correct position with a Wittig reaction. The ketone 5-bromo-2-pentanone was coupled to a phosphonium salt (prepared from bromopentane and triphenylphosphine) to form the alkenes (E) and (Z) 1-bromo-4-methyl-4-nonene. The alkene functionality was easily removed by reduction with hydrogen gas over the catalyst palladium. Finally, the bromide was converted to the desired alcohol functionality by an S_N2 reaction with potassium hydroxide, to form 4-methylnonanol. The overall yield of the desired product was very low (0.56% yield) (see DISCUSSION for explanation).

4-Methylnonanol has a retention time of 23.5 min. under the temperature program we used on the GC. The MS fragmentation pattern is similar to that reported by Tanaka et al., 1986. However, the GC-MS results show that 4-methylnonanol was not very pure (1%) (see FIGURE 14). Modifications of this method were used by Dr. Vanderwel to obtain a pure product in better yield (see FIGURE 15). The pure synthetic compound was used to identify the pheromone 4-methylnonanol from beetle volatiles.

II. ELUCIDATION OF THE BIOSYNTHETIC PATHWAY OF 4-METHYLNONANOL BY GC/MS ANALYSES

The numerous peaks in the GC trace (shown in FIGURE 16) indicate that the Porapak Q

FIGURE 13: Synthetic route to 4-methylnonanol

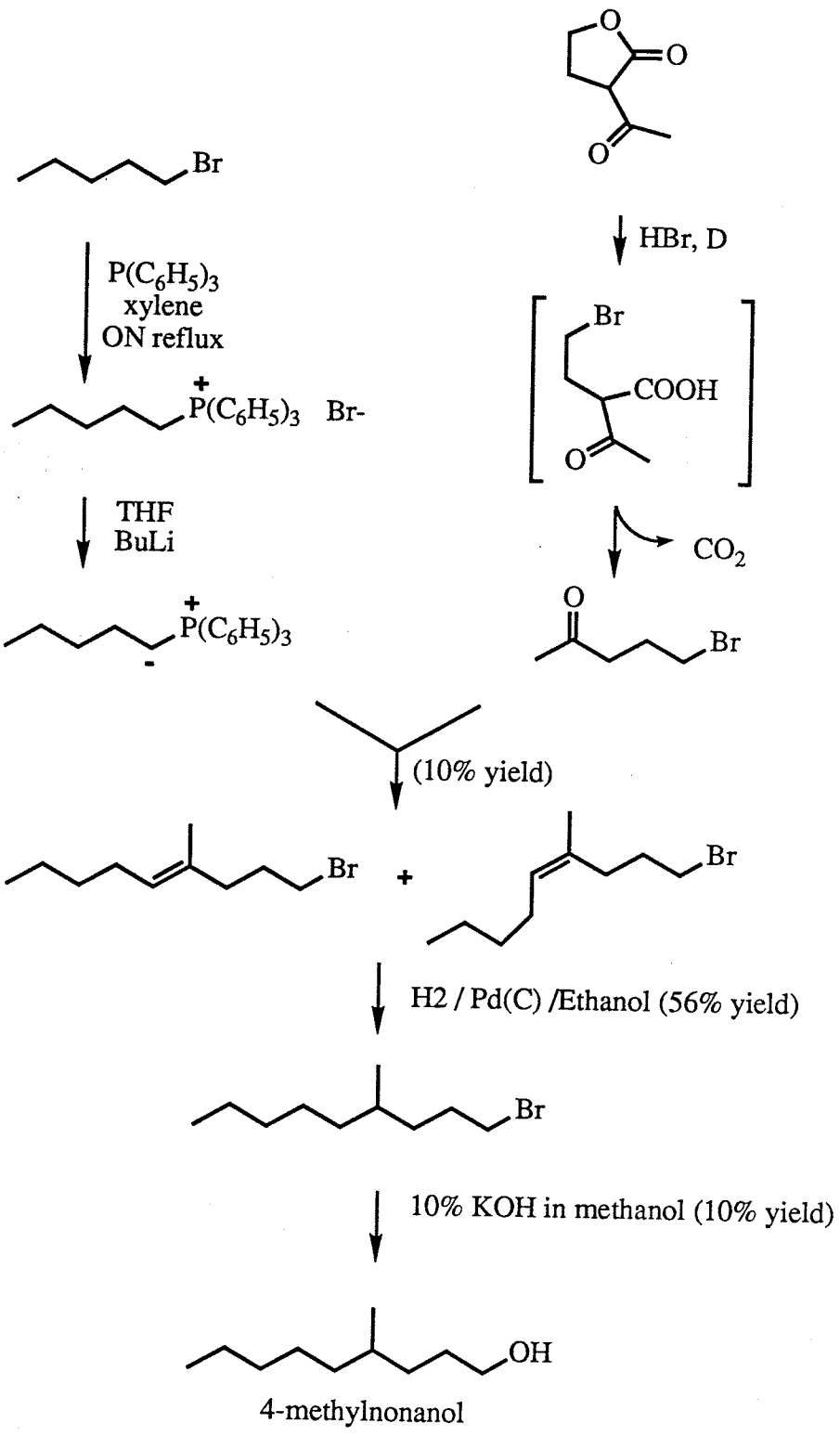


FIGURE 14: GC/MS of synthetic 4-methylnonanol (not pure)

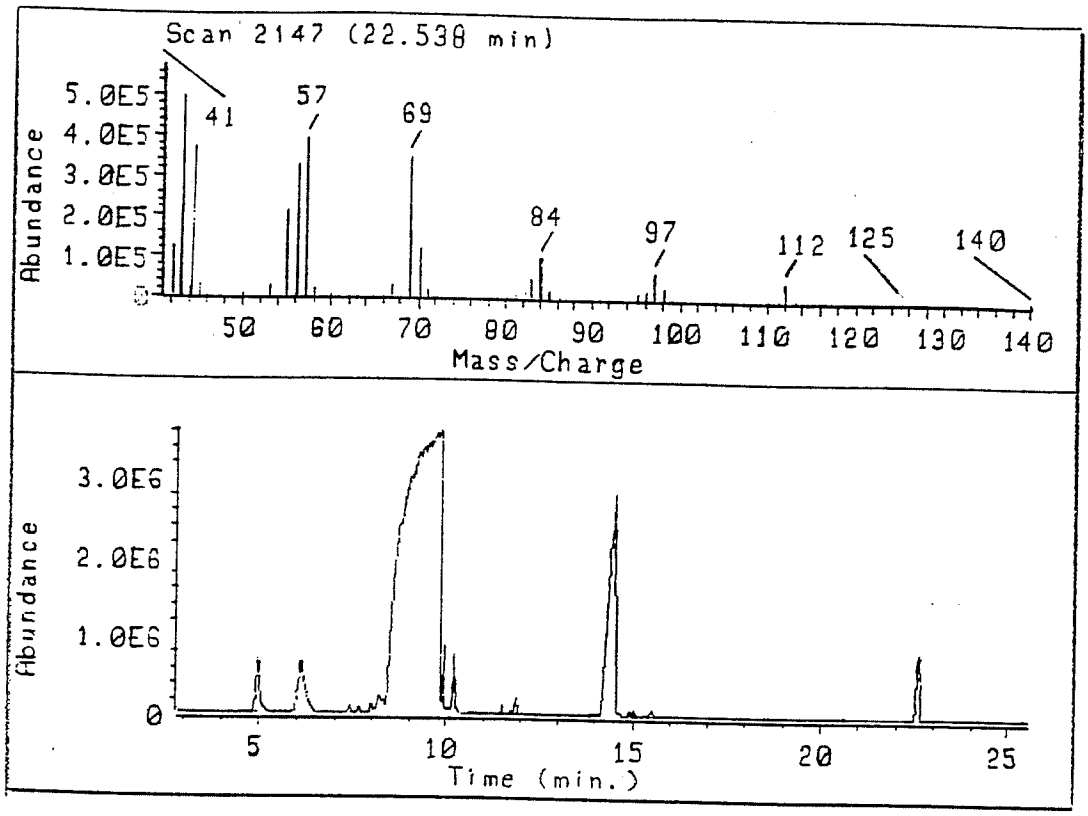


FIGURE 15: GC/MS of synthetic 4-methylnonanol (purified)

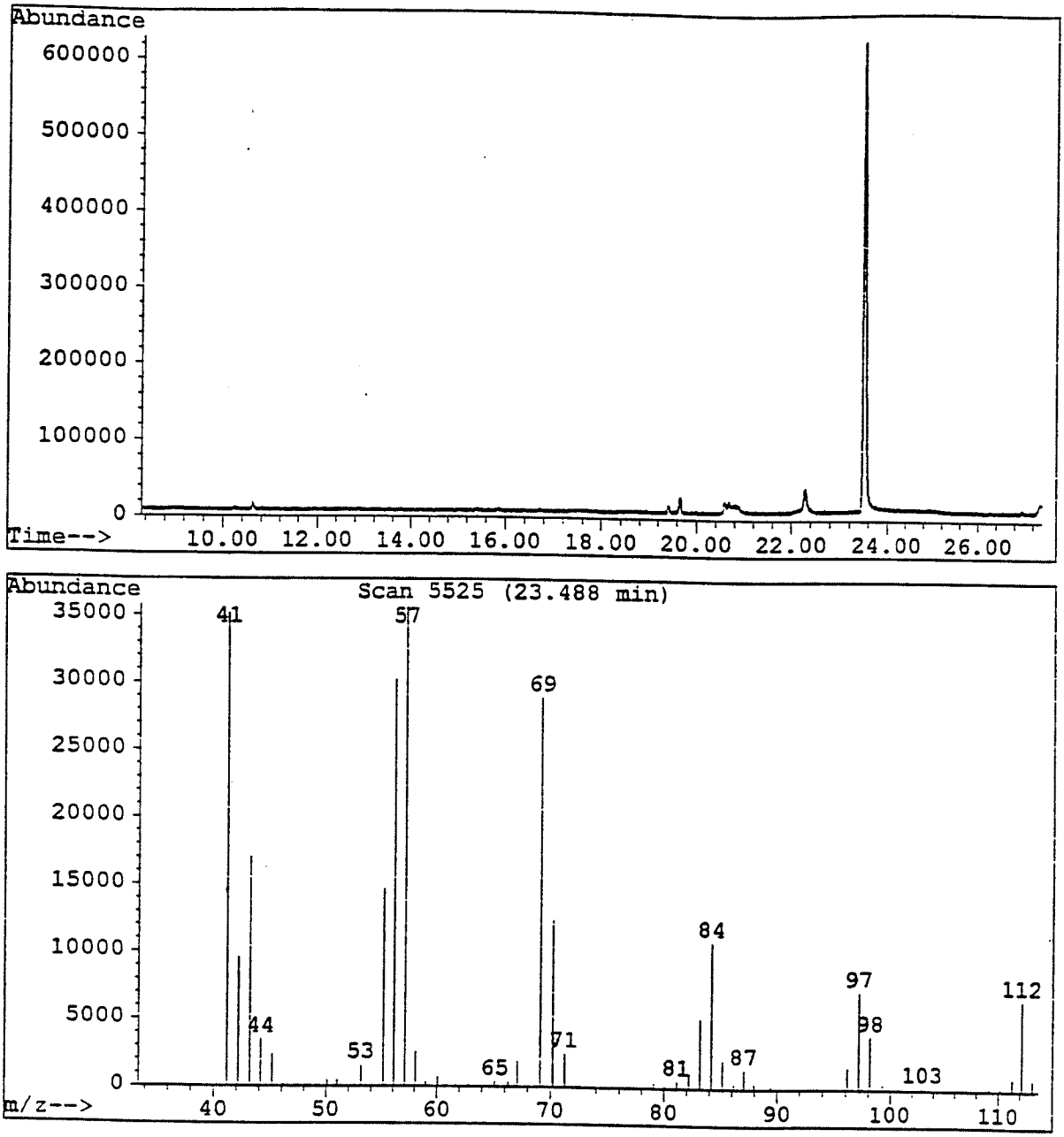
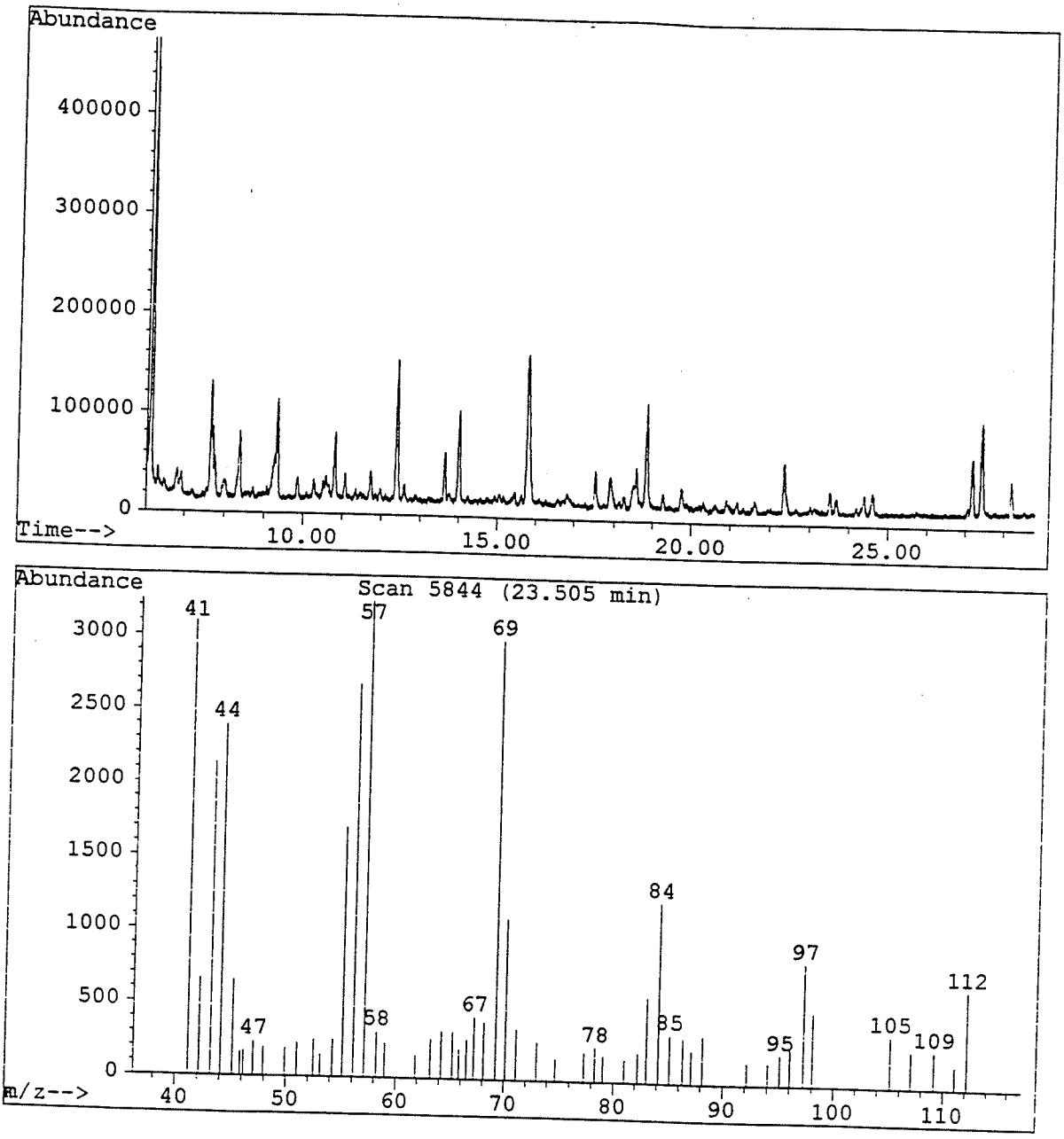


FIGURE 16: GC/MS of 4-methylnonanol in Porapak Q trapped volatiles of mature female yellow mealworm beetles



extract of *T. molitor* contains many compounds in addition to the pheromone. These likely include hydrocarbons from the cuticular surface of the insect and volatile compounds emitted in the frass. The pheromone peak was located through comparison of its retention time with the authentic 4-methylnonanol standard (23.5 min), and the identity was confirmed by comparison of the MS fragmentation patterns.

Incorporation of the putative precursors, labelled with stable isotopes, into the pheromone was determined by GC/MS. If ^{13}C or D labels were incorporated into the pheromone, some fragments would shift due to the increased mass of the stable isotope (depending on the location of the label on the putative precursor).

(A) Incorporation of [1-^{13}C]acetate:

According to the putative biosynthetic route (FIGURE 17), at most two acetate units would be incorporated into 4- methylnonanol. Since the acetate was labelled with ^{13}C at position 1, the ^{13}C label could be incorporated into positions 1 and/or 5 in the carbon chain of the pheromone. When the molecule fragments, C1 is lost almost immediately (the molecular ion peak is very small, as is the usual case with primary alcohols) and we were unable to detect the incorporation of ^{13}C from acetate into position 1 of the pheromone. Incorporation of ^{13}C into position 5 should be discernible by a shift of the m/e 69, 97, 112 fragments to m/e 70, 98, and 113 respectively (see FIGURE 17). The fragments of m/e 43 and 57 would have lost C5 and thus would not display a shift. Inspection of the GC-MS fragmentation pattern of the 4-methylnonanol produced in the presence of [1-^{13}C]acetate and comparison to the standard,

FIGURE 17: Expected labelling pattern in presence of [1-^{13}C]-acetate (^{13}C is represented by an asterisk)

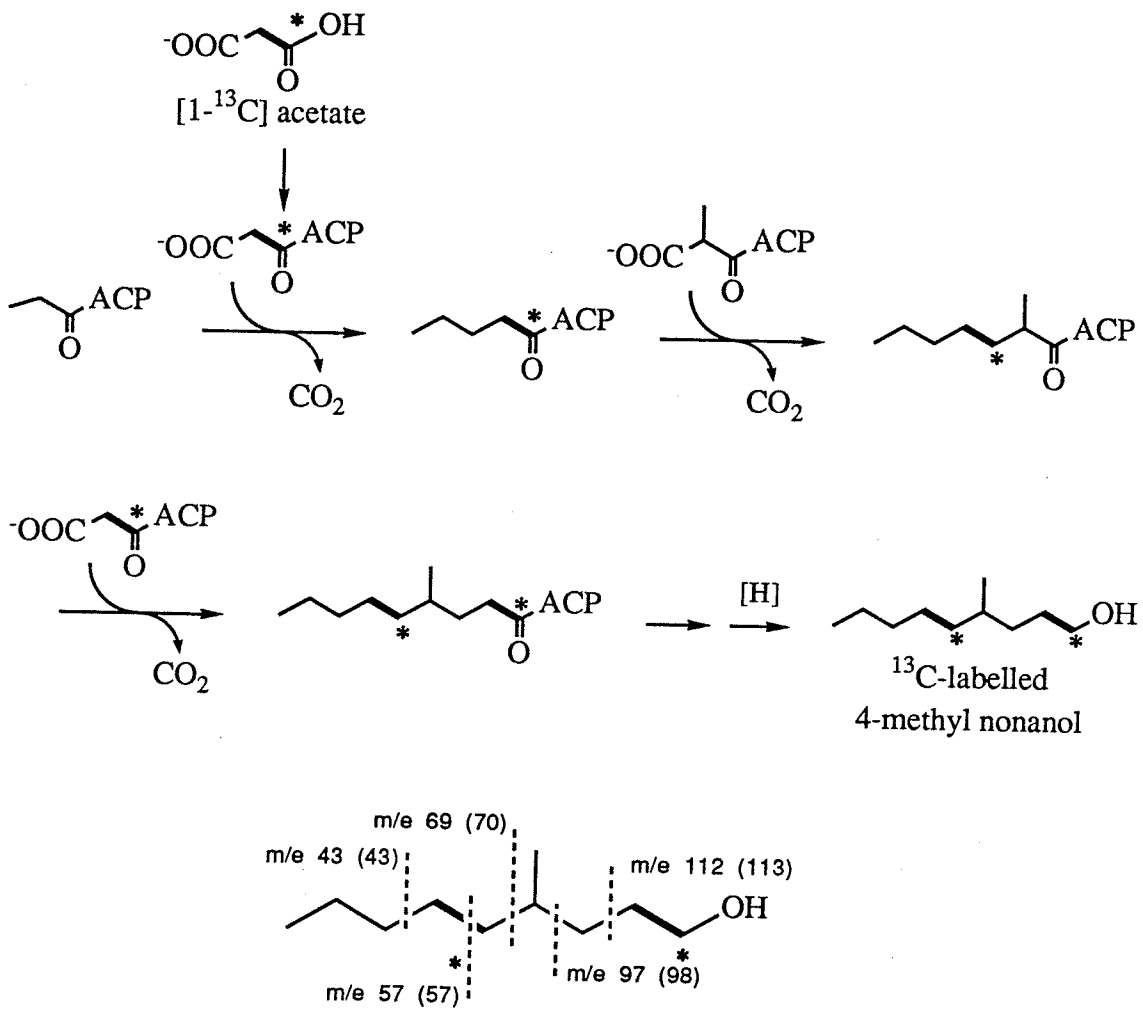
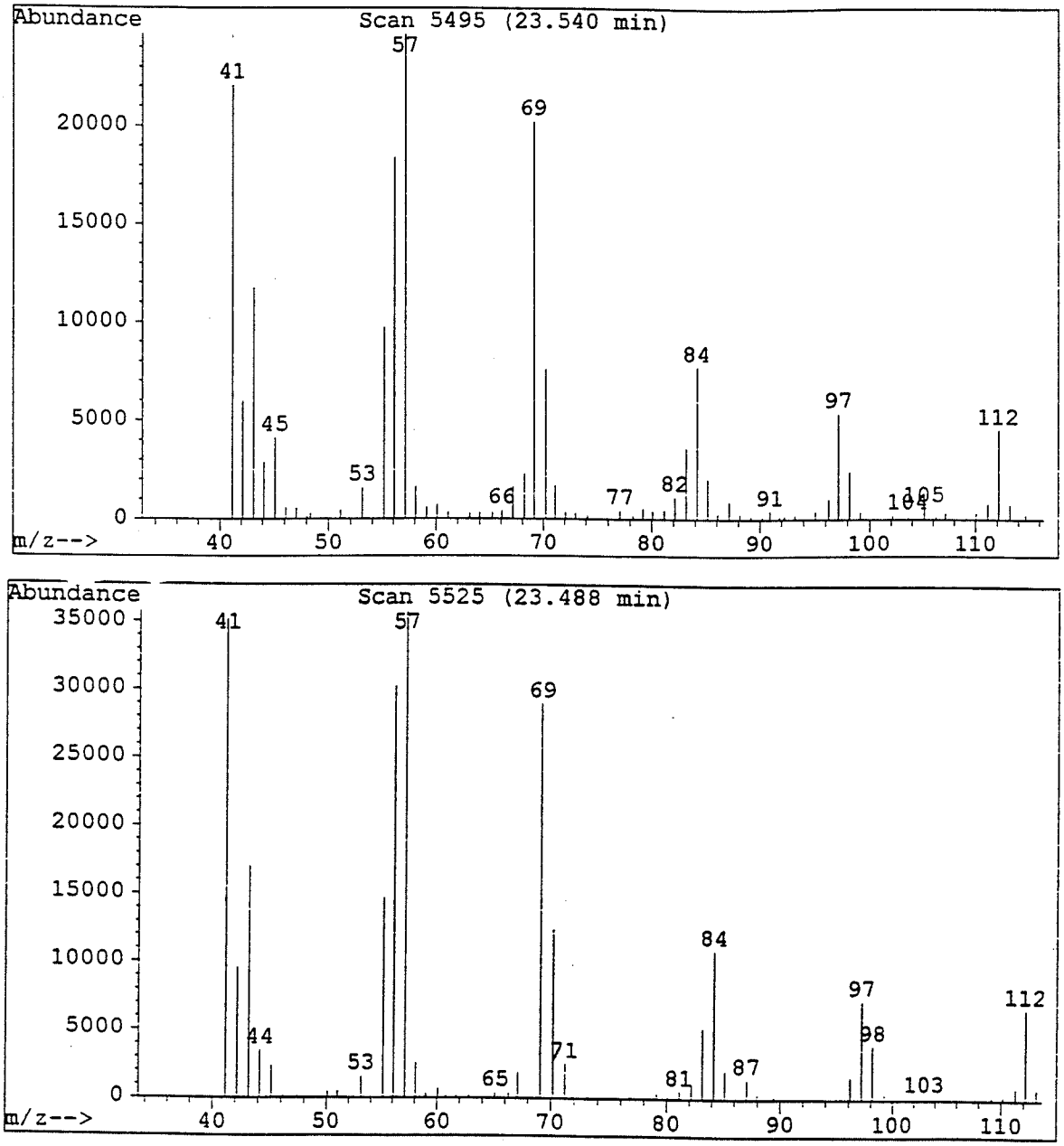


FIGURE 18: MS fragmentation pattern of 4-methylnonanol produced by female yellow mealworms exposed to [1-^{13}C]acetate (top), as compared to control



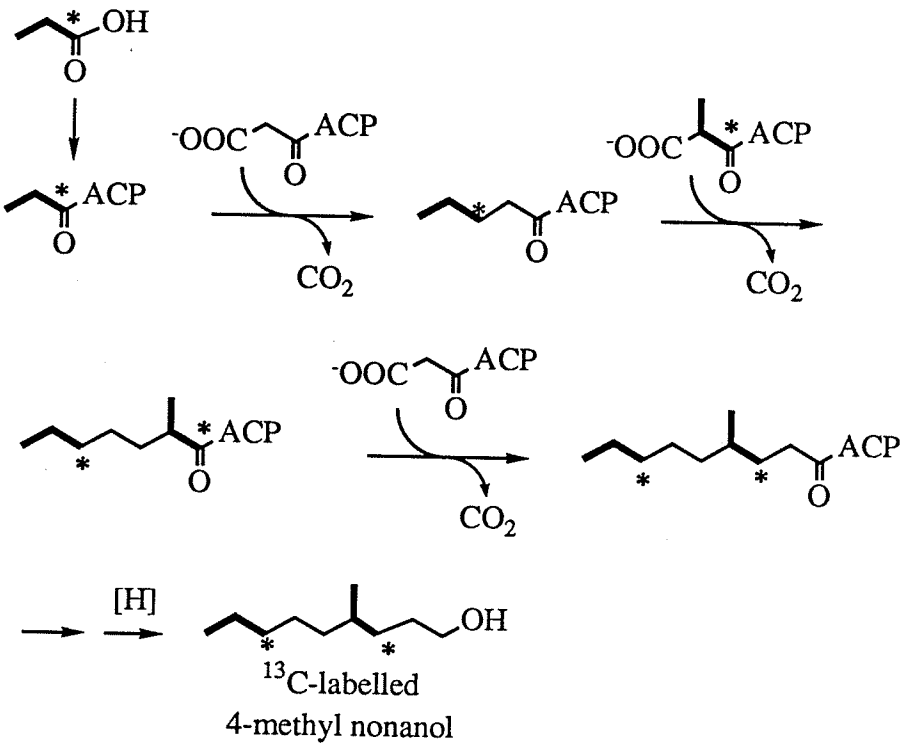
reveals no discernible shift in the fragments occurred (see FIGURE 18). This indicates that there was no appreciable incorporation of [1-^{13}C]acetate into the 4-methylnonanol.

We attempted to increase the sensitivity of the method using the technique of Selected Ion Monitoring (SIM). During SIM, only selected ions are scanned, so that greater sensitivity towards the fragment of interest is achieved. In the absence of a visible molecular ion peak we chose the 112 fragment to perform a SIM on, since it is quite distinct from other fragments as well as being the most abundant fragment with a high m/e ratio. Thus SIM was used to determine the ratio of the 112 to the 113 fragment (when label is incorporated the 113 fragment is enriched). Information from the SIM was used to calculate the percentage enrichment of the 113 fragment compared to the 112 fragment. In the unlabelled pheromone the 113 fragment has an abundance of 10.6%, due to the natural abundance of the ^{13}C isotope of carbon. Enrichment due to incorporation of label in the 113 fragment was at most about 1%. This shows that acetate was not efficiently incorporated into the pheromone, 4-methylnonanol.

(B) Incorporation of [1-^{13}C]propionate:

Propionate was the next precursor examined. As can be seen in FIGURE 19, the putative biosynthetic route to 4-methylnonanol biosynthesis would include at most two propionate units. Since the propionate is labelled at position 1, the ^{13}C label could be incorporated into positions 3 and 7 of the final product. Thus, based on the fragmentation pattern of 4-methylnonanol, we expected a shift in the fragment of m/e 112 to

FIGURE 19: Expected labelling pattern in presence of [1-^{13}C] propionate (^{13}C is represented by an asterisk)



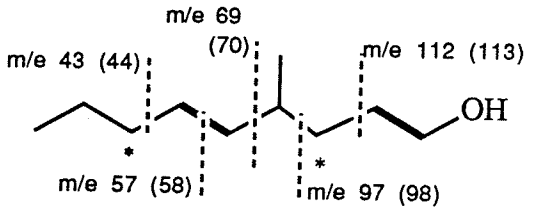
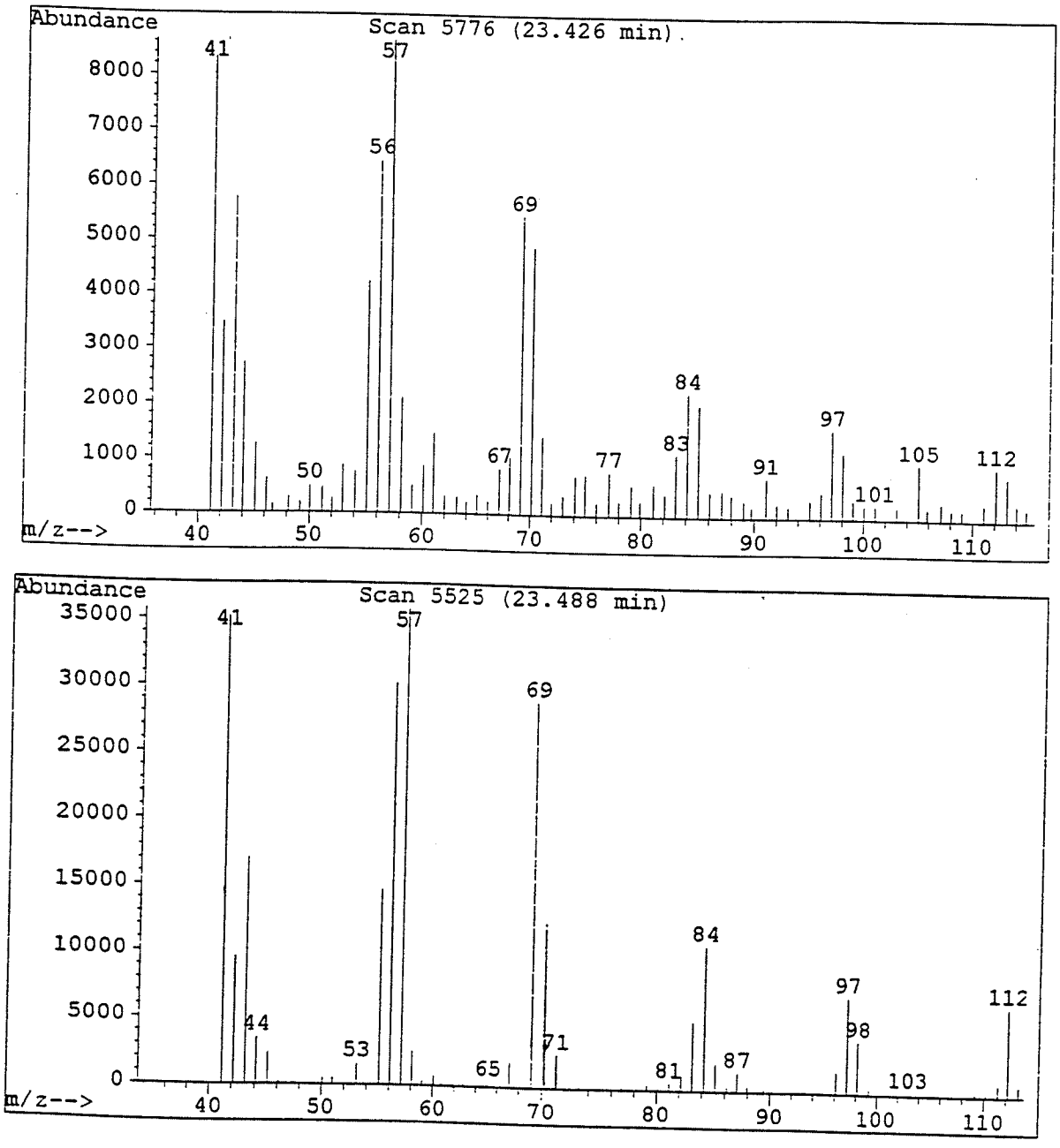


FIGURE 20: MS fragmentation pattern of 4-methylnonanol produced by female yellow mealworms exposed to [1-^{13}C]propionate (top), as compared to control



m/e 113 or 114 since this fragment could contain two ^{13}C labels. Incorporation of the labels should be discernible by a shift of the m/e 43, 57, 69, 97 fragments to m/e 44, 58, 70 and 98 respectively. The m/e 43, 57, 69 fragments would have lost the second label and so more than one shift in m/e units was not expected.

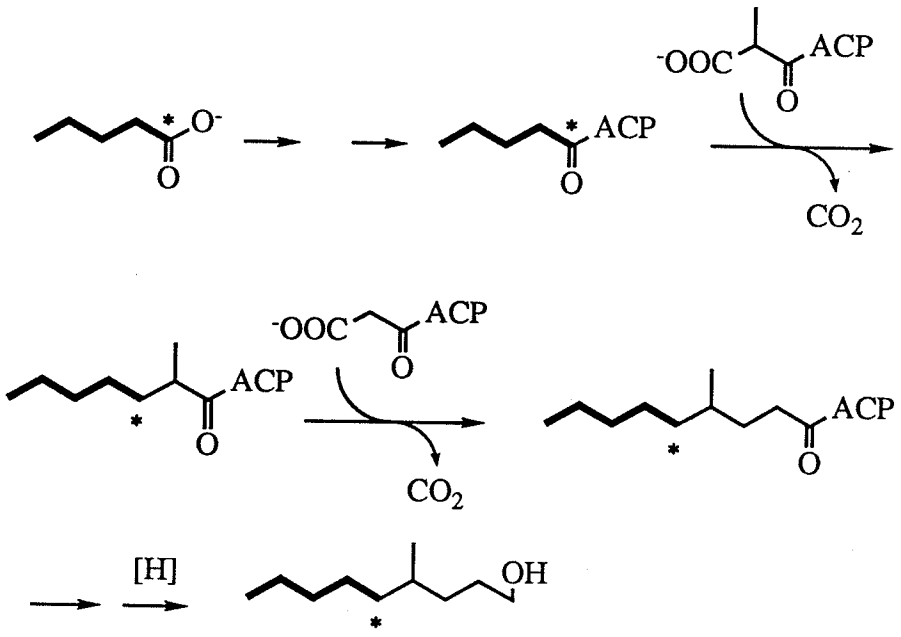
The MS of the pheromone produced by beetles exposed to [1-^{13}C]propionate was compared to that of unlabelled 4-methylnonanol in FIGURE 20. The m/e 112 peak is clearly shifted to m/e 113 and 114, indicating the incorporation of ^{13}C label into this fragment. Enrichment due to the incorporation of ^{13}C label in the 113 fragment was about 34%. Thus propionate was incorporated into the pheromone.

(C) Incorporation of [1-^{13}C]pentanoate:

According to the putative biosynthetic route (see FIGURE 21), at most one pentanoate unit would be incorporated into 4-methylnonanol. Since the pentanoate is labelled at position 1, the label would be incorporated into position 5 in the carbon chain of the pheromone. We expected a shift in the fragment of m/e 112 to m/e 113, since this fragment could contain one ^{13}C label. Incorporation of the label into position 5 should be discernible by a shift of the m/e 69, 97 and 112 fragments to 70, 98 and 113, respectively. The fragments of m/e 43 and 57 would have lost the C5 and thus would not display a shift.

As expected the m/e 69, 97 and 112 fragments all displaced a shift due to the incorporation of ^{13}C label (see FIGURE 22). The enrichment due to the incorporation of label into the 113

FIGURE 21: Expected Labelling Pattern in Presence of [1-^{13}C] Pentanoate (^{13}C is represented by an asterisk)



^{13}C-labelled
4-methyl nonanol

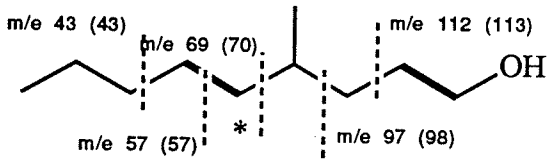
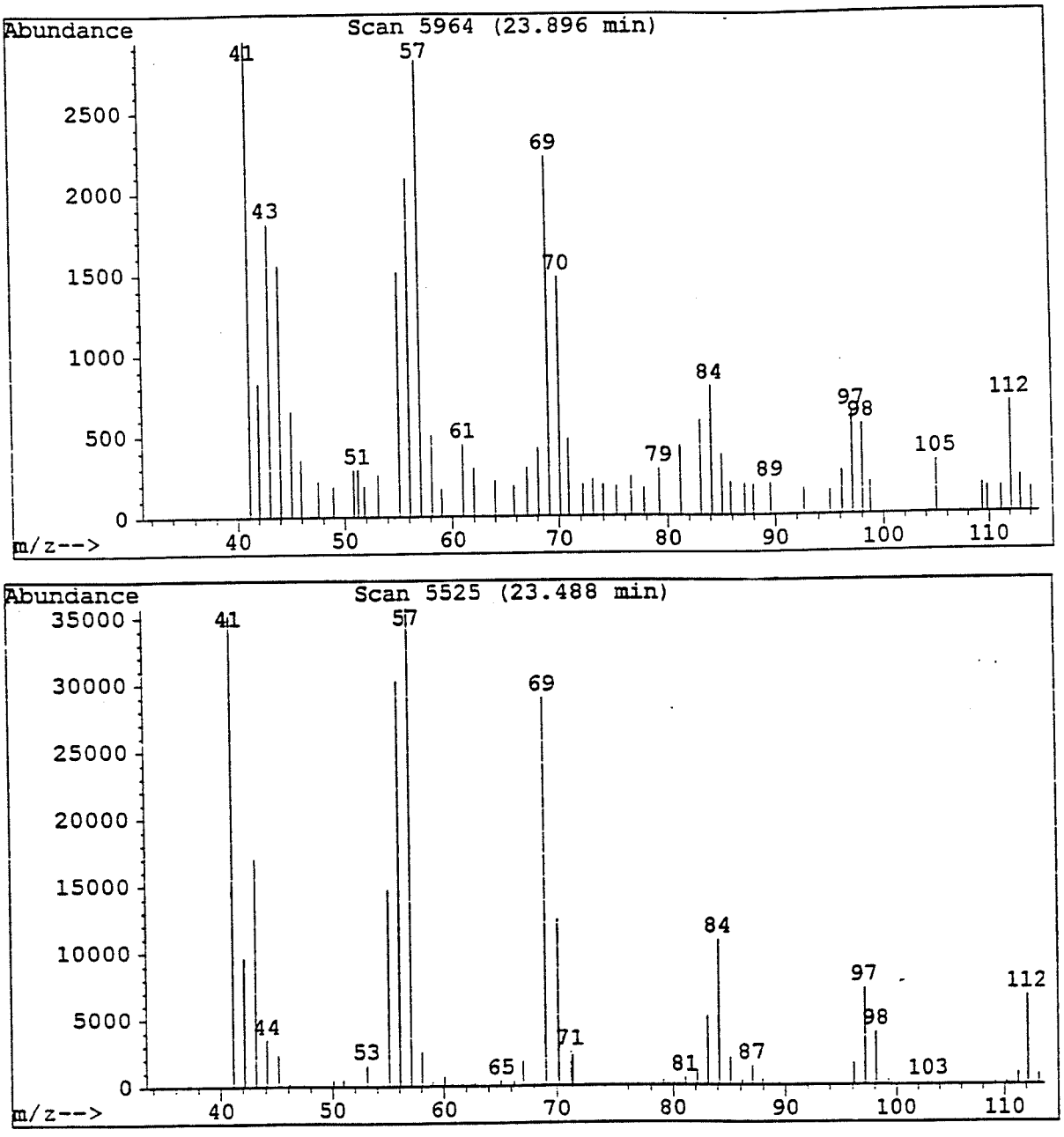


FIGURE 22: MS fragmentation pattern of 4-methylnonanol produced by female yellow mealworms exposed to [1-^{13}C]pentanoate (top), as compared to control



fragment was about 16%. Thus this precursor was incorporated into 4-methylnonanol.

(D) Incorporation of [1-^{13}C]2-methylheptanoate:

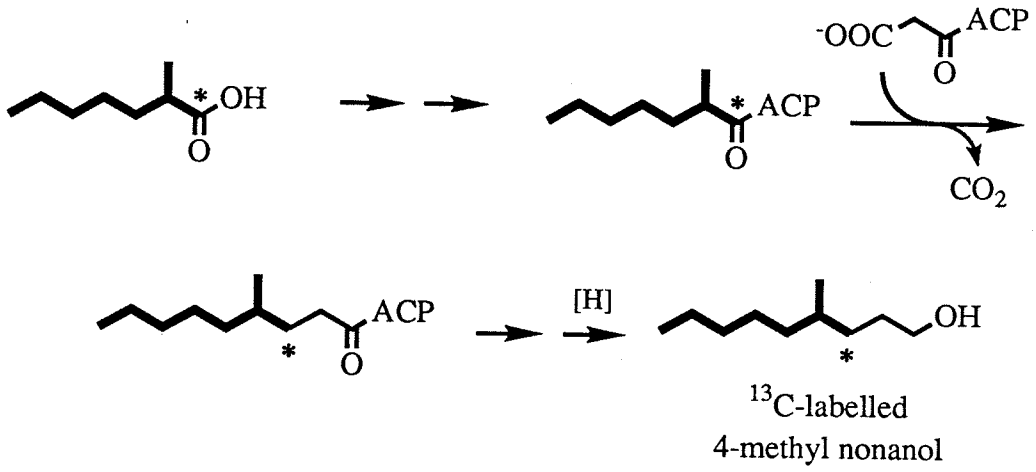
According to the putative biosynthetic route (FIGURE 23), at most one 2-methylheptanoate unit would be incorporated into 4-methylnonanol. Since the 2-methylheptanoate is labelled at position 1, the label could be incorporated into position 7 in the carbon chain of the pheromone. Thus, based on the fragmentation pattern of 4-methylnonanol, we expected a shift of the fragment m/e 112 to m/e 113, since this fragment contains one ^{13}C label. The fragment m/e 97 should shift to m/e 98. The smaller fragments (m/e 43, 57 and 69) should not be shifted since these fragments would have lost the label.

A dramatic increase was seen in the relative abundance of the 113 fragment (FIGURE 24). A shift of the m/e 97 fragment to m/e 98 was also observed. The 4-methylnonanol produced by beetles exposed to 2- methylheptanoic acid was about 45% enriched in ^{13}C .

(E) Incorporation of [3,4-D_2]4-methylnonanoic acid:

As seen in FIGURE 25 the putative biosynthetic route to 4-methylnonanol biosynthesis would include at most one 4- methylnonanoate. Deuterium labels, rather than ^{13}C labels were used to label the 4-methylnonanoic acid, since it was difficult to synthesize 4-methylnonanoic acid with ^{13}C label. Deuterium labels are stable isotopes of hydrogen and function in the same manner as the ^{13}C labels for our purpose. Since the 4-methylnonanoate is labelled at position 3 and 4, the labels could be incorporated into position 6 and 7 in the carbon chain of the

FIGURE 23: Expected labelling pattern in presence of [1-^{13}C] 2-methylheptanoic acid (^{13}C is represented by an asterisk)



^{13}C-labelled
4-methyl nonanol

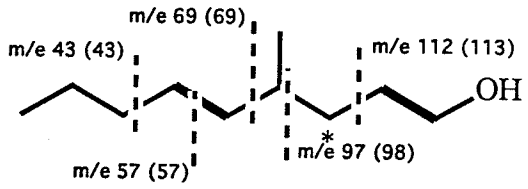
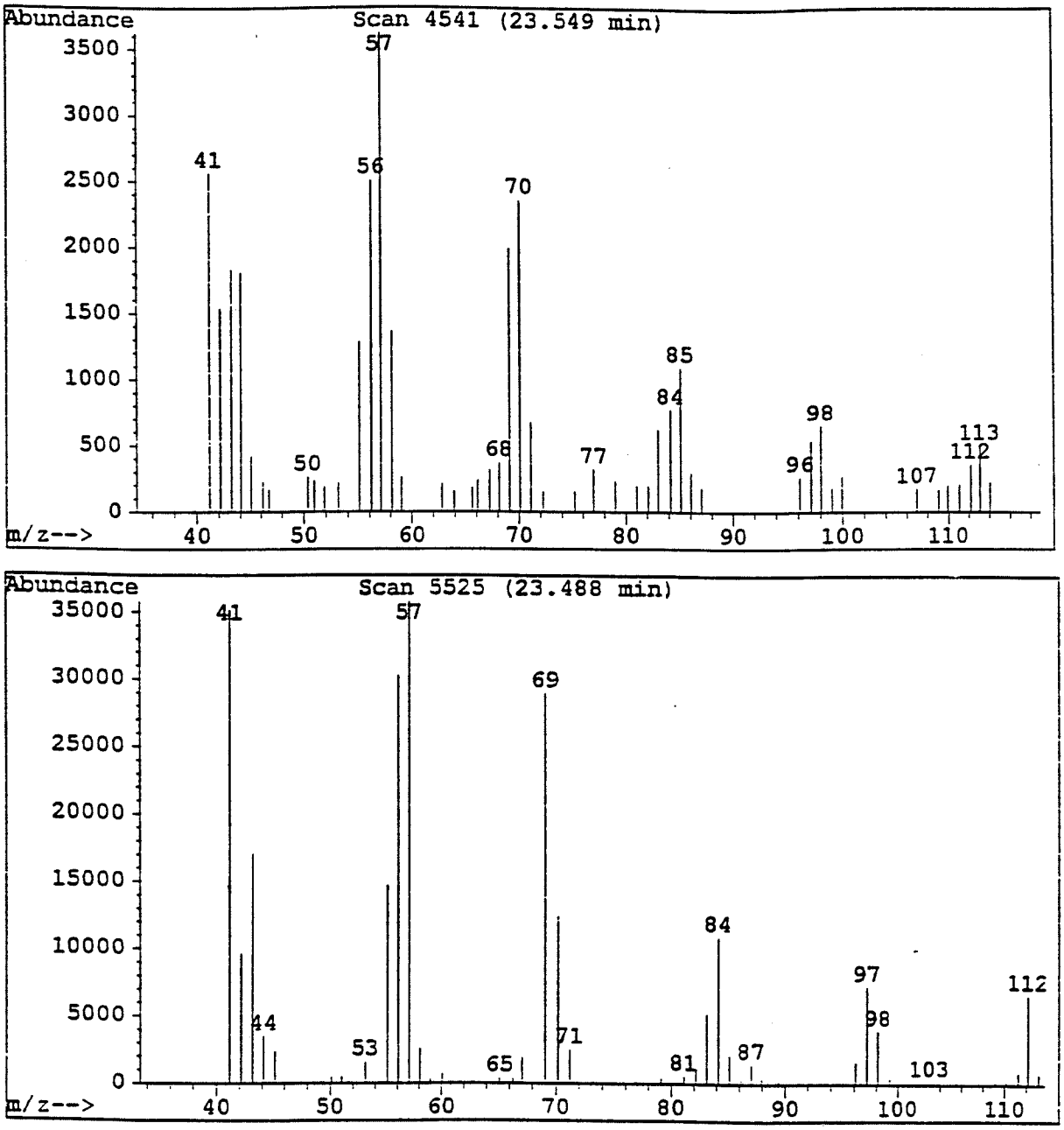


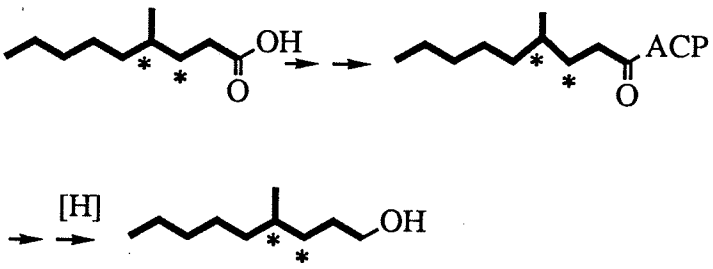
FIGURE 24: MS fragmentation pattern of 4-methylnonanol produced by female yellow mealworms exposed to [1-^{13}C]2-methylheptanoic acid (top), as compared to control



pheromone. A shift of fragment m/e 69 and 97 to m/e 70 and 98 (or 99) should occur. Since there are two deuterium labels m/e 112 could be shifted to both m/e 113 and 114 fragments.

When beetles were exposed to D_2-4-methylnonanoic acid, deuterium label(s) were indeed incorporated into m/e 112 shifting to 113 and 114 fragments (see FIGURE 26). Since the precursor was labelled with D_2, we studied the enrichment of the 114 fragment rather than the 113 fragment as done with the precursors labelled with carbon-13. The D_2-4-methylnonanoic acid precursor was only 55% D_2. When the beetles were exposed to this particular precursor, the pheromone was 30% D_2. This means that about 81% of the pheromone was produced from the supplied precursor (taking into account that D_2-4-methylnonanoic acid was not 100% D_2 in the first place). TABLE II shows a summary of the incorporation rates of all the putative precursors of 4-methylnonanol.

FIGURE 25: Expected labelling pattern in presence of [3,4-D_2] 4-methylnonanoic acid (D_2 is represented by an asterisk)



D_2-labelled
4-methyl nonanol

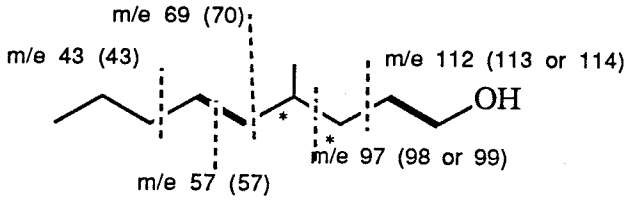


FIGURE 26: MS fragmentation pattern of 4-methylnonanol produced by female yellow mealworms exposed to [3,4-D_2]4-methylnonanoic acid (top), as compared to control

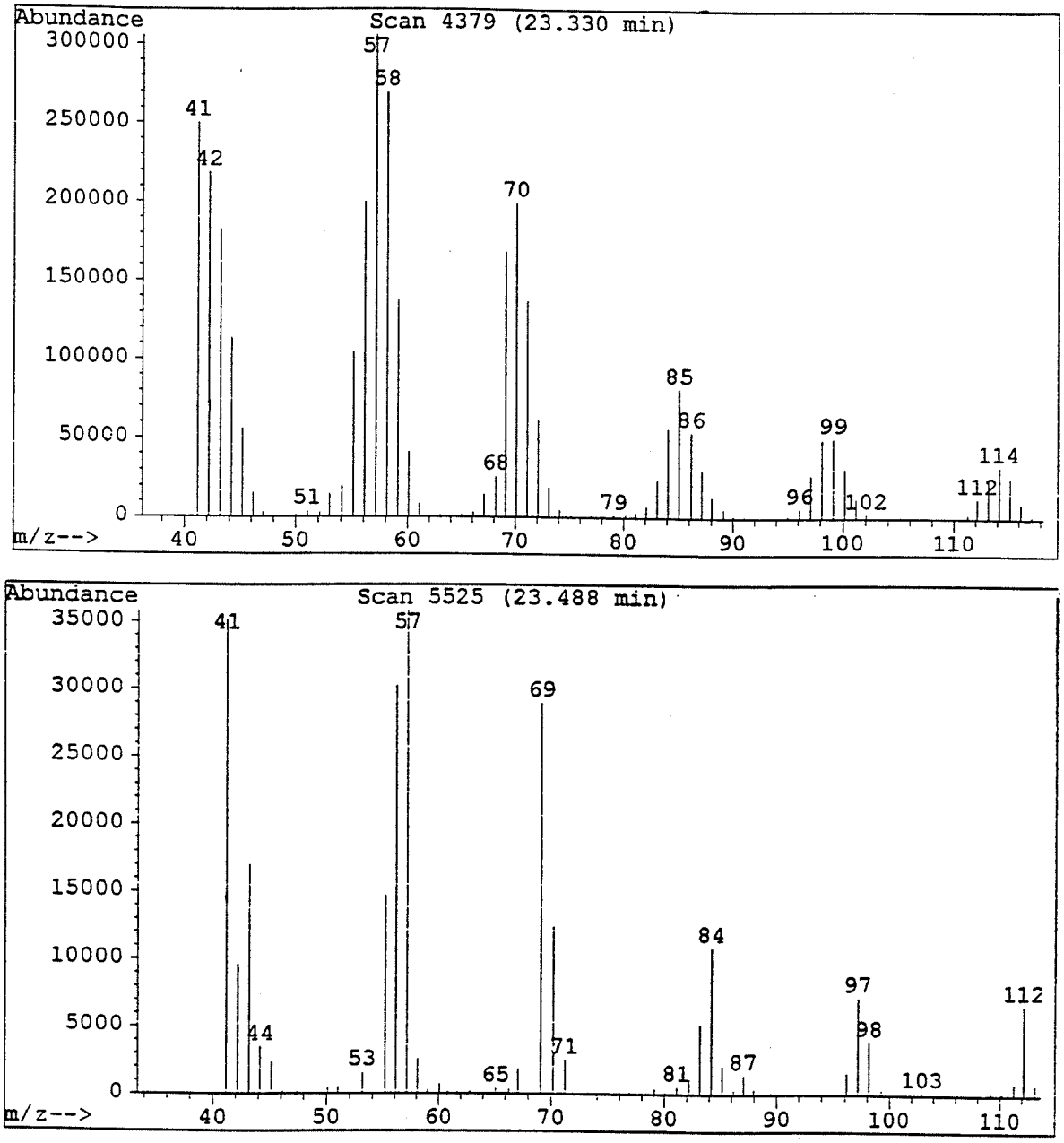
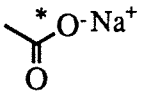
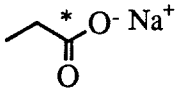
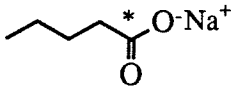
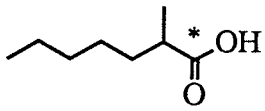
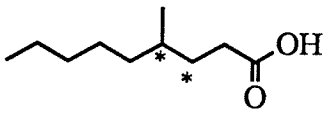


TABLE II: Summary of % incorporation of biosynthetic precursors into 4-methylnonanol

Precursor name	Structure	% incorporation into 4-methylnonanol
[1-^{13}C] Sodium acetate		< 1%
[1-^{13}C] Sodium propionate		34%
[1-^{13}C] Sodium pentanoate		16%
[1-^{13}C] 2-methylheptanoic acid		45%
[3,4-D_2] 4-methylnonanoic acid		81%

III. EFFECT OF METHOPRENE ON ATTRACTIVENESS OF EXTRACTS OF YELLOW MEALWORM BEETLES:

The effect of methoprene on the activity of extracts of female yellow mealworm beetles was determined by bioassay. Extracts prepared from decapitated mature female beetles (7-10 days old) provoked significantly ($p < 0.005$) fewer positive male responses than extracts prepared from control (untreated) mature female beetles (FIGURE 28). Topical treatment of the decapitated females with methoprene partially reversed this trend.

As shown in FIGURE 27, extracts prepared from immature beetles provoke far fewer responses from male beetles than extracts prepared from control (untreated) mature female beetles. Extracts prepared from immature females that were topically treated with methoprene evoked significantly ($p < 0.005$) more positive male responses.

We first attempted to quantify pheromone levels by GC, through comparison with an internal standard. Pheromone production from immature beetles treated with methoprene was approximately 1.7 ng/beetle and from the beetles that were untreated was approximately 1.0 ng/beetle (see FIGURE 29). Problems with this method are outlined in the DISCUSSION.

We used a radioactive assay to compare methoprene treated versus untreated beetles. HPLC analysis with authentic standards revealed that the pheromone eluted with pentane:ether (80:20) while the acid precursor did not elute until ethyl ether:acetic acid (100:1) was passed through the column (see FIGURE 30). We used this solvent system to separate the

FIGURE 27: Effect of methoprene on activity of immature (1-2 day post emergence) female yellow mealworm extracts (n=3, bars topped by different letters are significantly different, $p < 0.005$)

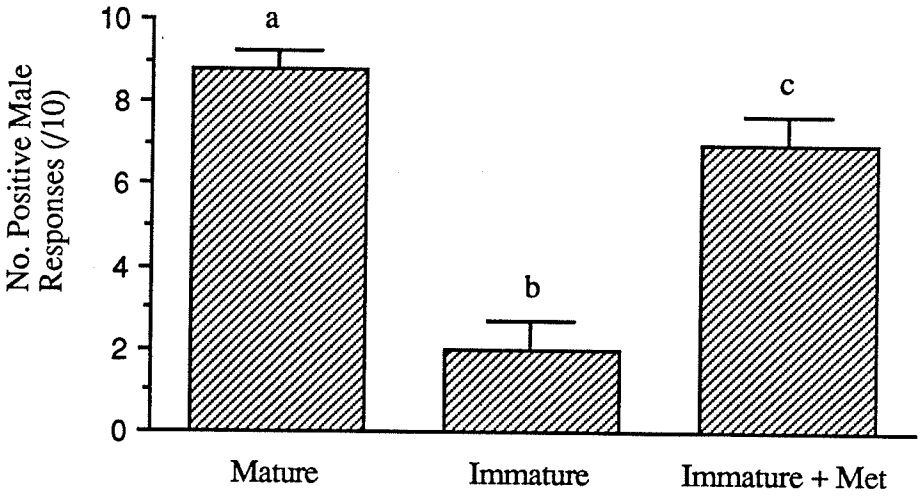


FIGURE 28: Effect of methoprene on activity of extracts of mature yellow mealworm beetles (n=3, bars topped by different letters are significantly different, $P < 0.05$)

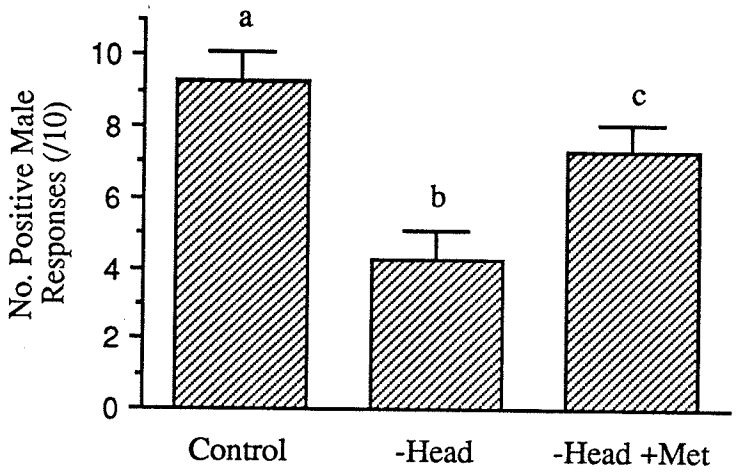
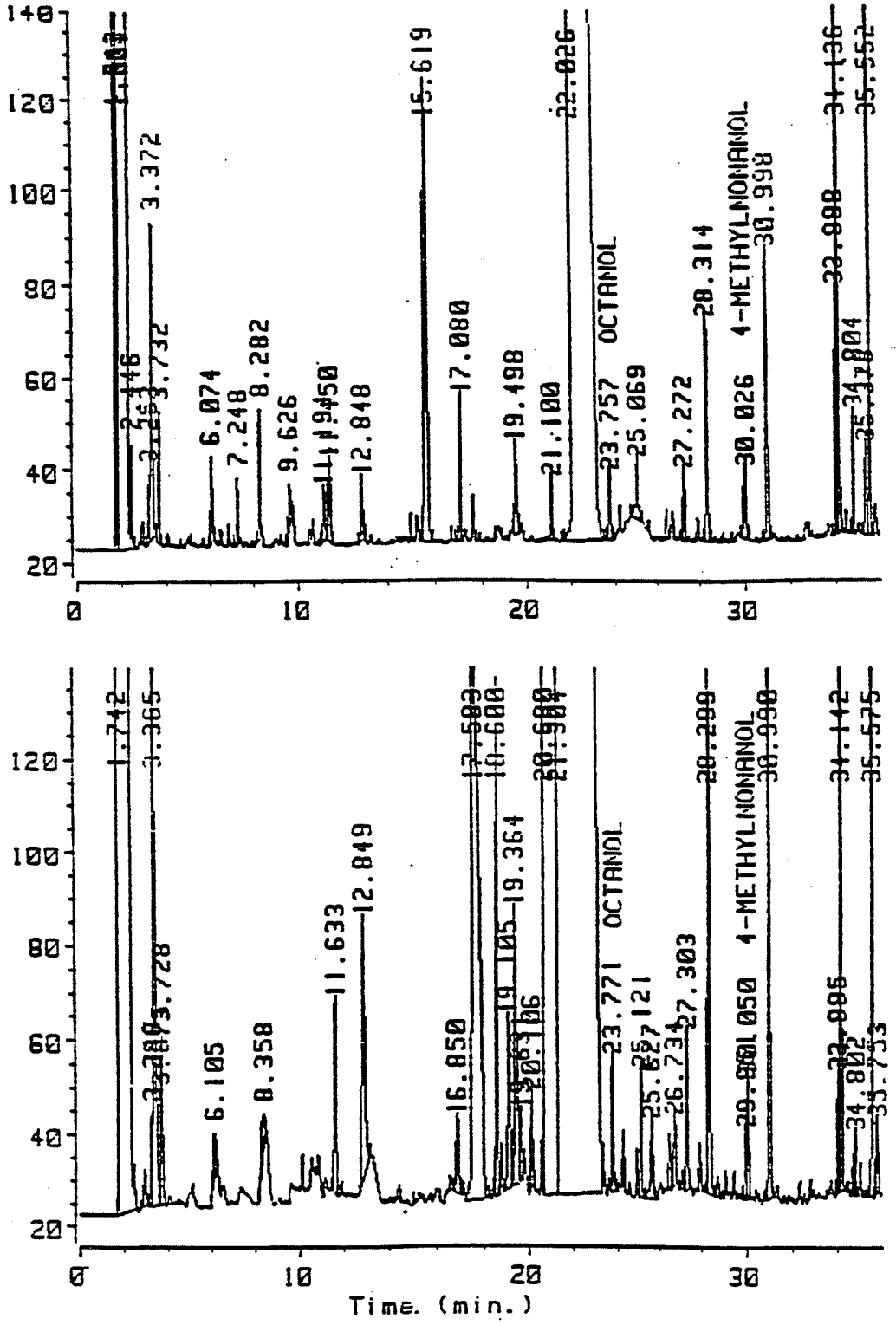


FIGURE 29: GC of 4-methylnonanol in Porapak Q trapped volatiles of methoprene treated (top panel)and untreated (bottom panel) female yellow mealworm beetles (0-2 days post emergence, aerated 6 days)



pheromone from its acid precursor.

Mature females were able to reduce significantly ($p < 0.05$) more precursor to pheromone in 3 h than the immature beetles were. However, in the presence of methoprene, the conversion of [^{3}H]4-methylnonanoic acid to 4-methylnonanol was significantly ($p < 0.05$) increased (see FIGURE 31). Thus, methoprene treatment stimulated the conversion of the acid precursor to the pheromone in immature beetles. This indicated that the reduction of 4-methylnonanoic acid to 4-methylnonanol is regulated by JH.

FIGURE 30: [^{3}H]4-methylnonanoic acid (in ethyl ether:acetic acid, 100:1) (above) and [^{3}H]4-methylnonanol (in pentane:ether, 80:20) (below) standards analyzed by HPLC

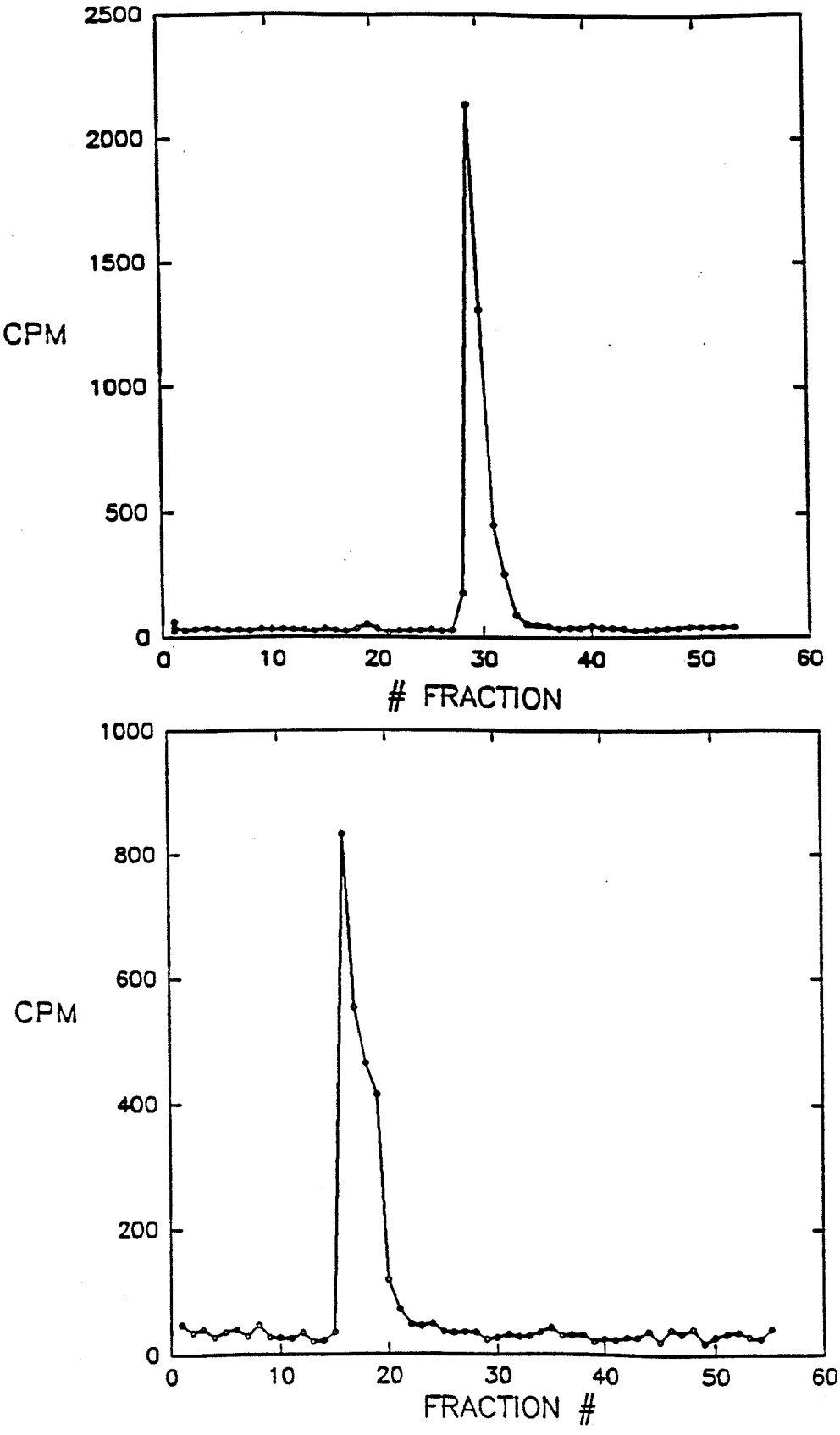
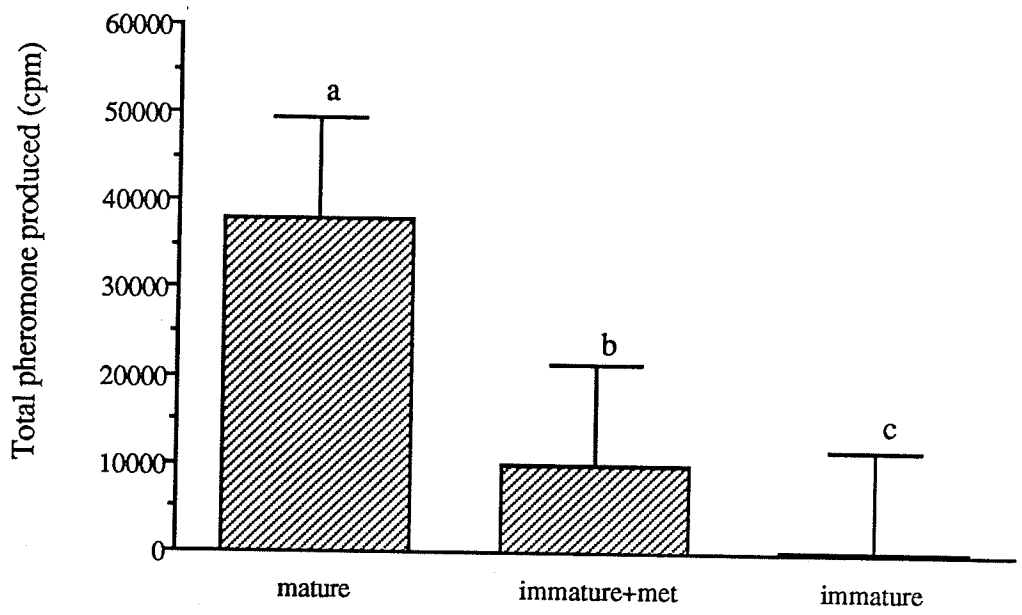


FIGURE 31: Effect of methoprene on pheromone production from [^{3}H]4-methylnonanoic acid by female yellow mealworm beetles (n=3, bars topped by different letters are significantly different, $p < 0.05$)



DISCUSSION

I. SYNTHESIS OF 4-METHYLNONANOL

An authentic sample of 4-methylnonanol was required to serve as a standard in the GC/MS analyses of pheromone production. Since 4-methylnonanol is not commercially available it was necessary to develop a synthetic route. 4-Methylnonanol has a relatively simple structure. The major synthetic problem is to introduce the methyl branch at the correct position. Our solution to this problem was to couple a methyl ketone (5-bromo-2-pentanone) to an alkyl bromide through a Wittig reaction (reviewed by Trippett, 1963). We anticipated that subsequent reactions would involve relatively straight forward functional group manipulations (reduction of a double bond and conversion of a bromide to an alcohol).

Unfortunately, this route yielded only impure product in poor yield. Two reactions were responsible for the low overall yield: the Wittig coupling and the conversion of the bromide to the alcohol. It may have been possible to increase the yield of the Wittig coupling by using more effective polar aprotic solvents such as hexamethylphosphoramide and N-methyl-2-pyrrolidine (Hutchins and Taffer, 1982). Although conversion of a bromide to an alcohol is a classical S_N2 reaction, in our hands the conversion of 4-methylnonyl bromide to 4-methylnonanol was problematic. We consistently obtained a mixture of the desired alcohol product and undesired alkenes (due to the competing E2 reactions). We tried to increase the leaving group ability (using iodide rather then bromide), and tried to activate the nucleophile using polar aprotic solvents such as DMSO and DMF, but could not obtain satisfactory yield

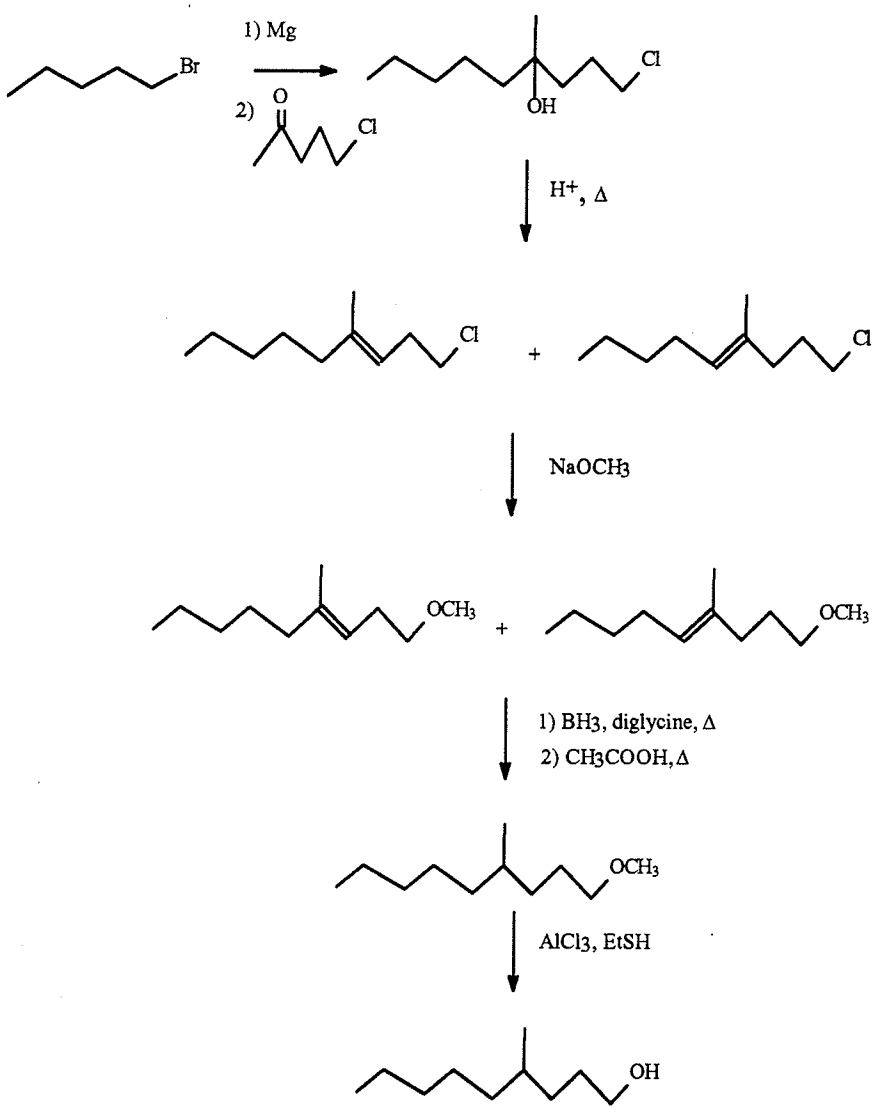
or purity. One major problem was the difference in polarity between the relatively hydrophobic 10-carbon bromide and the nucleophile, hydroxide ion: solvents that dissolved one did not dissolve the other. Use of a phase-transfer catalyst could have been attempted, but due to the numerous difficulties with this route we currently synthesize the compound by a different path.

Other workers in the laboratory subsequently modified the route to obtain the product in greater yield and purity. The problematic Wittig reaction was by-passed by using a Grignard reaction (reviewed by March, 1992) to couple the bromopentane to 5-chloro-2-pentanone. The chloride was converted to a methoxy group, which was easily converted to the alcohol with aluminum chloride in ethanethiol. The complete route is outlined in FIGURE 32. Simple modifications of this route were used to insert tritium or deuterium labels into putative precursors for other experiments. The pure 4-methylnonanol was used as a standard to identify the pheromone from the female beetle volatiles.

II. ELUCIDATION OF THE BIOSYNTHETIC PATHWAY

The results of this study are consistent with the biosynthetic route outlined in FIGURE 10. We determined the incorporation of stable isotope labelled substrates by GC/MS. Substrates were introduced by feeding the beetles "defatted" bran coated with the stable isotope labelled putative precursors. We attempted to reduce the dilution of the stable isotope-labelled precursors by fats present in the bran, by first extracting the bran with diethyl ether. Volatiles emitted from the feeding beetles were collected on Porapak Q (a solid adsorbent) columns

FIGURE 32: New synthetic route to 4-methylnonanol



for 6 days. The pheromone was obtained by extracting the Porapak Q columns. At this point the pheromone was analyzed by GC/MS.

The beetles efficiently converted D_2-4-methylnonanoic acid to 4-methylnonanol: about 81% of the pheromone was produced from the supplied precursor. Pheromone produced from [^{13}C] 2-methylheptanoic acid, [^{13}C] propionate and [^{13}C] pentanoic acid was about 45%, 34% and 16% enriched in ^{13}C, respectively. These precursors were also definitely being incorporated into 4-methylnonanol. The methyl branch apparently originated from propionate. In insects such as the cockroach it has been shown that propionyl-CoA can be converted to acetyl-CoA in the early stages of pheromone biosynthesis (Chase et al., 1992; Halarnkar et al., 1985). However, in the case of the yellow mealworm beetle since the ^{13}C label was in position 1 in the propionate, it would have been lost as $^{13}CO_2$ upon conversion to acetyl-CoA. Therefore the ^{13}C label incorporated in the presence of ^{13}C-propionate did not arise via acetyl-CoA.

Acetate, the first putative precursor in the pathway, was not efficiently incorporated into the pheromone (less than 1%). In our experiments, we let the yellow mealworm beetles feed on bran laced with stable isotope labelled acetate. Fatty acids are constantly being broken down and re-synthesized so there is a constant turnover of acetate (or acetyl-CoA) in the beetle, probably resulting in our labelled acetate being diluted in the presence of unlabelled acetate. In previous studies, when cucujid beetles were allowed to ingest radiolabelled acetate, the acetate was incorporated in the fatty acid derived pheromone at less than 0.1% (Vanderwel

et al., 1990). Similar incorporation rates would have been below our limit of detection (by GC/MS). Morse and Meighen (1984) found that the most efficient way of introducing radiolabelled acetate into the moth *Choristoneura fumiferana* was by topical treatment. However, even these incorporation levels would have been below our limit of detection (approximately 0.5-1% incorporation of the acetate into the pheromone).

The results of this study are clearly consistent with the proposed route outlined in FIGURE 10. However, the specific order of the pathway cannot be proven by this type of study. Propionate, 2-methylheptanoic acid and 4-methylnonanoic acid, all follow a trend where the incorporation rate increased as the precursor was closer to 4-methylnonanol. Pentanoic acid, on the other hand, was less efficiently incorporated than propionate. Similar results were obtained in other grain beetles, where a later precursor has a lower rate of incorporation in to the pheromone than an earlier precursor (Vanderwel et al., 1992). In the presence of pentanoic acid the beetles were apparently repelled by the strong odour of this compound, as they were trying to climb out of the food and out of the chamber. We tried to reduce the volatility of pentanoic acid by converting it to the potassium salt but the incorporation rate was still quite low. This precursor may act as an antifeedant, so the beetles do not ingest enough of the precursor for efficient incorporation. Alternatively, there may be variations in the rates of absorption (through the digestive tract or cuticle) and/or transportation of the compound through the hemolymph to the site of pheromone production. Gu et al. (1995) demonstrated that newly synthesized hydrocarbons and the methyl ketone sex pheromone in the German cockroach are transported to the target site by a carrier hemolymph lipophorin.

In case of the yellow mealworm beetle the site of pheromone production or the mode of transport is unknown but it may be very similar to that seen in the cockroaches. Lipophorin or any other carrier protein may not have the same capacity to transport all the precursors due to the differential solubilities and absorption properties of the precursors.

These labelling experiments show that 4-methylnonanol can be produced *de novo* from acetate and propionate. There is a possibility that the pheromone could be produced through chain shortening of longer fatty acids similar to the process in other insects (Vanderwel 1991; Vanderwel and Oeschlager, 1987; Bjostad et al., 1981). However, it is not likely that chain shortening is occurring in the yellow mealworm beetle. We observed extremely high incorporation rates (81%) of 4-methylnonanoic acid indicating it is an immediate precursor to 4-methylnonanol, rather than a precursor that must be first elongated to a longer chain and then chain shortened before forming the product.

III. REGULATION OF PHEROMONE BIOSYNTHESIS IN THE YELLOW MEALWORM BEETLE

Finally, we studied the process by which JH III regulates biosynthetic pathway of the pheromone. Since JH is unstable and very expensive, we used methoprene, a JH analogue, instead. The first step was to determine if methoprene would mimic the effect of JH III in the yellow mealworm beetles (ie., if it would increase the activity of the female extracts as determined by the ability to elicit a copulatory response from male beetles) (Menon, 1970, 1976). The bioassays indicated that methoprene significantly ($p < 0.005$) increased the

activity of the extracts of immature beetles. Methoprene also partially restored the activity of the extracts of decapitated adult female beetles (FIGURES 27 and 28).

The next step was to determine if the above effects were in fact due to an increase in the production of 4-methylnonanol and not due to other factors, such as an increase in the production of some other (as yet unidentified) sex pheromone; the reduction in the production of an "anti-sex pheromone" that may be present in the beetle; or even a decrease in the rate of degradation of 4-methylnonanol.

Our first approach to this problem was to attempt to quantify pheromone levels by GC/MS through comparison to an internal standard. We could not simply quantify the 4-methylnonanol present in the extracts of the females by GC, since < 40 nanogram of pheromone is produced by the female beetle (Vakili, 1994). We tried collecting the pheromone over longer period of time by aeration technique, but did not get significant differences. Methoprene-treated immature beetles did not produce significantly more pheromone than untreated beetles. The problem was that by the end of the aeration period (6 days) the "immature" beetles had matured, and thus were producing maximal levels of pheromone. We increased the sensitivity of the assay by using radiolabelled precursors whereby we could detect pheromone production after only 3h (rather than 6 days).

From the radiolabel studies we determined that methoprene regulates 4-methylnonanol production (FIGURE 31). These studies also indicated that the reduction of 4-

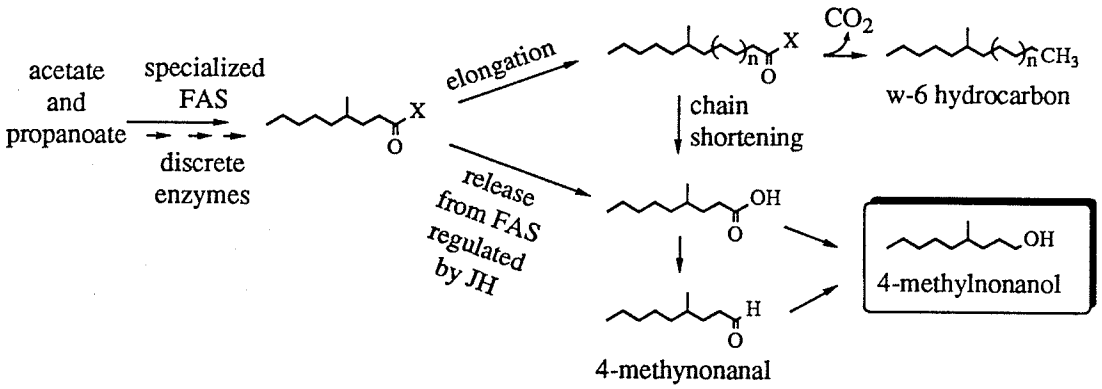
methylnonanoic acid to 4-methylnonanol is a regulated step in the production of the pheromone (there may be other regulated steps that we have not looked at due to the unavailability of compounds). From our stable isotope labelling studies we know that 4-methylnonanoic acid has a high incorporation rate (81%) into the pheromone indicating that this is a precursor close to the end of the pathway. Although it is unusual to regulate the last step of a biosynthetic pathway, there are precedents for such reactions in moths (Martinez et al., 1990; Arima et al.,1991). There could be more than one site of regulation in beetles but when the beetles need to produce the sex pheromone rapidly it is convenient to regulate the last step instead of the earlier steps. It would be interesting to look for stored precursors in immature beetles.

Several aspects of 4-methylnonanol biosynthesis remain to be investigated. The conversion of propionate to 4-methylnonanoic acid via intermediates may be catalyzed by enzymes that are associated in a multienzyme complex analogous to the ubiquitous fatty acid synthetase (FAS). FAS found in mammals synthesizes the 16-carbon fatty acyl derivative before the fatty acid is released. It does not release intermediates of shorter chain-length than 16-carbons (Lehninger et al., 1993). However, in the case of the yellow mealworm beetle JH might induce the production of a special enzyme to cleave off from the long chain fatty acids the growing substrate, once it attains 9-carbons in its backbone. Activation of such an enzyme would divert a specialized FAS from producing longer ω-6 branched fatty acids to produce the immediate precursor of the pheromone, in this case 4-methylnonanoic acid directly (see FIGURE 33). Long chain ω-6 cuticular hydrocarbons have not been detected

in the yellow mealworm beetle (Lockey, 1978), supporting the fact that chain shortening is not taking place.

In conclusion this study served to demonstrate the biosynthetic pathway and the regulation of the pathway in the yellow mealworm beetle. This is a first step towards understanding the regulation of pheromone biosynthesis in beetles. In future studies we plan to verify the synthetic route *in vitro* thereby avoiding problems of transport, absorption, digestion etc. There may also be other precursors involved such as an aldehyde 4-methylnonanal, which maybe an intermediate product between 4-methylnonanoic acid and 4-methylnonanol. In various organisms fatty acids are reduced to alcohols via aldehyde intermediates (Riendeau and Meighen, 1985). 4-Methylnonanal is quite volatile and it was difficult to coat it on the bran with the method we have developed, so we did not test whether or not it is a precursor. *In vitro* studies can easily show if the acid is first being reduced to an aldehyde intermediate before converting to an alcohol. A clear understanding of processes in this model insect will help us to study pheromone biochemistry in other beetles. Long term benefits could include the development of pest control strategies that are not detrimental to the environment.

FIGURE 33: Possibilities in the pathway of the yellow mealworm beetle



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