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Isolation, Determination of Absolute Stereochemistry, and Asymmetric Synthesis of Insect Methyl-Branched Hydrocarbons

A Dissertation submitted in the partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Chemistry

by

Jan Edgar Bello

June 2014

Dissertation Committee:
Dr. Jocelyn G. Millar, Chairperson
Dr. Thomas H. Morton
Dr. Catharine Larsen

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2014

The Dissertation of Jan Edgar Bello is approved:

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Committee Chairperson

University of California, Riverside

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This dissertation would not have been possible without the guidance, assistance, and support from both my academic and biological families. I would first and foremost like to thank my advisor Professor Jocelyn G. Millar, who has guided me through this rigorous process and has helped me become the chemical ecologist I am today. I would also like to thank my research group Dr. Steve McElfresh, Dr. Yunfan Zou, Dr. Rebeccah Waterworth, R. Max Collignon, Joshua Rodstein, Jackie Serrano, and Brian Hanley for all the suggestions, insect collecting, synthetic discussions, and experimental advise that have allowed me to complete this dissertation.

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The text and figures in Chapter 4, in part or in full, are a reprint of the material as it appears in *Insects* **2013**, *4*, 743-760. The article was printed open access with a creative commons license, which gives the authors copyright over the printed material. The co-author, Stephan Kuhbandner, performed the biological assays and statistical analyses of the results. The co-authors, Prof. Jocelyn G. Millar and Prof. Joachim Ruther, directed and supervised the research, which forms the basis for chapter 4.

Dedication

This dissertation is dedicated to my parents, Edgar and Nancy Bello, who have always encouraged me to follow my dreams. In addition, I would like to dedicate this work to my nieces, Abigail and Emilynn, and my nephew, Joshua, who remind me to look at the world with childish wonder and who inspire me to keep asking questions and searching for answers.

ABSTRACT OF THE DISSERTATION

Isolation, Determination of Absolute Stereochemistry, and Assymetric Synthesis of Insect Methyl-Branched Hydrocarbons

by

Jan Edgar Bello

Doctor of Philosophy, Graduate Program in Chemistry
University of California, Riverside, June 2014
Dr. Jocelyn G. Millar, Chairperson

Methyl-branched hydrocarbons (MBCHs) are ubiquitous components of insect cuticular lipids. Several have been shown to function as contact pheromones, and it is likely that many more remain to be discovered. The majority of insect-produced MBCHs are chiral, but there have been no studies to determine whether they are biosynthesized enantiospecifically. In fact, there have been only a handful of studies on the effects of chirality on the biological activities of MBCH contact pheromones. This is primarily a result of the small to vanishingly small specific rotations of MBCHs (~3° to a tiny fraction of a degree), which in the past made enantiomeric analysis through polarimetry impractical, particularly in light of the small amounts of hydrocarbons (ng to µg) that can be obtained from many insects. The problem was compounded by difficulties in isolation of individual MBCHs from the crude mixture, and the time-consuming synthesis of chiral MBCH standards, both of which have hindered research on MBCH chirality.

The first part of this dissertation describes a generic method for the isolation of insect MBCHs from crude cuticular hydrocarbon (CHC) extracts of insects. The isolation

of pure MBCH compounds required the initial fractionation of crude CHC extracts with $AgNO_3$-impregnated silica gel chromatography followed by 5 Å molecular sieves adsorption of saturated *n*-hydrocarbons. These simple fractionation techniques were then followed up by reverse phase HPLC, with non-aqueous solvent systems, using an evaporative light scattering detector to detect all components as they eluted. This combination enabled the separation of MBCHs by chain-length and branch point. Following the described protocol 36 MBCH compounds from 20 species of insects, spanning nine orders of the Insecta, were isolated. Stereochemical analysis of these isolated MBCHs with a digital polarimeter revealed that the absolute configuration of all these insect natural products is (*R*), and the stereochemistry conserved through at least the nine orders of Insecta studied regardless of methyl branch position or chain length.

The second part of this work describes the development of an efficient asymmetric synthesis of enantiopure methyl-branched hydrocarbons. Evans' alkylation was utilized to induce the asymmetry of the methyl branch point. A library of 45+ chiral methyl-branched hydrocarbons were synthesized to be used in collaborative studies testing their bioactivity in various insect systems, and as standards to confirm the polarimetric analyses of the previously isolated insect MBCH compounds. To date, the enantiopure compounds have been used to test the structure-bioactivity relationships of the MBCH contact pheromone of a parasitic wasp, *Lariophagus distinguendus*, and other studies are in progress.

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List of Abbreviations

ACP	acyl carrier protein
atm	atmospheric pressure (unit)
CHC	cuticular hydrocarbon
CoA	coenzyme-A
DCM	dichloromethane
DHP	dihydropyran
DMSO	dimethyl sulfoxide
ELSD	evaporative light scattering detector
Et_2O	diethylether
EtOAc	ethyl acetate
FAS	fatty acid synthase
FT-IR	fourier transform infrared spectroscopy
GC-MS	gas chromatography-mass spectrometry
HPLC	high performance liquid chromatography
$LiAlH_4$	lithium aluminum hydride
LiOH	lithium hydroxide
M	molar (concentration)
m-CPBA	meta-chloroperbenzoic acid
MBCHs	methyl-branched hydrocarbon
MS	mass spectrometry
n-BuLi	*n*-butyllithium

$NaBH_4$	sodium borohydride
NaHMDS	sodium hexamethyldisilazide
NaOEt	sodium ethoxide
NaOMe	sodium methoxide
NMP	n-methylpyrrolidinone
NMR	nuclear magnetic resonance spectroscopy
p-TsOH	*para*-toluenesulfonic acid
Pd/C	palladium on carbon
PPh_3	triphenyl phosphine
R	alkyl group
RMgBr	generic Grignard reagent
RP-HPLC	reverse-phase high performance liquid chromatography
rt	room temperature
TBS	*tert*-butyldimethylsilyl
Tf	triflate
Tf_2O	triflic anhydride
THF	tetrahydrofuran
THP	tetrahydropyran
TPP	thiamine pyrophosphate
Ts	tosyl
TsCl	tosyl chloride
UV	ultraviolet

Chapter 1: Introduction

Insect cuticular hydrocarbons (CHCs) are relatively non-volatile aliphatic compounds that constitute the waxy layer coating the exoskeleton of all insects. At first glance, the function of this wax layer may appear to be merely protective, preventing the dehydration of insects by acting as a hydrophobic barrier.[1,2] A closer look at the functions of these natural products reveals a secondary purpose; components of this complex blend of *n*-alkanes, methyl-branched alkanes, alkenes, and more polar compounds also function as short range or contact pheromones.[3] CHC components have been shown to mediate mate identification between solitary conspecific insects, elicit courtship between a copulating pair, and influence mate choice.[2-4] They are also known to have the unintended consequence of helping specialist predators identify their desired prey,[5] to mediate and maintain aggregations of overwintering ladybird beetles,[6] and to act as trail pheromones for certain species of cerambycid beetles.[7] The communicative roles of CHCs are even more evident for social insects, which rely on CHC profiles to distinguish nestmates from conspecifics of foreign colonies.[8-10] The ability to identify nestmates from genetically similar strangers is of substantial importance, not only to protect colonies from invaders, but also to ensure that resources and altruistic behaviors are shared only between members of the same colony.[11] Social insects also rely on CHCs to allocate tasks and differentiate between social castes within a colony.[12,13] CHC components function as fertility and dominance signals, allowing the worker class to determine the fecundity and health of the reproductive members of their colony.[14,15]

For various species of insects, methyl-branched hydrocarbons (MBCHs) have been identified as the active contact pheromone(s) in the CHC blend. For example, courtship of female cadavers of *Argillus planipennis*, the emerald ash borer beetle, by mature males was induced by treatment of the hexane-washed cadavers with a racemic mixture of 9-methyltricosane.[16] A similar result was seen in *Xylotrechus colonus,* a species of longhorned beetle, where copulation attempts were induced by treatment of female cadavers with a blend of *n*-pentacosane, 3-methylpentacosane, and 9-methylpentacosane.[17] Methyl- branched hydrocarbons, specifically 3-methylalkanes, have also been shown to act as queen primer pheromones, inhibiting worker ovarian development for several species of social hymenopterans.[18,19,20]

Thus, the study of methyl-branched hydrocarbons is important in understanding the reproductive and social behaviors of numerous insect species. Methyl-branched alkanes are chiral molecules, except where the branch point is in the 2-position or in the center of an odd numbered hydrocarbon chain due to symmetry. Thus, mono-methylalkanes can exist in two enantiomeric forms, and hydrocarbons with multiple branches can exist in a myriad of stereoisomeric forms. The biosynthesis of these natural products is likely to produce one enantiomer or stereoisomer, but it is not known whether different species might produce different stereoisomers.[1] To date, there has been very little research on the possible significance of stereochemistry in the biological activity of these compounds, or to determine if these molecules are biosynthesized in high stereochemical purity.[21]

The dearth of research into the role of stereochemistry in the bioactivities of MBCH contact pheromones or the stereospecificity of insect MBCH biosynthesis is due to a variety of factors. First, methyl-branched hydrocarbons lack functional groups that could act as contact points in chiral stationary phase columns to assist in stereoisomeric separation, making the resolution of MBCH stereoisomers by standard analytical separation methods impossible. Second, no robust, generic methods have been developed to isolate pure methyl-branched hydrocarbons from the CHC blend, so that the optical rotations of insect-produced compounds could be measured, and their biological activities assayed.[22] The determination of the absolute configurations of purified insect MBCHs is further complicated by their small specific rotations (~ 3º to a tiny fraction of 1º, depending on the location of the branch point) and small amounts of compounds (ng to μg) present on most insects.

The scarcity of studies assessing the effects of stereochemistry within the biological activities of MBCH contact pheromones can also be traced to the time, effort, and resources involved in synthesis of enantiopure methyl-branched hydrocarbons, particularly when a number of them may be required in order to completely define a contact pheromone.[22] Thus, almost all studies to date involving methyl-branched hydrocarbon contact pheromones have utilized racemic mixtures of MBCHs in their bioassays. Fortunately, in most cases the racemic mixtures have been shown to have at least some biological activity, but in many cases the racemic compounds appear to be less active than crude CHC extracts, suggesting that the chirality of the contact pheromone may be important.[23-26] The importance of chirality in the biological activities of volatile

semiochemicals has been well established.[27-29] Frequently, only one enantiomer or diastereomer of a pheromone is biologically active, whereas the other stereoisomer(s) are either benign or inhibit the biological activity of the active isomer. Despite the strong correlation of stereochemistry to the biological activities of volatile infochemicals, the role of chirality in MBCH contact pheromones remains largely unknown, and an area ripe for exploration.

1.2 Biosynthesis of Insect Methyl-branched Hydrocarbons

Cuticular hydrocarbon biosynthesis in insects, like the biosynthesis of all acetogenins, is initiated by glucose oxidation in the cytosol to form pyruvate.[30] Pyruvate is then decarboxylated, in a similar fashion to thiamine-catalyzed benzoin condensation, by pyruvate dehydrogenase and a thiamine pyrophosphate co-factor, to form hydroxyethyl-thiamine pyrophosphate (TPP). Lipoate transacetylase then catalyzes the addition of hydroxyethyl-TPP to lipoamide, which produces acetyldihydrolipoamide. Nucleophilic addition of coenzyme-A to acetyldihydrolipoamide, catalyzed by acetyl-CoA synthetase, forms acetyl-CoA.[31] Acetyl-CoA then undergoes an S-acetyltransferase catalyzed transthioesterification with S-acyl-carrier-protein (ACP) to form acetyl-ACP (Scheme1.1).

Scheme 1.1 Mechanism of acetyl-ACP biosynthesis.

The biosynthesis of insect CHCs occurs within cells associated with the epidermal layer called oenocytes, which utilize the fatty acid synthetase (FAS) biosynthetic pathway to form long-chain aliphatic hydrocarbons from cytosolic acetyl-ACP.[32] Saturated hydrocarbon formation is initiated by a 3-ketoacyl-ACP synthase catalyzed Claisen condensation of acetyl-ACP with malonyl-ACP substrates to produce 3-ketobutyl-ACP, which is subsequently reduced by 3-ketoacyl-ACP reductase to form 3-hydroxybutyl-ACP. β-Elimination of the resulting β-hydroxythioester by 3-hydroxyacyl-ACP dehydrase forms 2-butenoyl-ACP, which is subsequently reduced by an enoyl-ACP reductase-catalyzed, 1-4 hydride addition followed by α-protonation to form butyl-ACP (Scheme 1.2). Subsequent iterative FAS-mediated Claisen condensations with malonyl-ACP moieties, followed by NADPH mediated reductions, result in the elongation of the hydrocarbon chain and biosynthesis of straight-chain fatty acids.[33]

Scheme 1.2. Initiation of saturated CHC biosynthesis by acetyl-ACP and malonyl-ACP. Incorporation of methylmalonyl-ACP results in the biosynthesis of MBCHs.

The methyl branches found in MBCHs for all except the two position arise from the incorporation of methionine-derived propionate, in the form of methylmalonyl-ACP, at specific points during the chain elongation process (Scheme 1.2).[34] This incorporation results in mono- to multimethyl-branched hydrocarbons. By contrast, for MBCHs in which the methyl branch is found at the 2-position, biosynthesis is initiated by the transamination, decarboxylation, and thioesterification of leucine forming 3-methylbutyryl-CoA. Chain elongation is then propagated by malonyl-ACP, which leads to the formation of 2-methyl MBCHs with an odd number of carbons in the hydrocarbon backbone (Scheme 1.3 a).[35] For 2-methylalkanes that have an even-numbered hydrocarbon backbone, the biosynthesis originates with the oxidation of valine to form 2-propanoyl-CoA which then undergoes the same chain elongation sequence with malonyl-ACP substrates (Scheme 1.3 b).[36] This finding was supported by labeling studies

completed by Chase and coworkers, in which ^{13}C-labeled valine and methionine incorporated into the diet of the German cockroach, *Blatella germanica*, resulted in ^{13}C-labeled methyl-branched hydrocarbons.[37]

Scheme 1.3. Incorporation of amino acids to form precursors for MBCH biosynthesis (A) incorporation of leucine forms 2-methylalkanes with odd-numbered hydrocarbon backbones, and (B) incorporation of valine results in 2-methylalkanes with even-numbered hydrocarbon backbones.

For the few insect species whose diets are high in vitamin B_{12}, such as termites, the propionate utilized in MBCH biosynthesis arises from metabolized succinate.[38] In labeling experiments performed by Blomquist and coworkers, *Zootermopsis augusticollis* termite colonies whose diets were enriched with [2,3-^{13}C]-labeled succinate produced ^{13}C-labeled methyl-branched hydrocarbons. Conversely, in colonies of *Z. augusticollis* whose diets were enriched with ^{13}C-labeled valine and isoleucine, the labeled carbon atoms were not detectably incorporated into their MBCHs, providing evidence of a strictly succinate-derived methylmalonyl-CoA substrate in the hydrocarbon biosynthesis of this species.[38]

Microsomal chain elongation of MBCH-ACP intermediates using malonyl and methylmalonyl-ACP substrates results in the formation of long-chain methylated fatty

acyl-ACP moieties.[39] These long-chain fatty acyl-ACPs are then reduced to aldehydes, using NADPH, and then decarbonylated to form methyl-branched cuticular hydrocarbons.[40] The mechanism for the decarbonylation of long-chain fatty acyl-CoAs has been a topic of debate among insect biochemists and chemical ecologists. Early studies of branched hydrocarbon formation showed that tritium-labeled fatty acids were converted *in vivo* to hydrocarbons that contained one less carbon in the hydrocarbon chain.[38] Originally, the decarbonylation mechanism was believed to mimic hydrocarbon formation in algae, plants, and vertebrates, in which the fatty acyl-CoA is reduced to an aldehyde and, in the absence of co-factors, decarbonylated to form the MBCHs and carbon monoxide. However, Reed and coworkers have reported that incubation of [1-^{14}C]-tetracosenoyl-CoA with housefly microsomes resulted in the formation of labeled carbon dioxide, and not carbon monoxide. [41, 42]

Further work by Mpuru and coworkers showed that [9,10-^{3}H, 1-^{14}C]-octadecanoyl-CoA was converted, in the presence of oxygen and NADPH, into its corresponding aldehyde, which was later transformed into a C_{17} branched hydrocarbon product and CO_2.[43] This result led to the proposal of a new decarbonylation mechanism involving a cytochrome P450 mediated oxidative decarbonylation (Scheme 1.4). Recent work by Qiu and coworkers has shown that the oxidative decarbonylation mechanism in insects is performed by an insect-specific oxidative aldehyde decarbonylase.[44] RNAi knockouts of the Cyp4g1 gene, which encodes the insect decarbonylase enzyme, resulted in the increase of long-chain fatty acids and fatty acyl esters, and a large decrease in the

concentration of CHCs on the cuticle of mutated *Drosophila melanogaster* as compared to wild type (Table 1.1).

Scheme 1.4. Proposed cytochrome P450 mediated decarbonylation mechanism. Radical addition of cytochrome P450 to the carbonyl carbon of the aldehyde results in the formation of an iron carboxylate complex and radical hydrocarbon pair, which then forms the deuterated hydrocarbon and CO_2.[43]\

‘

	males			females		
	n	esters/acids	alkanes/alkenes	n	esters/acids	alkanes/alkenes
GAL4 parents	6	1.7 ± 0.2 a	427 ± 31 a	6	1.0 ± 0.1 a	475 ± 35 a
UAS parents	10	1.2 ± 0.2 a	537 ± 49 a	10	0.8 ±0.2 a	452 ± 143 a
CY4PG1 RNAi cross	20	695 ± 136 b	196 ± 7 b	19	943 ± 231 b	235 ± 9 b
CPR RNAi cross	17	570 ± 121 b	172 ± 9 b	19	762 ± 137 b	214 ± 10 b

Table 1.1. Effects of RNAi suppression of Cyp4g1 and CPR in oenocytes on cuticular lipids of *D. melanogaster*, GAL4 and UAS parents are wild type (Data taken from reference 44)

Although the biosynthetic route for insect methyl-branched hydrocarbons has been elucidated, the stereospecificity of this biosynthesis remains unknown. From a strictly organic chemistry aspect, asymmetric induction must occur during the reduction of 2-methyl-2-enoyl-ACP by the enoyl-ACP reductase domain of microsomal FAS (Scheme 1.5). The stereoselectivity of this reduction could be determined in two

complementary ways. The first involves the isolation and cloning of the genes which encode the enoyl-ACP reductase domain of FAS, followed by construction of recombinant enoyl-ACP reductases and labeled precursors, and finally analysis of labeled products formed by both the isolated enzymes and the recombinant mutants. The second, empirical method requires the isolation of pure MBCHs and the determination of their specific rotations. Use of either or both methods would help clarify the stereospecificity of methyl-branched hydrocarbon biosynthesis in insects.

NADPH + H^+
Enoyl-ACP Reductase

Scheme 1.5. Asymmetric reduction of Enoyl-ACP by the enoyl-ACP reductase domain of microsomal FAS.

1.3. Enantioselective Synthesis of Chiral Methyl-Branched Hydrocarbons

The first asymmetric synthesis of chiral methyl-branched cuticular hydrocarbons was performed by Ade and coworkers in 1980, in the synthesis of the stereoisomers of 17,21-dimethylheptatriacontane, the contact sex pheromone of the tsetse fly.[45] This synthesis utilized indirect resolution of chiral carboxylic acids as their diasteromeric amides with (*D*)- or (*L*)-phenylglycine, separating the amides by HPLC. With this method, 2-methyl- and 3-methylalkanoic acid amides were separated in high diastereomeric purity and yield (Scheme 1.6). 2-Methylalkanoic acids **1** were first treated with thionyl chloride to form the correponding acid chlorides **2**, which were subsequently

treated with a chiral amino acid to form the diastereomeric amides **3a** and **3b**. The diastereomeric amides were then separated via silica gel HPLC, and the purified amides reduced with $LiAlH_4$ to yield (*R*)- and (*S*)-2-methylalkanols **4a**. These alcohols were transformed to chiral 2-methylalkyl tosylates **4b** and subsequently alkylated to form the chiral methyl-branched hydrocarbons. This synthesis was hindered by the fact that HPLC was required for the isolation of the diastereomerically pure intermediates, which limited the scale of the syntheses.

Scheme 1.6. Synthesis of chiral methyl-branched hydrocarbons utilizing directed resolution of 2-methylcarboxylic acids *via* HPLC separation of diastereomeric amides. Reagents: (a) $SOCl_2$, pyridine, CH_2Cl_2, 0 ºC; (b) *D*- or *L*-phenylglycine, THF, -78 ºC; (c) HPLC separation; (d) $LiAlH_4$, THF, 0 ºC; (e)TsCl, pyridine, CH_2Cl_2, 0 ºC; (f) Li_2CuCl_4 (cat.), R"-MgBr, Et_2O, -78 ºC.

In 1984 Phillip Sonnet developed a more accessible method for the synthesis of chiral methylalkanes based on the fractional recrystallization of diastereomeric amides prepared by derivatization of racemic 2-methylalkanoic acids **5** and one enantiomer of α-methylbenzylamine **7** (Scheme 1.7)[46]. Recrystallization of the resulting adducts allowed isolation of optically pure diastereomeric amides **8**. Cleavage of the stereoisomerically pure amides with LiOH, followed by $LiAlH_4$ reduction resulted in the formation of chiral

2-methylalkanols intermediates **9**, which could then be alkylated in a plethora of ways to obtain chiral methyl-branched hydrocarbon products.

Scheme 1.7. Asymmetric synthesis of methyl-branched hydrocarbons developed by Phillip Sonnet via fractional recrystallization of diastereomeric amides. Reagents: (a) $SOCl_2$, pyridine, CH_2Cl_2; (b) (*R*)-α-methylbenzylamine, THF, 0 ºC; (c) recrystallization; (d) i. LiOH, THF, 0 ºC; ii. $LiAlH_4$, THF, 0 ºC; (e) TsCl, pyridine, CH_2Cl_2, 0 ºC; (f) $CuCl_2$(cat.) , R"-MgBr, THF, 0 ºC.

In the mid 1980s, the use of chiral synthons as the source of asymmetry in chiral MBCHs became the most popular method for the synthesis of these optically active molecules. Kuwahara and coworkers developed the first synthesis of enantiopure MBCHs prepared via a chiral synthon in 1983 (Scheme 1.8).[47] In this synthesis, (*R*)- or (*S*)-citronellic acid **10** was first reduced to (*R*)- or (*S*)-citronellol **11**, which was subsequently treated with tosyl chloride and pyridine to produce (*R*)- or (*S*)-citronellyl tosylate **12**. Compound **12** was then alkylated using a copper-catalyzed Grignard cross-coupling reaction to form the longchain alkene **13**. The resulting alkene **13** was then treated with *m*-chloroperbenzoic acid (MCPBA), and the resulting epoxide **14** was then oxidatively cleaved with sodium periodate to form the corresponding 4-methylalkanal **15**. Wittig olefination of aldehyde **15** with various n-alkyltriphenylphosphonium ylides,

followed by hydrogenation, gave the desired chiral methyl-branched hydrocarbons **17**. The use of optically active citronellol for the synthesis of chiral MBCHs is still popular today, with several groups publishing variants of this synthesis using other citronellol-derived chiral synthons.[48-51]

Scheme 1.8. Preparation of chiral MBCHs from (*R*)- or (*S*)-citronellic acid by Kuwahara and coworkers (1983). Reagents: (a) $LiAlH_4$, THF, 0 ºC; (b) TsCl, pyridine, CH_2Cl_2, 25 ºC; (c) Li_2CuCl_4, R'MgBr, Et_2O, -40 ºC; (d) *m*-CPBA, CH_2Cl_2; (e) $NaIO_4$, THF, rt; (f) R"-PPh_3Br, *n*-BuLi, THF, 0 ºC; (g) H_2, Pd/C, 1 atm, hexanes, rt.

In 1998, Fukusaki and coworkers developed a synthesis of chiral methyl-branched hydrocarbons using a double-ended approach, with chiral synthons derived from the enantiomers of methyl 3-hydroxy-2-methylpropanoate (Scheme 1.9).[52] The use of this synthetic method provided the added convenience that the chiral synthon could first be protected on one side of the chiral center and then alkylated on the other to give an easily separable polar alcohol intermediate after deprotection. This synthesis involved THP-protection of (*R*)- or (*S*)-methyl 3-hydroxy-2-methylpropanoate to form (*R*)- or (*S*)-

methyl 2-methyl-3-((tetrahydro-2*H*-pyran-2-yl)oxy)propanoate **18**, which was reduced to the corresponding alcohol **19**. The alcohol was then transformed into a sulfonate ester **20** and alkylated using a Grignard reagent with Li_2CuCl_4 catalysis to form a chiral methyl-branched synthon **21**. Deprotection and subsequent alkylation of the tosylate of the resulting chiral alcohol produced a chiral methyl-branched hydrocarbon **22**. This synthetic method has the added advantage of requiring only one chiral synthon for the formation of both enantiomers of any desired monomethyl-branched hydrocarbon. That is, alkylation of one side of the chiral synthon with an alkyl Grignard reagent A and alkylation of the opposite side with a different alkylating agent B results in the formation of one enantiomer, whereas reversing the order of alkylation produces the antipode. Schlamp and coworkers followed a similar synthetic approach, but exploited an even more convenient (albeit expensive) pair of chiral synthons, the enantiomers of 3-bromo-2-methyl-1-propanol, to make chiral methyl-branched products.[53]

Scheme 1.9. Preparation of chiral methyl-branched hydrocarbons using the enantiomers of methyl-3-hydroxy-2-methylpropanoate as the source of stereochemistry. Reagents: 3,4-dihydro-2H-pyran, *p*-TsOH, CH_2Cl_2, rt; (b) $LiAlH_4$, THF, 0 ºC; (c) TsCl, pyridine, CH_2Cl_2; (d) Li_2CuCl_4 (cat.), R'-MgBr, Et_2O, -40 ºC.

Kenji Mori developed a new synthesis of chiral MBCHs from (*R*)-3-methyl-butyrolactone **23**, in an attempt to determine the absolute configuration of the coffee leaf miner moth pheromone, 5,9-dimethylpentadecane (Scheme 1.10).[54] In this synthesis, (*R*)-3-methylbutanolide **23** was reduced with diisobutylaluminum hydride to afford lactol **24**, which underwent a Wittig olefination with an alkyltriphenylphosphonium ylide to give the alkene **25** as a mixture of *E*/*Z* isomers. The alkene was then hydrogenated to form the (*R*)-2-methylalkanol **26**. The corresponding tosylate of **26** underwent a Finkelstein reaction with sodium iodide to afford (*R*)-2-methylalkyl iodide **27.** A number of alkylation steps then were used to produce the dimethyl-branched pheromone. (*S*)-3-Methylbutanolide was utilized in the same reaction sequence to afford the opposite enantiomer.

Scheme 1.10. Kenji Mori's synthesis of chiral methyl-branched hydrocarbons from the enantiomers of 3-methylbutanolide. Reagents: (a) $NaBH_4$, Et_2O, -10 ºC; (b) R'-PPh_3Br, *n*-BuLi, THF, 0 ºC; (c) H_2, Pd/C, hexanes, rt; (d) TsCl, pyridine, CH_2Cl_2, rt; (e) NaI, THF, reflux; (f) Li_2CuCl_4(cat.), R''-MgBr, Et_2O, -40 ºC.

One of the more complex syntheses of chiral methyl-branched hydrocarbon pheromones was completed by Lamers and coworkers in 2003, where the total synthesis of these simple natural products was started from the polycyclic sesquiterpinoid (+)-

aromadendrene (Scheme 1.11).[55] In this synthesis, (+)-aromadendrene **30** was first converted to the chiral alcohol **31** in four steps. After several unsuccessful attempts at Baeyer-Villiger oxidation of **31**, Lamers and coworkers decided to first brominate **31** with PPh_3/CBr_4 to form the bicyclic bromoketone **32**, which was then treated with MCPBA to form the bromolactone **33**. Treatment of **33** with EtOH and *p*-TsOH resulted in ring opening of the lactone to form bromoester **34**. Simultaneous Grob fragmentation and reduction of **34** with NaOEt and $NaBH_4$ afforded the alcohol **35**, which was then hydrogenated to afford ethyl (*S*)-10-hydroxy-7-methyl decanoate **36**. The preparation of chiral MBCHs from **36** then utilized the same synthetic steps as the double-ended chiral synthons described earlier in this chapter.

Scheme 1.11. Preparation of chiral methyl-branched hydrocarbons from the sequiterpenoid (+)-aromadendrene. Reagents: (a) PPh_3, CBr_4, CH_2Cl_2, 0 ºC; (b) *m*-CPBA, CH_2Cl_2, rt; (c) EtOH, *p*-TsOH, rt; (d) 1. NaOEt, THF, rt; 2. $NaBH_4$, THF, 0 ºC; (e) H_2, Pd/C, hexanes, rt.

Unlike the previously described syntheses for chiral MBCHs that rely on the stereochemistry of a chiral synthon to prepare enantiomerically enriched methyl-branched hydrocarbon pheromones, a recently published synthesis route by Taguri and coworkers utilized stereospecific inversions of secondary tosylates, derived from the enantiomers of propylene oxide, to dictate the stereochemistry of their methyl-branched hydrocarbon products (Scheme 1.12).[56] Taguri and coworkers first alkylated (*R*)-propylene oxide **37** using a $CuCl_2$-catalyzed Grignard cross coupling reaction forming the secondary alcohol **38**, which was immediately tosylated to afford the secondary tosylate **39**. Clean S_N2 displacement of the tosylate with dimethyl malonate inverts the stereochemistry of the methyl branch-point and forms compound **40**, which then undergoes a Krapcho decarboxylation to afford alkyl ester **41**. The methyl-branched ester **41** is reduced to the corresponding 3-methylalkanol **42**, which is then treated with Ph_3PI_2 to afford the 3-methylalkyl iodide 43. Alkylation of the alkyl iodide **43** with a number of alkylating agents, via Li_2CuCl_4-catalyzed Grignard cross coupling reactions, produces any desired methyl-branched alkane product.

(*R*)-**37** —a→ **38** (R, OH) —b→ **39** (R, OTs) —c→ **40** (R, CO_2Me, CO_2Me)

—d→ **41** (R, CO_2Me) —e→ **42** X=OH; f → **43** X=I —g→ **44** (R, R')

Scheme 1.12. Synthesis of chiral MBCHs via stereospecific inversions of secondary tosylates. Reagents: (a) R-MgBr, $CuCl_2$, Et_2O, 0 ºC; (b) TsCl, pyridine, CH_2Cl_2, rt; (c) dimethylmalonate, NaOMe, THF, 0 ºC; (d) LiCl, DMSO, reflux; (e) $LiALH_4$, THF, 0 ºC; (f) PPh_3I_2, THF, -10 ºC; (g) Li_2CuCl_4 (cat.), R'-MgBr, Et_2O, 0 ºC.

Although several published syntheses of chiral methyl-branched hydrocarbons have been described, there is still room for improvement, in terms of developing an efficient and generic synthetic route that can produce either enantiomer of any desired methyl-branched hydrocarbon in good yield and high enantiomeric purity. Each of the syntheses described above has one or more limitations which makes preparation of a large number of chiral MBCHs arduous. For example, many of these syntheses utilize expensive chiral synthons (citronellol, methyl 3-hydroxy-2-methylpropanoate, (+)-aromadendrene, 3-bromo-2-methyl-propan-1-ol), which makes the gram scale preparation of chiral MBCHs costly and renders their use in biological assays less appealing than racemic MBCHs. Some of the described syntheses also proceed via easily racemized intermediates (Lamers et al. 2003; Taguri et al. 2012) that could ruin the stereochemistry of the final MBCH products and negatively affect bioassay results. Also, all of the syntheses described above have overall yields of less than 25% and require at least eight steps to prepare the desired products. Thus, the development of a new efficient synthesis of chiral MBCHs using cheap or cheaply made starting materials could make enantiopure compounds more easily available for use in bioassays, which in turn would remove one of the major bottlenecks hindering research to elucidate the importance of stereochemistry in relation to the biological activities of methyl-branched hydrocarbon contact pheromones.

1.4. Significance of Chirality in the Biological Activities of Methyl-Branched Hydrocarbon Contact Pheromones.

Some of the most complicated and diverse relationships between structural configuration and biological activity occur with volatile pheromones used by insects. For example, in 1974 Silverstein and coworkers synthesized the enantiomers of the alarm pheromone, 4-methyl-3-heptanone, of the leaf cutter ant, *Atta texana*.[57] They found that (*S*)-4-methyl-3-heptanone was approximately 400 times more bioactive than (*R*)-4-methyl-3-heptanone, but (±)-4-methyl-3-heptanone was also active, suggesting that the (*R*)-enantiomer did not inhibit the bioactivity of the correct stereoisomer. This result, which demonstrated that insects detect and respond to specific stereoisomers, now has been shown with numerous insects since Silverstein's first demonstration of this phenomenon.[28,29]

(*S*)-4-methyl-3-hexanone

(*R*)-4-methyl-3-hexanone

Figure 1.1. Enantiomers of 4-methyl-3-hexanone, the alarm pheromone of *Atta texana,* the active pheromone (*S*)-4-methyl-3-hexanone is boxed.

The relationship between stereochemistry and biological activity in the context of insect pheromones has now been revealed to be much more complex in many cases than the one bioactive stereoisomer/other stereoisomers inactive model described above, with a plethora of distinct stereochemistry-bioactivity relationships now being known. For example, in some species of insects, an exact ratio of pheromone enantiomers is naturally produced and required to obtain optimal biological activity, with changes in the natural ratios resulting in partial or total loss of activity. For example, the male-produced aggregation pheromone of the ambrosia beetle, *Gnathotrichus sulcatus*, was shown to be composed of a 1:1 enantiomeric mixture of 6-methyl-5-heptene-2-ol (sulcatol) by Borden and coworkers after enantiomerically pure (*R*)- and (*S*)-sulcatol were shown to be inactive in bioassays.[58] A similar result was seen for the volatile pheromones produced by the female Douglas fir beetle, *Dendroctonus pseudotsugae*, whose natural pheromone was determined to be comprised of a 55:45 ratio of (*R*)- to (*S*)-1-methylcyclohex-2-ene-1-ol.[59] For some organisms, the stereoisomeric purity of their pheromones is crucial for activity, with the presence of even trace amounts of other stereoisomers rendering the otherwise active semiochemical unattractive.[60-65] There have even been cases in which males and females of the same species produce opposite enantiomers of the same compound to specifically attract members of the opposite sex.[66,67]

Figure 1.2. Aggregation pheromone of the ambrosia beetle, *Gnathotrichus sulcatus,* is a 50:50 mixture of both (*R*)-sulcatol and (*S*)-sulcatol.

There are many more cases that show the complicated stereochemistry-bioactivity relationships of volatile pheromones, but there have been only a handful of studies that have tried to identify the importance of stereochemistry in relation to the biological activities of non volatile MBCH contact pheromones. In fact, it is still virtually unknown if insects can discriminate between MBCHs with similar structures (i.e. same branch point and different chain lengths, or same chain length and different branch point), let alone between enantiomers of MBCHs. In 1985, McDowell and coworkers performed the first analyses of the stereochemistry of methyl-branched hydrocarbon sex pheromones, in which they investigated the absolute stereochemistry of the sex pheromone of the tsetse fly, *Glossina pallidipes,* 13,23-dimethylpentatriacontane. The active pheromone was shown to be the *meso*-isomer, (13*R,*23*S*)-dimethylpentatriacontane, whereas the other stereoisomers were unattractive.[68] Duff and coworkers saw a similar result with the sex pheromone components of the spring hemlock looper moth, *Lambdina athasaria*, 7-methylheptadecane and 7,11-dimethylheptadecane, where a mixture of (*S*)-7-methylheptadecane and *meso*-7,11-dimethylheptadecane were the only bioactive stereoisomers of the pheromone.[69] However, in this case, it must be noted that the pheromone components, although hydrocarbons, are volatile attractants rather than contact pheromones.

More recently, Schlamp and coworkers indirectly identified the absolute stereochemistry of the female-produced contact sex pheromone components of the peach

twig borer moth, *Anarsia lineatella*.[70] Using female decoys baited with a single enantiomer or a racemic mixture of 11-methyltricosane and octadecyl acetate, Schlamp and coworkers showed that males attempted copulation only with decoys treated with (±)-11-methyltricosane and octadecyl acetate. The female decoys treated with (*S*)-11-methyltricosane alone did not elicit a significant increase in copulatory response compared to a solvent treated lures, whereas lures treated with the (*R*)-enantiomer inhibited the contact response of males. Silk and coworkers also performed similar bioassays to try to determine the absolute stereochemistry of the contact sex pheromone of the cerambycid beetle *Tetropium fuscum*, which utilizes a blend of 11-methylheptacosane and (*Z*)-9-heptacosene as its contact sex pheromone.[71] Using solvent-extracted female cadavers treated with either (*R*)- or (*S*)-11-methylheptacosane and (*Z*)-9-heptacosene, Silk et al. showed that males attempted copulation only with extracted cadavers treated with (*S*)-11-methylheptacosane, whereas those treated with the (*R*)-enantiomer were unattractive. However, the reported specific rotation for the synthetic (*S*)-11-methylheptacosane utilized in the study, $[\alpha]_D$= -0.06 (c=3.33, hexanes), actually matches the specific rotation of the opposite enantiomer, (*R*)-11-methylheptacosane, suggesting that the stereochemical identification of these compounds may be incorrect. Ablard and coworkers completed another recent study attempting to identify the stereochemistry of methyl-branched contact sex pheromone components for the parasitic wasp *Ooencytrus kuvanae*. Female *O. kuvanae* produce a blend of 5-methylheptacosane and 5,17-dimethylheptacosane that acts as a contact sex pheromone responsible for mate recognition.[72] Although both sexes produce the compounds comprising the contact

pheromone blend, it was shown with the use of synthetic chiral MBCHs, that (*S*)-5-methylheptacosane and (5*R*,17*S*)-dimethylheptacosane were likely to be the active female-produced stereoisomers, whereas the combination of (*R*)-5-methylheptacosane and (5*R*,17S)-dimethylheptacosane inhibited attraction. The other stereoisomers of the two compounds comprising the contact pheromone blend were unattractive. These results suggest that male and female *O. kuvanae* respectively might biosynthesize the opposite enantiomers of 5-methylheptacosane, to allow males, or possibly both sexes, to differentiate each other. Unfortunately, none of the previously reported studies have isolated the insect-produced MBCHs to perform direct stereochemical identifications, so the absolute stereochemistries of these natural products have not yet been proven by unequivocal analytical chemistry methods.

In total, there have been very few studies of the stereochemistry-bioactivity relationships of nonvolatile methyl-branched hydrocarbon contact pheromones, and all studies to date have been correlative, with none of the MBCH contact pheromones in the publications discussed above being isolated to determine the absolute configurations of the insect-produced compounds. Absolute stereochemistries have been assigned only via the responses of insects to synthetic compounds. As discussed, the limited number of reports involving stereochemistry and biological activities of MBCHs are a direct result of the lack of : 1) methods for isolation of pure MBCHs from the CHC extracts, 2) instrumental limitations which make the stereochemical characterization of purified MBCHs difficult, 3) and the tedious and inefficient routes to produce long-chain chiral MBCHs. The development of straightforward methods for the isolation, stereochemical

identification, and synthesis of insect-produced MBCHs is needed, so that the relevance of the absolute stereochemistries of insect MBCH contact pheromones can be thoroughly and properly assessed.

1.5. Isolation of Pure MBCH Compounds from CHC Extracts of Insects

The isolation of individual methyl-branched hydrocarbons from the cuticular hydrocarbon blend is potentially a tedious and difficult task because insect cuticular hydrocarbons are composed of a mixture of olefins, long-chain *n*-hydrocarbons, methyl branched hydrocarbons, and more polar compounds. Whereas most of the components of CHCs can be separated by analytical gas chromatography, isolation by preparative gas chromatography becomes awkward because of the high temperatures required and the difficulty in eliminating cold spots at the interface of the GC column with the collection apparatus, which can ruin the separation. Preparative gas chromatography is also limited in scale. In addition, isolating and identifying each individual methyl-branched hydrocarbon in the blend is hampered by the difficulty of separating long-chain methyl-branched hydrocarbons with varying lengths and branch points when using standard liquid chromatographic techniques.

As a start, methyl-branched hydrocarbons and long-chain *n*-hydrocarbons can be separated from alkenes and more polar compounds in cuticular lipid extracts using silver nitrate impregnated silica gel flash chromatography columns. This chromatographic method is based on the ability of transition metals, specifically silver, to complex

olefins.[73] This property allows the saturated hydrocarbons to be eluted quickly with a hexane or a similar alkane solvent, whereas unsaturated hydrocarbons, and any functionalized compounds, are retained. The unsaturated hydrocarbons can then be recovered by elution with 20% cyclohexene in hexanes, followed by elution with ether or more polar solvents to recover the more polar constitutents.

In a second purification step, *n*-alkanes can then be separated from methyl-branched hydrocarbons by adsorption of the former in molecular sieves. This separation is possible because *n*-hydrocarbons fit into and become entrapped in the pores of 5Å molecular sieves, whereas branched hydrocarbons are excluded.[74] Even molecules with methyl branches near the end of a long straight chain, i.e. 2-methyldocosane, are not adsorbed because "the entire molecule must enter the apertures guarding the pores of the sieves and become physically entrapped within the crystal lattice before adsorption occurs."[75] Thus, the presence of any methyl branch prevents adsorption. This procedure requires use of a branched hydrocarbon solvent, typically isooctane, to prevent the sieves from adsorbing only solvent and little, if any, long chain *n*-alkanes. In sum, these simple steps allow fractionation of a crude cuticular extract into fractions containing linear alkanes, branched alkanes, alkenes, and functionalized compounds.

Separation of the various methyl-branched hydrocarbons in each blend by chain length and branch point is potentially challenging because of the lack of polar functional groups in each molecule. Thus, normal phase liquid chromatography on silica gel or some other polar stationary phase is useless. In contrast, reverse phase high performance liquid chromatography (RP-HPLC) using non-aqueous solvent systems has excellent

potential for isolating CHCs from complex blends, because the separation mechanism depends primarily on hydrophobic interactions between the solutes and the stationary phase. Thus, homologous alkanes and alkenes should be readily separable on the basis of chain length, the presence or absence of double bonds, and branching in the chains. However, this leads to the second problem, one of detection, because most HPLC detectors are poorly sensitive or insensitive to CHCs. This includes UV detectors because the CHCs have no chromophores, and coupled HPLC/mass spectrometry, in which saturated alkanes and alkenes are only poorly ionized or not ionized at all by electrospray or other ionization methods that are normally used with HPLC/MS, and so are invisible to the detector.

In contrast, evaporative light-scattering detectors (ELSD) are essentially universal detectors that can detect any non-volatile molecule. With this detector, the column effluent is passed through a nebulizer where it is combined with a nitrogen gas flow to produce a fine mist of droplets. The mist then passes through an evaporation chamber, where the mobile phase is evaporated leaving behind fine particles of the target compound. When irradiated with a laser, the particles scatter the incident light, with angled photodiodes or photomultiplier tubes detecting the scattered photons.[76] Thus, the signal obtained by the ELSD is not dependent on the spectrochemical properties of the analyte, but rather is directly related to the mass of analyte that is passed through the laser beam.[77] Therefore, the use of ELSD in the HPLC separations of CHCs should allow the detection and fractionation of all semivolatile to nonvolatile compounds in an extract. However, the ELSD is a destructive detector, and its use in CHC isolations thus requires

the introduction of an effluent splitter between the column and detector to isolate the bulk of the purified CHCs.

Once isolated, the branched hydrocarbons can then be unequivocally identified by coupled gas chromatography-mass spectrometry (GC-MS).[23] Specifically, the 70 eV EI mass spectra of MBCHs show diagnostic ions from α-cleavage on either side of the branch point, and the molecular ion confirms the chain length (Figure 1.3).[1] Where the molecular ion is not visible, chain length can usually be deduced by calculation of the Kovat's retention index relative to straight chain hydrocarbon standards.[23]

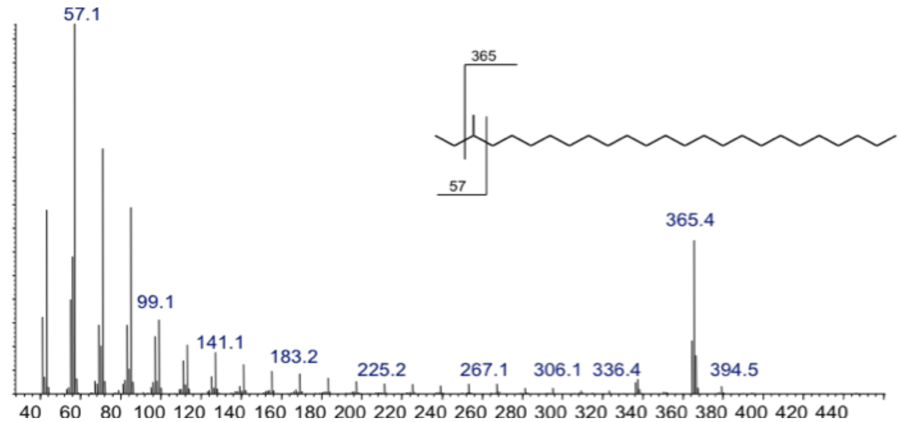

Figure 1.3. Mass spectrum of 3-methylpentacosane showing the enhanced diagnostic ions from α-cleavage on either side of the methyl branch point

1.6. Determination of the Absolute Stereochemistry of Insect MBCHs by Analytical Chemistry Techniques

The primary methods that have been used in the determination of the absolute configurations of semiochemicals are gas or liquid chromatography with chiral stationary phases, which permits the resolution of the enantiomers or other stereoisomers of many chiral compounds, on microgram to nanogram scale. Absolute stereochemistry is then

assigned by comparisons of the chromatographic retention times of synthetic standards of known absolute configuration with those of the naturally produced compounds.[77] However, to date chiral stationary phase chromatography columns have been unable to resolve the enantiomers of MBCHs due to their lack of functional groups or other significant structural variations that can interact strongly with moieties on the stationary phase to allow resolution.[22]

An alternative method for determination of the absolute configurations of insect produced MBCHs might be to measure the specific rotations of isolated pure compounds with a high accuracy digital polarimeter. The absolute stereochemistry of the MBCHs can then be determined by comparing the specific rotations of enantiopure synthetic standards, to those of the insect-produced compounds, assuming each of those compounds can be isolated in pure form. Because the specific rotations of long-chain MBCHs are only a few degrees for 3-methylalkanes and decrease to a small fraction of a degree as the methyl branch is moved towards the middle of the chain, this may require isolation of milligram quantities of each chiral MBCH. Sufficient quantities may be available from extractions of relatively few large insects with considerable amounts of CHCs, or by extracting large numbers of small insects.

1.7. Conclusion

Methyl-branched hydrocarbons now are known to mediate numerous interactions for both solitary and social insects, but to date, detailed research into their chemistry and biological functions have been limited by several cumulative factors, described in detail above. Thus, the overall goals of this dissertation are:

1. To develop a generic method for the isolation of pure individual insect cuticular hydrocarbons from crude extracts, with a particular focus on methyl-branched hydrocarbons;

2. To determine the absolute stereochemistry of the isolated insect methyl-branched compounds to gain insight into the stereospecificity of branched hydrocarbon biosynthesis;

3. To develop an efficient and generic asymmetric synthesis of methyl-branched hydrocarbons, to provide quantities of chiral MBCHs sufficient for use as analytical standards, and as test compounds in biological assays;

4. To synthesize a large library of chiral methyl-branched hydrocarbons to use as standards for the stereochemical assignment of the previously isolated MBCHs;

5. To work with collaborators to determine the stereochemical and structural specificity of MBCH contact pheromone responses for various insect species. To date only bioassays with one species, the parasitic wasp, *Lariophagus distinguendus,* have been completed.

The overall goal of my research was to determine the significance of chirality in relation to the biological activity of insect-produced methyl-branched hydrocarbons and develop methods for their isolation, stereochemical identification, and efficient asymmetric synthesis. Specifically, Chapter 2 describes methods for isolation and determination of the absolute configuration of insect-produced methyl-branched alkanes, and provides examples from 20 species in nine different orders of insects. Chapter 3 describes methods for the chemical synthesis of chiral MBCHs, as well as the pitfalls that

occurred in initial attempts to synthesize these compounds by copper-catalyzed alkylations with Grignard reagents. Finally, Chapter 4 focuses on deciphering the stereochemical and structural specificity of the contact pheromone response of a parasitic wasp *Lariophagus distinguendus.*

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Chapter 2: Isolation and Determination of Absolute Configuration of Insect-Produced Methyl-Branched Hydrocarbons

2.1. Introduction

The use of chemical signals is highly developed within the insects, with various types of pheromones mediating a wide variety of inter- and intraspecific interactions. Volatile pheromones, such as sex and aggregation pheromones, are the most well known and well-studied types of pheromones used by insects. It is now apparent that insects also utilize non-volatile molecules, in the cuticular lipids of insects as close-range or contact pheromones.[1-4] Cuticular lipids are a complex blend of long-chain aliphatic *n*-alkanes, methyl-branched alkanes, alkenes, and lesser amounts of more polar lipids that comprise the protective waxy layer on the exoskeletons of insects. Primarily, this layer acts as a waterproofing barrier to prevent desiccation,[5] but components of cuticular lipids have evolved secondary roles as contact pheromones, mediating a variety of behaviors and physiological changes in different types of insects.[1,2,6] For example, solitary insects utilize cuticular hydrocarbon (CHCs) to identify the species and sex of possible mates,[7,8] whereas in social insects, CHCs have additional roles in mediating identification of nestmates,[9,10] recognition of members of various castes, and task allocation within the colony.[11] Recent work also has demonstrated that CHCs are used by social insect queens to signal their fecundity and dominance status within the colony, and to inhibit development of workers into reproductives.[12-14]

Determination of the roles of specific CHCs as contact pheromones has been hindered by three interlinked problems. First, the cuticular lipids of a given insect species typically consist of a large number (tens to >100) of compounds, which can be

difficult to isolate in pure form to test their individual bioactivities. Specifically, the CHCs have very similar polarity and so are not separable by liquid chromatography on silica gel or other polar chromatographic media. Conversely, the compounds are so hydrophobic that they are not soluble in the aqueous-organic solvents typically used with reverse phase chromatography. Whereas most of them can be separated by analytical gas chromatography, isolation by preparative gas chromatography becomes awkward because of the high temperatures required and the difficulty in eliminating cold spots at the interface of the GC column with the collection apparatus, which can ruin the separation.

As an alternative, reverse phase high performance liquid chromatography (RP-HPLC) using non-aqueous solvent systems has excellent potential for isolating CHCs from complex blends because the separation mechanism depends primarily on hydrophobic interactions between the solutes and the stationary phase. Thus, homologous alkanes and alkenes should be readily separable on the basis of chain length, the presence or absence of double bonds, and branching in the chains. However, this leads to the second problem, one of detection, because most HPLC detectors are poorly sensitive or insensitive to CHCs. This includes UV detectors, because the CHCs have no chromophores within the accessible range, and coupled HPLC/mass spectrometry, in which saturated alkanes and alkenes are only poorly ionized or not ionized at all by electrospray or most other ionization methods normally used with HPLC/MS, and so are invisible to the detector.

The third problem concerns the inherent chirality of all of the methyl-branched CHCs except those with a branch in the 2-position, or those with a methyl branch in the

exact center of the chain such that the two remaining alkyl groups are identical. Thus, most monomethyl-branched CHCs can exist in one of two enantiomeric forms. The problem is compounded when more than one methyl branch is present, because the number of possible stereoisomers increases geometrically with the number of branch points. It has long been known that the correct stereochemistry is integral to the activity of biologically relevant molecules, in large part due to biological receptor proteins being inherently chiral because they are composed of chiral amino acids. Insects are no exception, with numerous examples known of the naturally produced stereoisomer of a volatile pheromone eliciting the desired biological activity, whereas other stereoisomers can vary in activity in different systems and contexts, from eliciting hyperactivity through to strongly inhibiting the bioactivity of the natural stereoisomer.[3,4]

Methyl-branched cuticular hydrocarbons (MBCHs) now have been identified as contact pheromones in a number of insect species.[31-43] Despite the growing number of such reports, most studies involving MBCHs have ignored the issue of stereochemistry and utilized only racemic mixtures of synthesized MBCHs in bioassays to assess their functional roles. The question as to whether insects biosynthesize MBCHs enantiospecifically, or whether receiving individuals can detect and discriminate between the stereoisomeric forms of MBCHs is still largely unknown.[15] This is due in large part to the difficulties in determining the absolute configurations of the insect-produced compounds, particularly when they are produced in only nanogram to microgram amounts per individual.

The primary methods that have been utilized to determine absolute configurations of semiochemicals are gas or liquid chromatography with chiral stationary phases, which permits the resolution of the enantiomers or other stereoisomers of many chiral compounds on microgram to nanogram scale. Absolute stereochemistry is then assigned by comparisons of the chromatographic retention times of synthetic standards of known absolute configuration with those of the naturally produced compounds.[16] However, to date, chiral stationary phase columns have been unable to resolve the enantiomers of MBCHs due to their lack of functional groups or other significant structural features that interact strongly with moieties on the stationary phase to permit resolution.[17]

An alternative method for determination of the absolute configurations of insect-produced MBCHs would be to measure the specific rotations of isolated pure compounds with a sensitive digital polarimeter. The absolute stereochemistry of the MBCHs can then be determined by comparing the specific rotations of enantiopure synthetic standards, generated by straightforward methods, to those of the insect-produced compounds (See chapter 3). However, the polarimeter measures the optical rotation of the entire contents of the optical cell, so that this method is only useful if compounds can be isolated in pure form. Because the specific rotations of long-chain MBCHs are only a few degrees for 3-methylalkanes and decrease to a small fraction of a degree as the methyl branch is moved towards the middle of the chain, this may require isolation of milligram quantities of each chiral MBCH. Thus, development of a method of isolating individual, pure MBCHs in milligram amounts is a prerequisite to the determination of their stereochemistry.

In the present study we describe a straightforward solution to this nested series of problems. Crude extracts of insect cuticular lipids were first separated into straight-chain alkanes, methyl-branched alkanes, alkenes, and more polar compounds by previously known fractionation methods.[20-22] Reverse phase HPLC with nonaqueous solvent systems and an evaporative light-scattering (ELS) detector (a "universal" detector that detects essentially any nonvolatile molecule) then allowed the isolation of individual components from these fractions. This methodology was used to isolate pure MBCHs from crude cuticular extracts of 20 insect species from 9 orders, including adults of both sexes from both holometabolous and hemimetabolous species, and of several different life stages. The specific rotations of the isolated compounds then were determined, and compared with those of a synthesized library of enantiopure standards to determine the absolute configurations of the insect-produced compounds. We report here that the absolute stereochemistry of 36 such monomethyl-branched hydrocarbons was conserved throughout all these samples, with the absolute configurations of all 36 compounds being (*R*).

2.2. Results

2.2.1. Isolation of Pure Compounds from Cuticular Hydrocarbon Extracts.

The American cockroach, *Periplaneta americana,* was chosen as a model species to determine the feasibility of isolating pure MBCHs from an insect cuticular extract. Both male and female *P. americana* have similar cuticular lipid profiles,[23] which contain a blend of approximately 25 compounds (Figure 2.1A). Because males and females might

produce different stereoisomers of individual MBCHs, the CHCs of each sex were analyzed separately. Thus, hexane extracts of the cuticular lipids from 14 males and 16 females respectively were first fractionated on a silver nitrate-impregnated silica gel chromatography column. Sequential elution of the column with hexanes followed by 5% cyclohexene in hexanes and then diethyl ether yielded fractions containing saturated hydrocarbons (Fig. 2.1B), unsaturated hydrocarbons, and more polar compounds, in sequence. After concentration and reconstitution in isooctane, the saturated hydrocarbon fraction was then treated with activated 5Å molecular sieves, resulting in adsorption of the straight-chain hydrocarbons into the sieve matrix. Removal of the molecular sieves by filtration gave a filtrate containing only branched-chain alkanes (Fig. 2.1C). After concentration, this fraction was taken up in ethyl acetate and further fractionated on a C18 reverse phase HPLC column, eluting with a nonaqueous, isocratic solvent system of ethyl acetate/methanol (1:1). The column effluent was split 80:20 between fraction collection and an ELS detector (Fig. 2.1E), and isolated compounds were conclusively identified by GC-MS. The most abundant methyl-branched hydrocarbon, 3-methylpentacosane, was collected in multimilligram amounts, in 99% chemical purity (Fig. 2.1D, mass spectrum can be seen in figure S2.1), from adults of both sexes (Table 1).

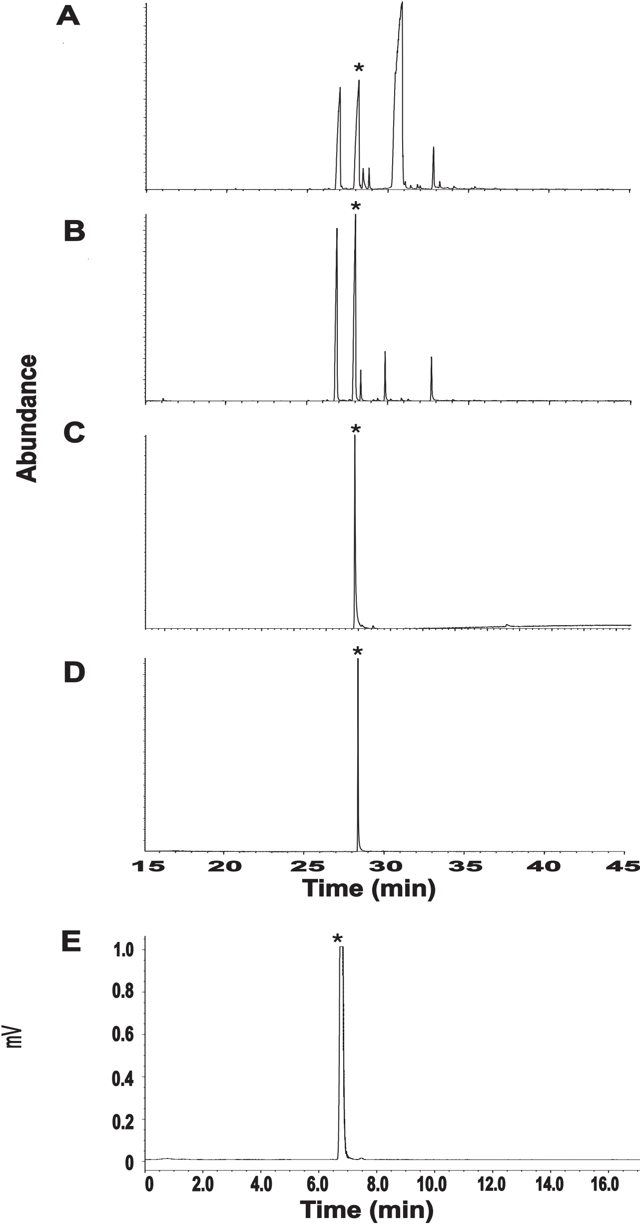

Figure 2.1. Chromatographic representation of MBCH isolation methods with ♀ *Periplaneta americana* as model system. Chromatograms A-D are total ion chromatograms from GC-MS analyses. (A) total CHC extract of *P.americana*; (B) alkanes fraction of ♀*P. americana* CHC extract after $AgNO_3$-impregnated silica gel fractionation; (C) Methyl-branched hydrocarbons from the ♀*P. americana* CHC extract after adsorption of *n*-alkanes with 5Å molecular sieves; (D) 3-methylpentacosane* (3MeC25) isolated after reverse-phase HPLC-ELSD separation of the MBCH fraction. (E) HPLC-ELSD chromatogram of the purified 3MeC25. The desired compound is indicated in all chromatograms with a * symbol.

We then tested the methodology with extracts prepared from insects with more complex CHC profiles than those of *P. americana.* Thus, the crude hexane extract of females of the cave dwelling cockroach *Blaberus giganticus* was fractionated using the preliminary steps described above, followed by reverse phase HPLC of the resulting MBCH fraction. Isocratic elution with a slightly more polar solvent mixture (2:3 ethyl acetate/methanol) was required to obtain adequate separation of peaks, because of the increased complexity of this fraction in comparison to those from the American cockroaches. The results of the separation sequence are shown in Figs. 2.2A-D, with multimilligram amounts of 3-methylhentriacontane being obtained from adults of both sexes. The mass spectrum of the isolated 3-methylhentriacontane can be seen in Figure S.2.2.

Crude hexane extracts of adults of the cerambycid beetle *Monochamus titillator* proved to be even more complex than those of *B. giganticus* (Fig. 2.3A-F). In this case, separation of the components of the MBCH fraction with an isocratic solvent system was unsatisfactory, but elution with a straightforward gradient solvent system using increasing amounts of ethyl acetate in methanol provided adequate resolution (Fig. 2.3F), allowing isolation of 9-methylpentacosane (Fig. 2.3C and S2.3), 9-methylheptacosane (Fig. 2.3D and S2.4), and 3-methylhentriacontane (Fig. 2.3E and S2.5) in one pass.

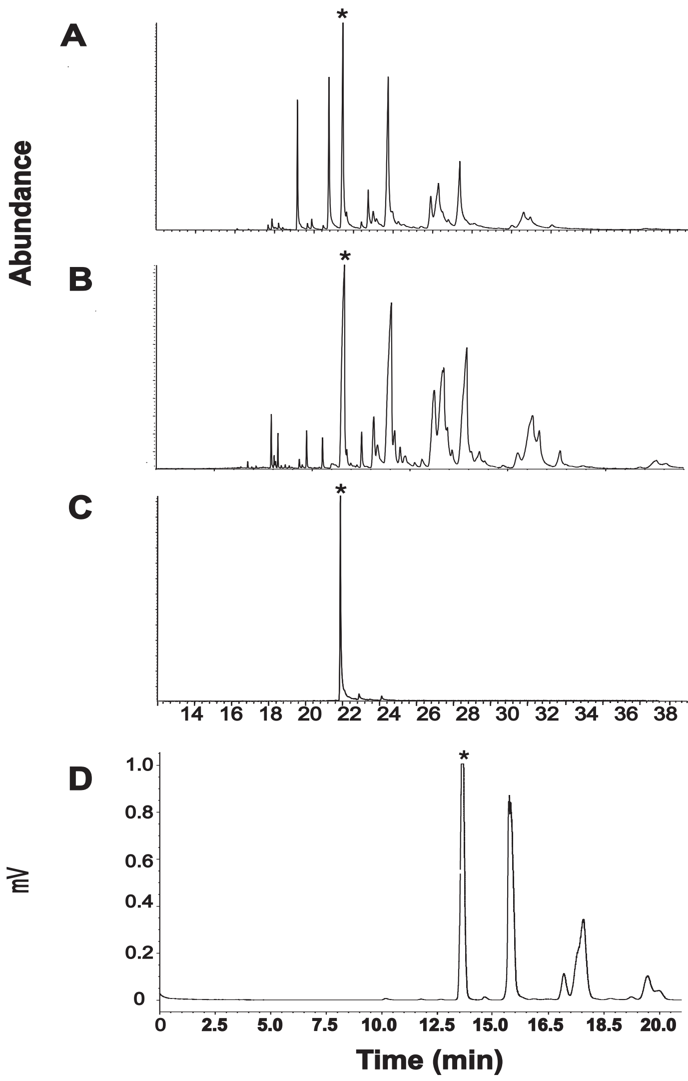

Figure 2.2. Isolation of 3-methylhentriacontane (3MeC31) from ♀ *Blaberus giganticus* CHC extracts. Total ion chromatograms of (A) alkanes fraction of ♀ *B. giganticus* CHC extract after $AgNO_3$-impregnated silica gel fractionation; (B) methyl-branched hydrocarbons from the ♀ *B. giganticus* CHC extract after adsorption of *n*-alkanes with 5Å molecular sieves; (C) 3MeC31* isolated by HPLC-ELSD separation of the MBCH fraction. (D) Chromatogram of the HPLC-ELSD separation of the MBCH fraction of ♀ *B.giganticus.* The desired compound is indicated in all chromatograms with a * symbol.

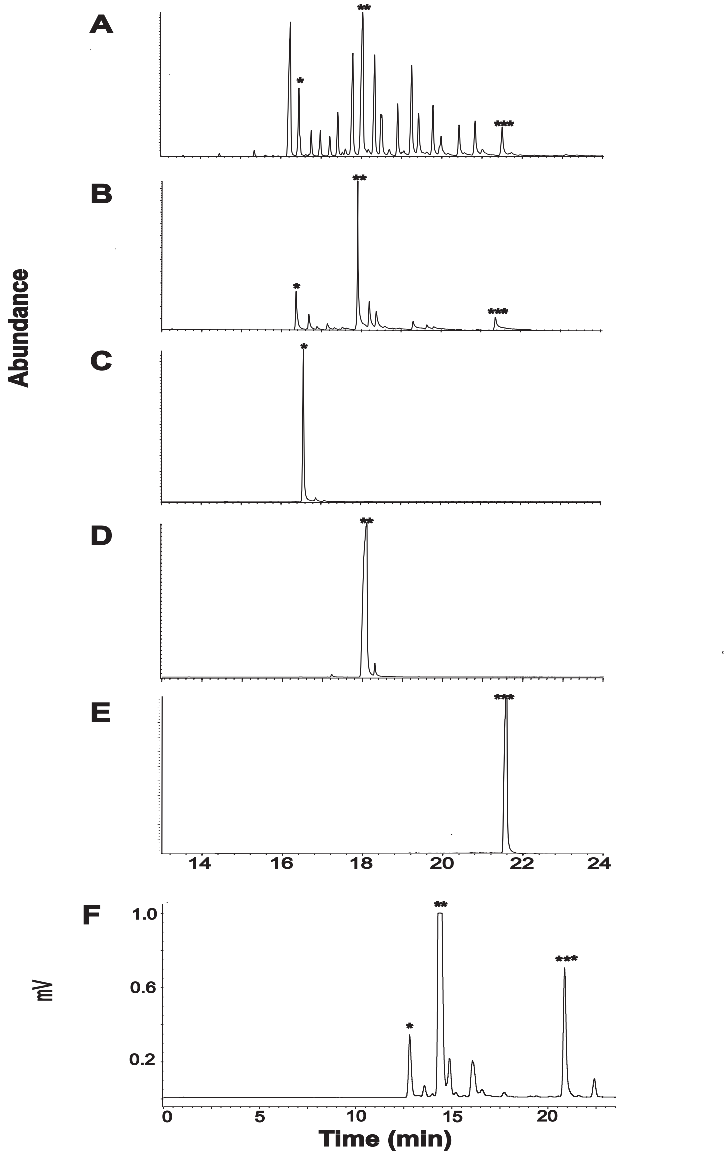

Figure 2.3 Isolation of 9-methylpentacosane*, 9-methylheptacosane**, and 3-methyl-hentriacontane*** from ♂ *Monochamus titillator* CHC extracts. Traces A-E show GC-MS total ion chromatograms of (A) alkanes fraction of ♂ *M. titillator* CHC extract after $AgNO_3$-impregnated silica gel fractionation; (B) methyl-branched hydrocarbons from the ♂ *M.titillator* CHC extract after 5Å molecular sieve adsorption of *n*-alkanes; (C) 9-methylpentacosane* (9MeC25) isolated after HPLC-ELSD separation of the MBCH fraction; (*D*) 9-methylheptacosane** (9MeC27) isolated after RP-HPLC-ELSD separation of the MBCH fraction; (E) 3-methylhentriacontane*** (3MeC31) isolated after HPLC-ELSD separation of the MBCH fraction; (F) ELSD chromatogram of the HPLC separation of the MBCH fraction of ♀ ♂ *M. titillator.* The desired compounds are indicated in all chromatograms with a *, **, or *** symbol.

The method was then extended to cuticular extracts from a variety of insect species from nine orders (Table 1), including examples of both holometabolous and hemimetabolous species, adults of both sexes of some species, and other life stages. The details of each separation are described in section 2.5.2. In total, 36 chiral MBCHs were isolated from 20 different species in amounts ranging from 0.7 mg to 13.7 mg (Table 2.1). For all species tested, one or more MBCHs were isolated in high chemical purity, in microgram to milligram amounts suitable for determination of absolute configurations.

2.2.2. Determination of the Absolute Configurations of Insect-Produced Methyl-branched Hydrocarbons.

With a large number of isolated MBCHs in hand, we then turned our attention to determination of their absolute configurations, using a high-sensitivity digital polarimeter fitted with a small-volume optical cell. Scattered reports in the literature had suggested that synthetic monomethyl-branched long-chain alkanes with (*R*)-absolute configurations all exhibited negative rotations. This was confirmed by measuring the specific rotations of enantiopure synthetic standards of both (*R*)- and (*S*)-configurations, with chain lengths from 25 to 35 carbons and methyl branches on carbons 3 to 13 (table of synthetic standards in chapter 3). Measurement of the specific rotations of the 36 MBCHs isolated from the various insect species determined that every one of the 36 compounds had the (*R*)-configuration (Table 2).

Genus	Species	Order	Sex/ life stage	Isolated compound	Amount (mg)	# Insects of extracted
Periplaneta	*americana*	Blattodea	♀	3MeC25	13.7	16
Periplaneta	*americana*	Blattodea	♂	3MeC25	12.3	14
Periplaneta	*brunnea*	Blattodea	♂	3MeC23	3.7	8
Periplaneta	*australasiae*	Blattodea	♂	3MeC23	2.7	8
Periplaneta	*fuliginosa*	Blattodea	♂	3MeC23	1.9	8
Blaberus	*giganticus*	Blattodea	♀	3MeC31	6.3	10
Blaberus	*giganticus*	Blattodea	♂	3MeC31	3.7	7
Blaberus	*discoidalis*	Blattodea	nymph	3MeC29 3MeC31	1.8 2.5	85
Blaberus	*discoidalis*	Blattodea	♀	3MeC29 3MeC31	3.2 4.0	8
Blaberus	*discoidalis*	Blattodea	♂	3MeC29 3MeC31	2.5 3.6	8
Incisitermes	*minor*	Isoptera	worker	3MeC23	2.3	65
Extatosoma	*tiaratum*	Phasmatodea	♀	11MeC23	2.9	14
Thasus	*neocalifornicus*	Heteroptera	♀	3MeC29	3.1	30
Thasus	*neocalifornicus*	Heteroptera	♂	3MeC29	2.5	27
Brothylus	*gemmulatus*	Coleoptera	♀	3MeC25	1.9	20
Tenebrio	*molitor*	Coleoptera	♀	11MeC23	2.6	175
Tenebrio	*molitor*	Coleoptera	♂	11MeC23	1.7	130
Xylotrechus	*colonus*	Coleoptera	♀	3MeC25	1.5	65
Monochamus	*clamator*	Coleoptera	♀	3MeC23	1	22
Monochamus	*titillator*	Coleoptera	♀	9MeC25 9MeC27 3MeC31	1.5 5.1 3.1	52
Monochamus	*titillator*	Coleoptera	♂	9MeC25 9MeC27 3MeC31	1.1 4.5 2.5	38
Calosoma	*protector*	Coleoptera	♀	3MeC25	0.9	65
Zophobas	*morio*	Coleoptera	larvae	3MeC25 3MeC27	1.2 3.0	245
Linepathema	*humile*	Hymenoptera	worker	3MeC25 3MeC27	1.1 0.8	3000
Hemileuca	*eglanterina*	Lepidoptera	♂	5MeC27	1.8	125
Musca	*domestica*	Diptera	♂	3MeC25	0.8	1500
Myrmeleon	*sp.*	Neuroptera	♂	3MeC25	0.7	20

Table 2.1. Methyl-branched hydrocarbons isolated from CHC extracts of 20 species of insects.

Genus	Species	Sex/ life stage	Isolated compound	Conc. (g/100 mL)	Specific Rotation $[\alpha]_D^{25}$	Absolute Stereochemistry
Periplaneta	*americana*	♀	3MeC25	5.48	-3.19 ± 0.01	(*R*)
Periplaneta	*americana*	♂	3MeC25	4.92	-3.17 ± 0.01	(*R*)
Periplaneta	*brunnea*	♂	3MeC23	1.48	-3.25 ± 0.03	(*R*)
Periplaneta	*australasiae*	♂	3MeC23	1.08	-3.21 ± 0.04	(*R*)
Periplaneta	*fuliginosa*	♂	3MeC23	0.76	-3.23 ± 0.03	(*R*)
Blaberus	*giganticus*	♀	3MeC31	2.52	-2.91 ± 0.01	(*R*)
Blaberus	*giganticus*	♂	3MeC31	1.48	-2.95 ± 0.03	(*R*)
Blaberus	*discoidalis*	nymph	3MeC29 3MeC31	0.72 1.0	-2.99 ± 0.03 -2.89 ± 0.07	(*R*) (*R*)
Blaberus	*discoidalis*	♀	3MeC29 3MeC31	1.28 1.60	-2.96 ± 0.03 -2.85 ± 0.01	(*R*) (*R*)
Blaberus	*discoidalis*	♂	3MeC29 3MeC31	1.00 1.44	-2.98 ± 0.07 -2.85 ± 0.03	(*R*) (*R*)
Incisitermes	*minor*	worker	3MeC23	0.92	-3.23 ± 0.01	(*R*)
Extatosoma	*tiaratum*	♀	11MeC23	1.16	-0.11 ± 0.05	(*R*)
Thasus	*neocalifornicus*	♀	3MeC29	1.24	-2.93 ± 0.08	(*R*)
Thasus	*neocalifornicus*	♂	3MeC29	1	-2.89 ± 0.06	(*R*)
Brothylus	*gemmulatus*	♀	3MeC25	0.76	-3.15 ± 0.08	(*R*)
Tenebrio	*molitor*	♀	11MeC23	1.04	-0.10 ± 0.01	(*R*)
Tenebrio	*molitor*	♂	11MeC23	0.68	-0.08 ± 0.03	(*R*)
Xylotrechus	*colonus*	♀	3MeC25	0.6	-3.19 ± 0.10	(*R*)
Monochamus	*clamator*	♀	3MeC23	0.4	-3.11 ± 0.12	(*R*)
Monochamus	*titillator*	♀	9MeC25 9MeC27 3MeC31	0.76 2.04 1.24	-0.19 ± 0.02 -0.27 ± 0.01 -3.01 ± 0.07	(*R*) (*R*) (*R*)
Monochamus	*titillator*	♂	9MeC25 9MeC27 3MeC31	0.60 1.80 1.00	-0.20 ± 0.03 -0.29 ± 0.02 -3.11 ± 0.07	(*R*) (*R*) (*R*)
Calosoma	*protector*	♀	3MeC25	0.36	-3.15 ± 0.09	(*R*)
Zophobas	*morio*	larvae	3MeC25 3MeC27	0.48 1.20	-3.19 ± 0.03 -3.01 ± 0.07	(*R*) (*R*)
Linepathema	*humile*	worker	3MeC25 3MeC27	0.60 0.32	-3.15 ± 0.09 -2.99 ± 0.10	(*R*) (*R*)
Hemileuca	*eglanterina*	♂	5MeC27	0.72	-0.67 ± 0.03	(*R*)
Musca	*domestica*	♂	3MeC25	0.32	-3.09 ± 0.04	(*R*)
Myrmeleon	*sp.*	♂	3MeC25	0.28	-3.13 ± 0.10	(*R*)

Table 2.2. Specific rotations $[\alpha]_D^{25}$ of isolated methyl-branched hydrocarbons. Values of $[\alpha]_D^{25}$ are means ± SD from 10 measurements.

2.3. Discussion

Cuticular hydrocarbons have been shown to mediate a variety of behavioral and social interactions in a number of insect species, and they have been implicated as contact pheromones and kairomones in many more. Cuticular hydrocarbon profiles have also been used in studies examining speciation. However, to date, the lack of methods for the isolation of pure compounds from insect cuticular extracts has resulted in discrepancies in the rigor with which specific compounds could be unequivocally linked to particular functional roles. Thus, many studies have been correlational only, showing statistically significant differences in the amounts and/or types of compounds present, often using principal components analyses to assess multiple compounds simultaneously, without being able to confirm that any one or any group of these compounds actually function as chemical signals. Other studies have used synthesized CHCs to probe the roles of specific compounds in bioassays with considerable success, [31-38] but this approach may be limited by the large number of compounds present in cuticular hydrocarbon extracts. Thus, a single study might require the syntheses of a substantial number of authentic standards in order to test rigorously the possible functional roles of candidate contact pheromone components. The problem is compounded when contact pheromones consist of blends of components,[38-40] each of which may have no or only partial activity when presented individually.[41-43]

As an alternative, we have shown that isolation of pure compounds from the often complex CHC extracts of insects is entirely feasible, using a combination of simple fractionation methods followed by reverse phase HPLC analysis. For cases in which the

pheromone consists of multiple components, subtractive recombination of individual isolated CHCs or small groups of compounds should render the elucidation of the subset of CHCs that are both necessary and sufficient for full biological activity straightforward.[23] Whereas we used extracts prepared from large numbers of individuals in some cases, these numbers were dictated by the sensitivity of the digital polarimeter used to determine the specific rotations of MBCHs from various species, rather than by the amounts required for assays of biological relevance, which would typically be one insect-equivalent per replicate or less. For smaller quantities, the separation methodology is limited only by the sensitivity of the ELS detector. At the other end of the scale, the methodology is amenable to separation of quantities larger than a few milligrams by batchwise operation, or simply by switching to larger diameter columns. It also should be noted that although our focus was on isolation of MBCHs, the methodology is also ideally suited to the isolation of members of other types of CHCs such as long-chain alkenes, as demonstrated by analysis of the alkenes fraction from *Blaberus giganticus* (Fig. S17) and *Cotinis mutabilis* (Fig. S18). In addition, members of other lipid classes, such as triglycerides, are readily separated by reverse phase HPLC with nonaqueous solvent systems.[55] Triglycerides and related compounds have recently been identified as contact pheromones for *Drosophila melanogaster*, and it is very likely that similar nonvolatile lipids will be discovered as contact pheromones in other insect species.[56,57]

The determination of the absolute configurations of pure MBCHs from CHC extracts of insects provided insight into the longstanding question of the stereochemical course of biosynthesis in chiral MBCHs. Our 20 study species were chosen on the basis

of availability and to some extent size, rather than on the basis of taxonomy. Each of the 36 compounds isolated, regardless of chain length or branch-point, was unequivocally shown to have the (*R*)-configuration. This conserved stereochemistry was found for both eusocial and solitary insects of different species, sexes, and life stages, and in both hemimetabolous and holometabolous insects, which are separated by millions of years of evolution. The fact that all these compounds all have the same configuration, despite being isolated from 20 essentially randomly chosen insect species in 9 orders, provides strong evidence that the biosynthetic pathway for these molecules is highly conserved throughout the Insecta.

In most insects, MBCHs are biosynthesized in special cells called oenocytes found on the inner surface of the abdominal cuticle in a process analogous to vertebrate fatty acid biosynthesis.[44] The hydrocarbon chains are formed by microsomal 3-ketoacyl-ACP synthase-catalyzed Claisen condensations of malonyl-CoA substrates, with the methyl branch-points being introduced by the insertion of a methyl malonyl-CoA moiety into the hydrocarbon chain by the same condensation mechanism.[45] The stereochemistry of the methyl branch-point is likely controlled by a stereoselective NADPH-catalyzed reduction of the resulting α,β-unsaturated branched thioester by the enoyl-ACP reductase domain of FAS (Fig. 2.4).[46] Subsequent elongation steps followed by a cytochrome-P450 mediated decarbonylation result in the synthesis of the internally branched chiral MBCHs.[2,47] Although the enoyl-ACP reductase domain has yet to be isolated from insects and there have been only a few cases of microsomal FAS isolation,[45,48] FAS isolated from various fungal species show highly conserved NADPH binding sites and

genetic homology within the enoyl reductase domain,[49] implying that this domain may also be highly conserved throughout the class Insecta. Furthermore, the conservation of the microsomal enoyl reductase FAS domain in insects would explain the lack of stereochemical diversity in our isolated MBCH compounds and corroborate the source of enantiospecificity in the biosynthesis of MBCHs. It must be noted though, that if the stereochemistry of the methyl branch point is controlled by the enoyl-ACP reductase reduction, compounds whose branch points are inserted early in the biosynthetic process would have the same spatial orientation as those with branch points inserted after the center of the hydrocarbon chain but would be assigned opposite stereochemical configurations due to nomenclature rules ((*R*) vs. (*S*)). Further insight into the stereospecificity of insect methyl-branched hydrocarbon biosynthesis would require the isolation and expression of microsomal FAS and its enoyl-ACP reductase domain from insects, and functionalize these enzymes with ^{14}C or ^{2}H labeled substrates to confirm the stereochemical specificity of the enoyl-reduction.

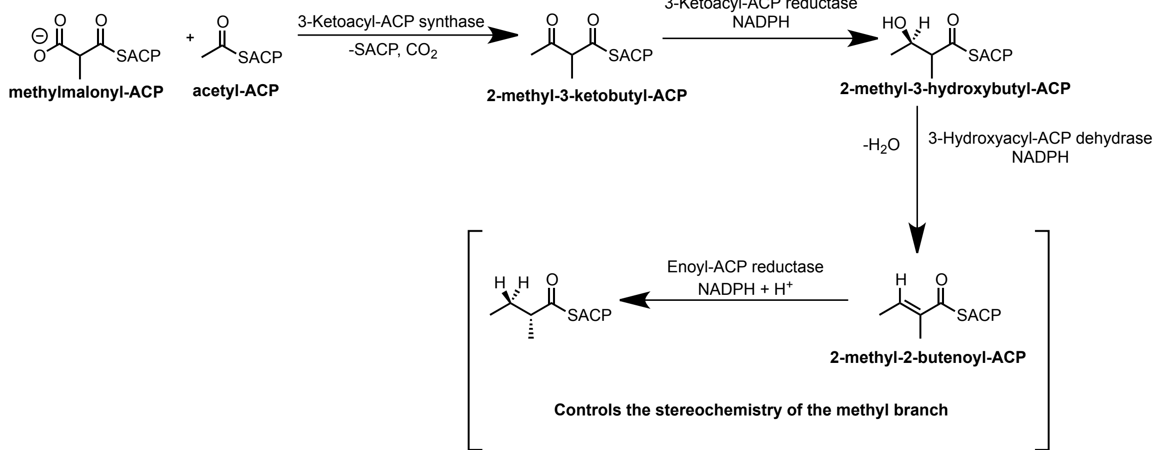

Figure 2.4. Overview of methyl-branched hydrocarbon biosynthesis by microsomal fatty acid synthase. Absolute stereochemistry of methyl-branched hydrocarbons is hypothesized to be controlled by the NADPH-catalyzed enoyl-reductase reduction of the α,β-unsaturated branched thioester.

It was pointed out more than two decades ago, and reiterated much more recently, that the possible importance of stereochemistry in MBCHs used as contact pheromones was essentially unknown.[2] To date, there have been only a handful of studies that have attempted to probe the effects of chirality in the context of the behavioral responses elicited by branched hydrocarbon semiochemicals. For example, the parasitic wasp *Ooencytrus kuvanae* utilizes a blend of 5-methylheptacosane and 5,17-dimethylheptacosane as a contact sex pheromone for mate recognition.[50] Superficially, both sexes seem to produce the same two compounds that apparently constitute the contact pheromone blend. However, bioassays with enantiopure standards of the synthetic MBCHs suggested that the pheromone consisted of (*S*)-5-methylheptacosane and (5*R*, 17*S*)-dimethylheptacosane, while the combination of (*R*)-5-methylheptacosane and (5*R*, 17*S*)-dimethylheptacosane inhibited attraction. The other possible stereoisomers

of the contact pheromone components were inactive in bioassays. Because the (*S*)-configuration is opposite to that of the 36 insect-derived MBCHs described above, these results suggest that female *O. kuvanae* might biosynthesize the (*S*)-enantiomer of 5-methylheptacosane to differentiate it from the typical (*R*)-MBCHs produced by most other insects, including the male of this species. It has not yet been possible to corroborate these findings by unequivocal determination of the absolute configurations of the compounds produced by the males and females respectively.

In similar fashion, the results of bioassays with synthetic standards have suggested that females of the cerambycid beetles *Tetropium fuscum* and *Tetropium cinnamopterum* utilize a mixture of (*S*)-11-methylheptacosane and (*Z)*-9-heptacosene as their contact sex pheromones.[51] However, the specific rotation reported for the synthetic (*S*)-11-methylheptacosane utilized in the study, $[\alpha]_D$= -0.06 (c=3.33, hexanes), actually matches the specific rotation of the opposite enantiomer (*R*)-11-methylheptacosane, suggesting that the stereochemical identification of these compounds may be incorrect. As with the previous study, the insect-produced 11-methylheptacosane has not yet been isolated to determine its absolute configuration by analytical methods.

Although our generic methods were successful in isolating a number of pure chiral MBCHs from a variety of insect species, there are limitations. The isolation of pure compounds from simple to moderately complex cuticular extracts is possible, but as the cuticular profiles become more complex (>100 compounds), the analyses become more difficult. To some extent, these issues can be addressed by optimization of the solvent gradient in combination with using high-resolution RP-HPLC columns with <2µm

stationary phase particle sizes (so called Ultra-HPLC). The methodology could also be improved if HPLC were used in combination with a polarimeter adapted for use as an HPLC detector by incorporation of a small volume flow cell instead of the static measuring cell. This would allow the determination of optical rotations "on the fly", in real time, as part of the HPLC analysis, and would eliminate the need for isolation of individual pure compounds for subsequent measurement of their specific rotations. Our original plans called for using such a detector, known as an advanced laser polarimeter HPLC detector (PDR Chemical, Lakeland, FL), which is claimed to be able to measure specific rotations of chiral compounds at very low concentrations (≥ 5 µg/mL).[52] However, our attempts to work with the single company in the world that produces these detectors have not yet been successful, due to problems getting run time on the instrument. The use of this detector in future studies would have the potential to simplify greatly further analyses of MCBHs by allowing determination of the sign of rotation, and thus the absolute configuration, of most if not all methyl-branched hydrocarbons in an extract in one pass.

2.4. Methods

2.4.1. Insects and CHC Extraction.

Periplaneta americana, *Periplaneta australasiae, Periplaneta. brunnea, Periplaneta fuliginosa, Blaberus giganticus, Blaberus discoidalis* adults, *Blaberus discoidalis* nymphs, *Extatosoma tiaratum, Tenebrio molitor, Incistermes minor* workers, *Zophobas molto* larvae, and *Musca domestica* were obtained from laboratory populations

maintained in various research groups within the Department of Entomology at the University of California, Riverside. *Linepathema humile* workers were obtained from colonies collected from a citrus grove on the University of California, Riverside campus. *Brothylus gemmulatus* and *Monochamus clamator* were collected during the late spring of 2013 from cerambycid beetle monitoring traps deployed in the San Bernardino National Forest, CA. *Xylotrechus colonus* were collected during the summer of 2013 from cerambycid monitoring traps near Urbana, IL. *Hemileuca eglanterina* were collected in springs of 1999-2003 from pheromone baited traps in the San Bernardino National Forest, CA, and had been frozen at -20ºC until extracted. *Thasus neocalifornicus*, *Calosoma protector,* and an unidentified *Mymeleon* sp.* were collected in the late summer of 2013 from Sedona, AZ. *Monochamus titillator* was collected in the summer of 2010 using pheromone baited traps in Kisatchie National Forest, Catahoula Ranger District, LA. Each insect species was separated by sex prior to extraction. The cuticular lipids were extracted by soaking composite samples of specimens in *n*-hexane (50 mL) for 5 min, followed by a second hexane rinse (50 mL) for 3 min. The two extracts were combined for further fractionation. The number of specimens extracted for each species is listed in Table 2.1.

2.4.2. Fractionation and Analysis of Cuticular Extracts.

The hexane extracts were concentrated under reduced pressure, reconstituted in ~500-1000 µL of hexanes, and loaded onto small liquid chromatography columns prepared from Pasteur pipettes filled with 300-500 mg of silica gel impregnated with 10% wt/wt silver nitrate (+230 mesh; Aldrich Chemical Co.). Each column was eluted

successively with four rinses of hexanes (1 ml each) to isolate the saturated alkanes, then with four rinses of 1:19 cyclohexene/hexanes (1 ml each) to isolate alkenes, and then 2 rinses of 100% diethyl ether (1 ml each) to recover more polar compounds from the extract. The components of each fraction were identified by coupled gas chromatography-mass spectrometry (GC-MS), using an HP6890 gas chromatograph (Hewlett-Packard, now Agilent, Santa Clara CA) equipped with a DB-17MS capillary column (25 m × 0.20 mm, 0.33 film thickness; J&W Scientific, Inc., Folsom CA) coupled to an HP5973 mass selective detector run in full scan mode, with electron impact ionization (70 eV). Compounds were identified by their retention indices in relation to straight-chain alkane standards and interpretation of their mass spectra, using a combination of their molecular ions and the enhanced ions from cleavage on either side of branch points. [58,59]

2.4.3. Separation of Methyl-Branched from Straight-chain Alkanes.

The hexane rinses from the silica-silver nitrate chromatography were pooled in a tared 20-dram vial, concentrated under reduced pressure, and weighed to determine the mass of the saturated alkanes. A 1 cm stir bar was added to each vial, followed by 5 mL of dry isooctane and 100 mg of activated 5Å molecular sieves (Aldrich Chemical Co.) per mg of sample. The vials were flushed with argon, capped with a Teflon™ lined cap, and stirred overnight. The resulting slurry was then transferred to a 20 mL glass centrifuge tube and the molecular sieves were pelleted via centrifugation with a safety-head centrifuge (Clay Adams) set to speed setting 4 for 10 min. The supernatant containing the isolated MBCHs was removed, filtered through a glass wool plug, and

placed in a tared 20-dram vial. The pellet was resuspended in 10 mL of fresh isooctane, the slurry was centrifuged, and the supernatant was filtered through a glass wool plug and combined with the original supernatant. The compounds in the combined solution were identified by GC-MS as described above.

2.4.4. RP-HPLC Isolation of Pure MBCHs.

After removal of *n*-hydrocarbons from the alkanes fraction by adsorption of the former into 5Å molecular sieves, the fraction containing only MBCHs in isooctane was concentrated *in vacuo,* reconstituted in EtOAc (500 μL), and transferred to 1 mL conical auto-sampler vial. The components of each fraction were then separated on an Infinity 1220 HPLC (Agilent Technologies) coupled to a 380-ELSD (Agilent Technologies). The HPLC was equipped with an Eclipse XDB-C18 reverse phase column (5 μm particle size, 4.6 mm i.d. x 250 mm; Agilent Technologies) and a 100 μL sample loop (25 μL injection volume was used.) The column oven was set to 50 ºC and the ELSD was set to nebulize at 40 ºC, evaporate at 70 ºC, and the gas flow rate was set to 1.20 SLM. Each separated component was collected using an 80:20 fraction splitter between the HPLC column and ELSD. The collected fractions were analyzed by GC-MS to determine purity, then pooled and concentrated *in vacuo* prior to stereochemical analysis by polarimetry. The methods utilized for compound isolation are shown in Table 2.3.

Insect Species	Sex	Solvent System	Flow Rate	Oven Temp (ºC)	Injection Volume (µL)	# of Injections	Isolated Compound	Retention Time (min)	Figure
Periplaneta americana	m	1:1 EtOAc/Methanol	1 mL/min (Isocratic)	60 º	25 µL	20	3-methylpentacosane	6.7-6.9	2.1
Periplaneta americana	f	1:1 EtOAc/Methanol	1 mL/min (Isocratic)	60 º	25 µL	20	3-methylpentacosane	6.7-6.9	2.1
Periplaneta brunnea	m	2:3 EtOAc/Methanol	1 mL/min (Isocratic)	60 º	25 µL	20	3-methyltricosane	5.8-6.0	S2.6
Periplaneta fuliginosa	m	2:3 EtOAc/Methanol	1 mL/min (Isocratic)	60 º	25 µL	20	3-methyltricosane	5.8-6.0	data not shown
Periplaneta austalasiae	m	2:3 EtOAc/Methanol	1 mL/min (Isocratic)	60 º	25 µL	20	3-methyltricosane	5.8-6.0	data not shown
Blaberus giganticus	m	2:3 EtOAc/Methanol	1 mL/min (Isocratic)	60 º	25 µL	20	3-methylhentriacontane	13.5-13.8	2.2
Blaberus giganticus	f	2:3 EtOAc/Methanol	1 mL/min (Isocratic)	60 º	25 µL	20	3-methylhentriacontane	13.5-13.8	2.2
Blaberus discoidalis	m	2:3 EtOAc/Methanol	1 mL/min (Isocratic)	60 º	25 µL	20	3-methylnonacosane 3-methylhentriacontane	11.7-11.9 13.4-13.7	S2.7
Blaberus discoidalis	f	2:3 EtOAc/Methanol	1 mL/min (Isocratic)	60 º	25 µL	20	3-methylnonacosane 3-methylhentriacontane	11.7-11.9 13.4-13.7	S2.7
Blaberus discoidalis	nymph	2:3 EtOAc/Methanol	1 mL/min (Isocratic)	60 º	25 µL	20	3-methylnonacosane 3-methylhentriacontane	11.5-11.8 13.5-13.8	data not shown
Incistermes minor	worker	2:3 EtOAc/Methanol	1 mL/min (Isocratic)	60 º	25 µL	20	3-methyltricosane	5.6-5.8	S2.8
Extatosoma tiaratum	f	1:1 EtOAc/Methanol	1 mL/min (Isocratic)	60 º	25 µL	20	11-methyltricosane	4.8-5.1	data not shown
Thasus neocalifornicus	m	2:3 EtOAc/Methanol	1 mL/min (Isocratic)	60 º	25 µL	20	3-methylnonacosane	11.6-11.9	S2.9
Thasus neocalifornicus	f	2:3 EtOAc/Methanol	1 mL/min (Isocratic)	60 º	25 µL	20	3-methylnonacosane	11.6-11.9	S2.9
Brothylus gemmulatus	f	1:5 EtOAC/Methanol ➔2:3 EtOAC/Methanol	1.4 mL/min (10 min) ➔1.0 mL/min (20 min)	60 º	25 µL	20	3-methylpentacosane	12.9-13.1	S2.10
Tenebrio molitor	m	1:1 EtOAc/Methanol	1 mL/min (Isocratic)	60 º	25 µL	20	11-methyltricosane	4.7-5.0	S2.11
Tenebrio molitor	f	1:1 EtOAc/Methanol	1 mL/min (Isocratic)	60 º	25 µL	20	11-methyltricosane	4.7-5.0	S2.11
Xylotrechus colonus	f	1:5 EtOAC/Methanol ➔2:3 EtOAC/Methanol	1.4 mL/min (10 min) ➔1.0 mL/min (20 min)	60 º	25 µL	20	3-methyltricosane	10.6-10.9	data not shown
Monochamus titillator	m	1:5 EtOAC/Methanol ➔2:3 EtOAC/Methanol	1.4 mL/min (10 min) ➔1.0 mL/min (20 min)	60 º	25 µL	20	9-methylpentacosane 9-methylheptacosane 3-methylhentriacontane	12.5-12.9 14.5-14.8 20.3.-20.6	2.3
Monochamus titillator	f	1:5 EtOAC/Methanol 2:3 EtOAC/Methanol	1.4 mL/min (10 min) ➔1.0 mL/min (20 min)	60 º	25 µL	20	9-methylpentacosane 9-methylheptacosane 3-methylhentriacontane	12.5-12.7 14.7-14.8 20.3.-20.6	2.3
Calosoma protector	f	2:3 EtOAc/Methanol	1 mL/min (Isocratic)	60 º	25 µL	20	3-methylpentacosane	7.6-7.9	data not shown
Zophobas morio	larvae	1:5 EtOAC/Methanol ➔2:3 EtOAC/Methanol	1 mL/min (Isocratic)	60 º	25 µL	20	3-methylpentacosane 3-methylheptacosane	12.8-13.1 14.9-15.2	S2.12
Linepathema humile	worker	2:3 EtOAc/Methanol	2:3 EtOAc/Methanol	60 º	25 µL	20	3-methylpentacosane 3-methylheptacosane	7.5-7.8 9.4-9.7	S2.13
Hemileuca eglanterina	m	1:1 EtOAc/Methanol	1 mL/min (Isocratic)	60 º	25 µL	20	5-methylheptacosane	8.3-8.6	S2.14
Musca domestica	m/f	1:5 EtOAC/Methanol ➔2:3 EtOAC/Methanol	1.4 mL/min (10 min) ➔1.0 mL/min (20 min)	60 º	25 µL	20	3-methylpentacosane	12.6-13.0	S2.15
Myrmeleon sp.	m	2:3 EtOAc/Methanol	2:3 EtOAc/Methanol	60 º	25 µL	20	3-methylpentacosane	7.5-7.8	data not shown

Table 2.3. RP-HPLC methods utilized in the isolation of pure compounds from the CHC extracts of insects. The method utilized for each insect species is specified, showing solvent composition, flow rate, column oven temp, injection volume, and retention time of isolated compound.

2.4.5. Synthesis of Chiral Standards for Polarimetric Comparisons.

Stereochemically pure methyl-branched hydrocarbon standards were synthesized as reported by Bello et al.[54] and Kuhbander et al.[55] (listed in table 3.1 in chapter 3).

2.4.6. Polarimetric Analysis of Isolated Methyl-branched Hydrocarbons.

Specific rotations of the isolated MBCH compounds were obtained with an Autopol IV Digital Polarimeter (Rudolph Research Analytical, Hackettstown NJ) operated in high accuracy specific rotation mode at 25 ºC. The polarimeter light source was set to a wavelength of 589 nm. Each isolated MBCH was dissolved in 250 µL of chloroform and transferred to a T32 micro sample cell (0.25 mm i.d. × 50 mm length × 250 µL volume; Rudolph Research Analytical). Sample concentrations (g/100 mL) were entered into the Autopol IV sampling program prior to analysis, and the specific rotation values were obtained 10 times and averaged. The sample was removed from the sample cell by syringe and the cell was rinsed 5 times with chloroform and dried with compressed air before being used for another sample.

2.4.7. Gas Chromatography-Mass Spectrometry (GCMS) of CHC Extracts

Mass spectra of the CHC extracts, isolated fractions, and purified MBCH compounds were obtained with a Hewlett-Packard (HP) 6890 GC (Avondale, PA) interfaced to an HP 5973 mass selective detector, in EI mode (70 eV) with helium as carrier gas. The GC was equipped with a DB17-MS column (25 m × 0.20 mm i. d., 0.33 µm film) and operated on splitless injection mode. The oven was programmed from 100-

280 ºC at either 5 ºC/min or 10 ºC/min after an initial time delay of 1 minute. The GC oven was held at 280 ºC for 20 mins, and the injector temperature was set to 280 ºC.

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2.7 Appendix

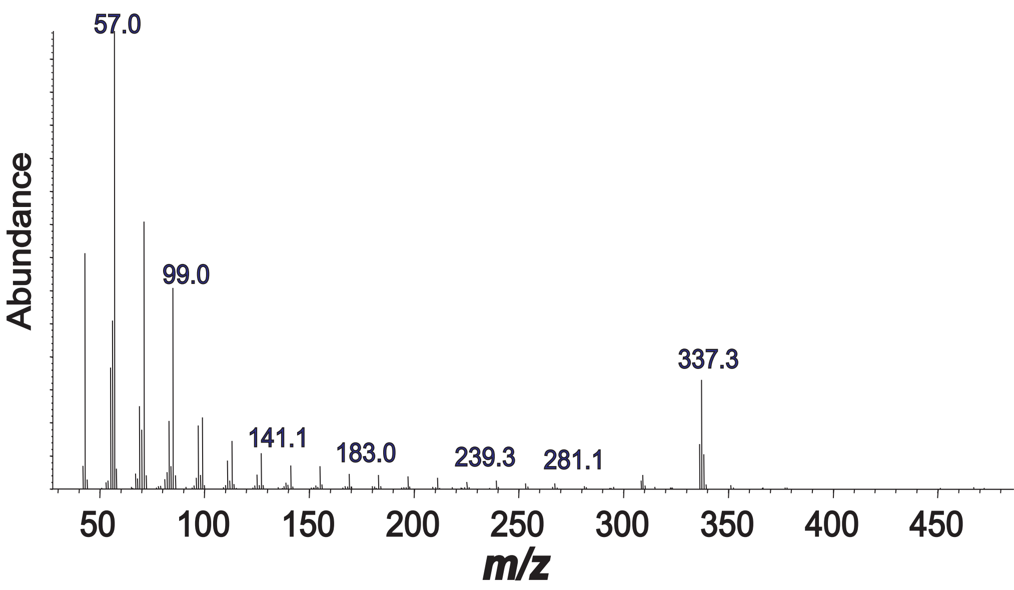


Figure S2.1: Mass spectrum of 3-methylpentacosane isolated from female *P. americana*

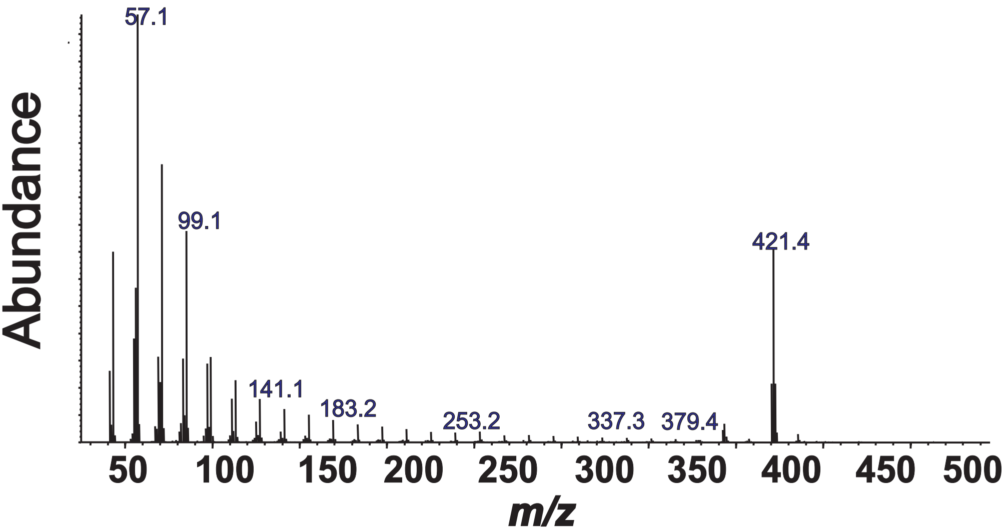


Figure S2.2: Mass spectrum of 3-methylhentriacontane isolated from female *B. giganticus*.

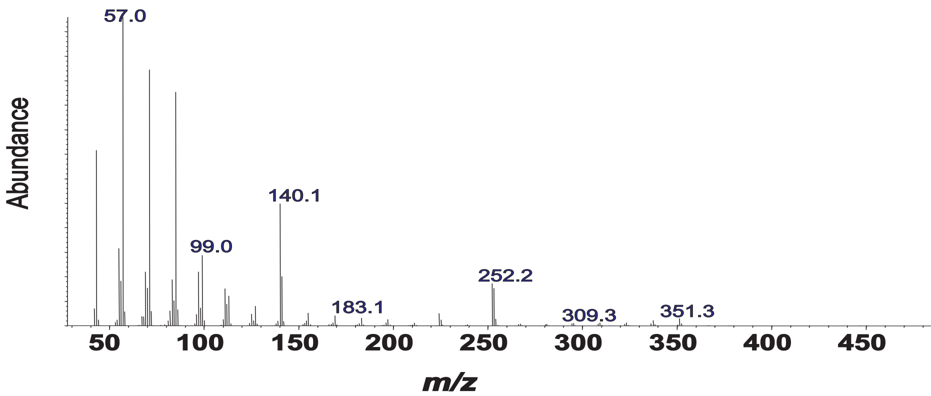


Figure S2.3: Mass spectrum of 9-methylpentacosane isolated from male *M.titillator*.

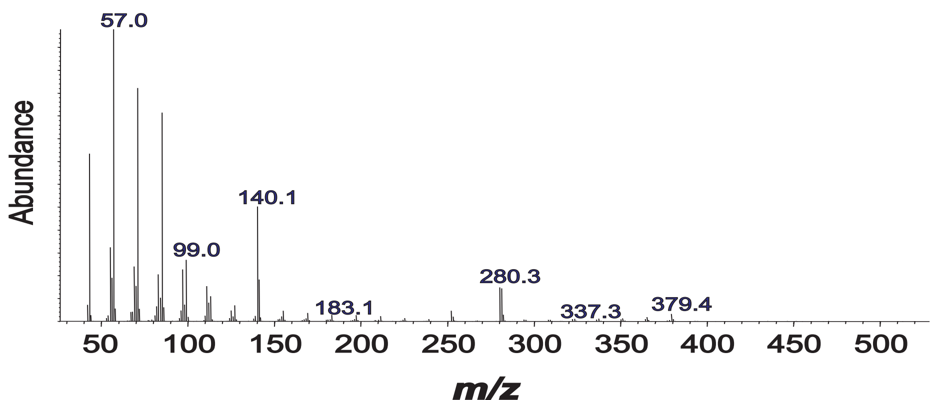


Figure S2.4: Mass spectrum of 9-methylheptacosane isolated from male *M. titillator*.

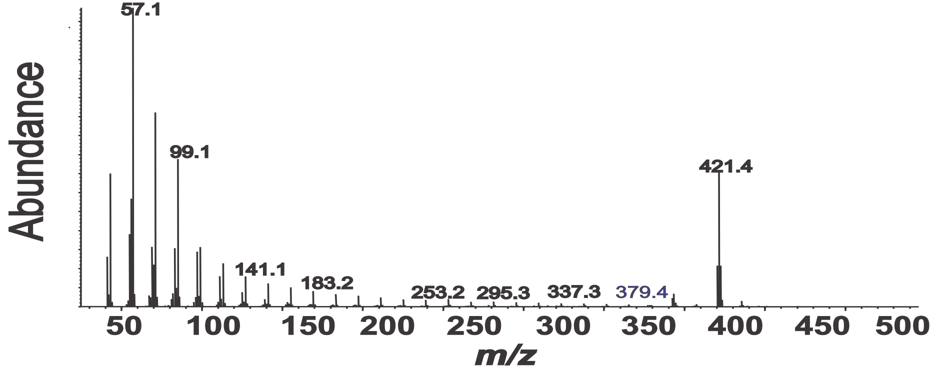


Figure S2.5: Mass spectrum of 3-methylhentriacontane isolated from male *M. titillator.*

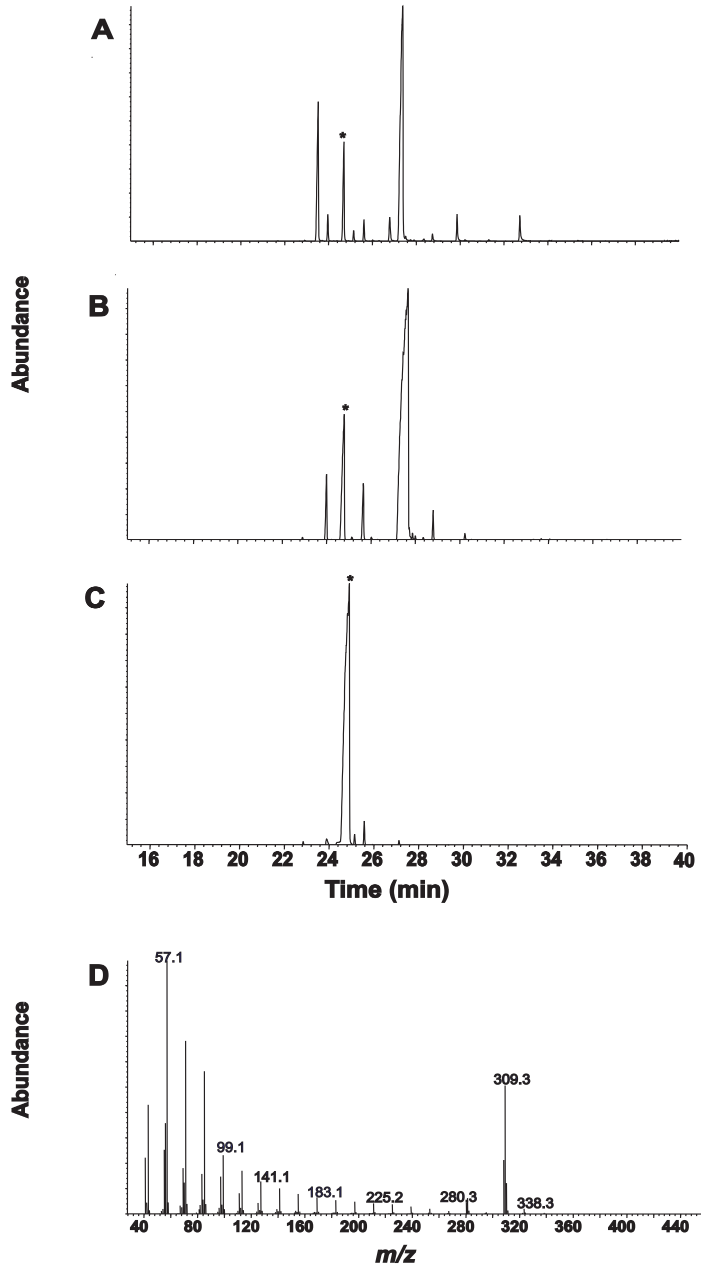


Figure S.2.6: (A) Total ion (TIC) chromatogram of alkanes fraction of male *P. brunnea* CHC extract, (B) TIC-Chromatogram of MBCH fraction of *P. brunnea* CHC extract. (C) TIC-chromatogram of 3-MeC23 isolated from male *P. brunnea* in high purity. (D) Mass spectrum of 3-MeC23 isolated from male *P. brunnea.* 3-methyltricosane is marked with a (*) in all of the shown chromatograms.

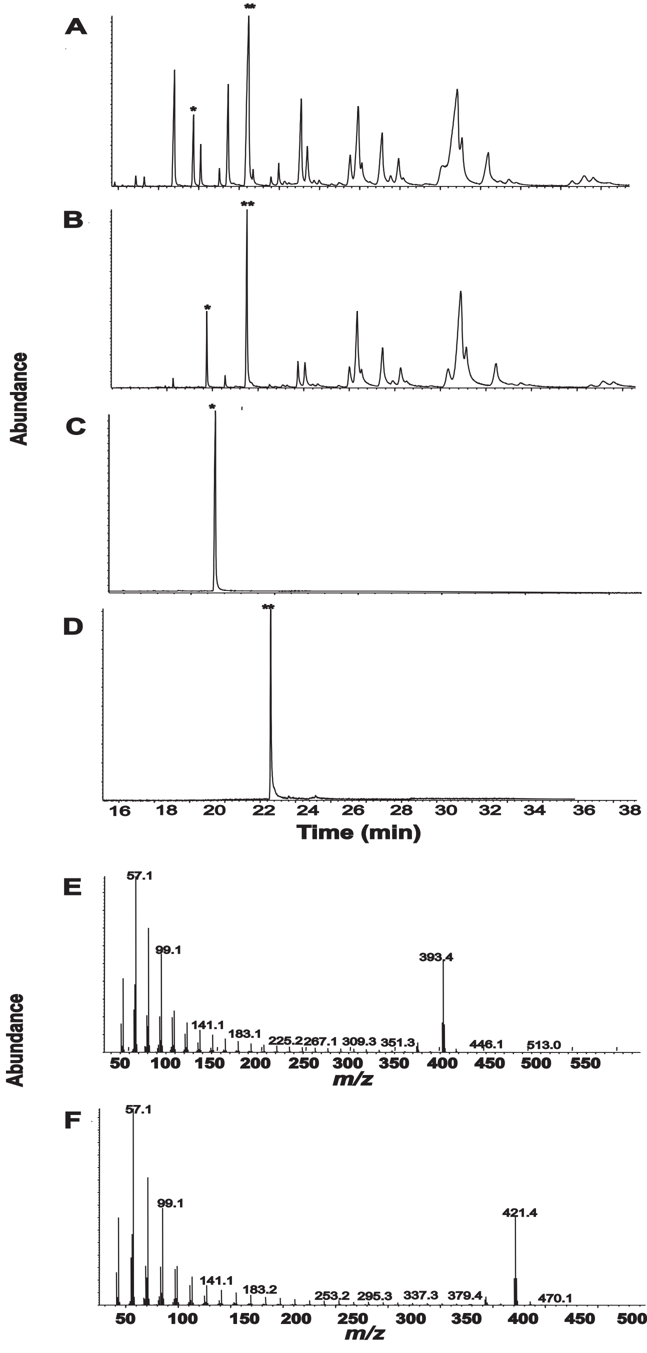


Figure S2.7: (A) TIC-chromatogram of alkanes fraction of female *B. discoidalis* CHC extract. (B) TIC-chromatogram of MBCH only fraction of *B. discoidalis* CHC extract. (C) TIC-chromatogram of 3-methylnonacosane (*) from *B. discoidalis* adults. (D) TIC-chromatogram of 3-methylhentriacontane (**) isolated from *B. discoidalis* in high purity. (E & F) Mass spectra of the isolated 3-methylnonacosane and 3-methylhentriacontane respectively.

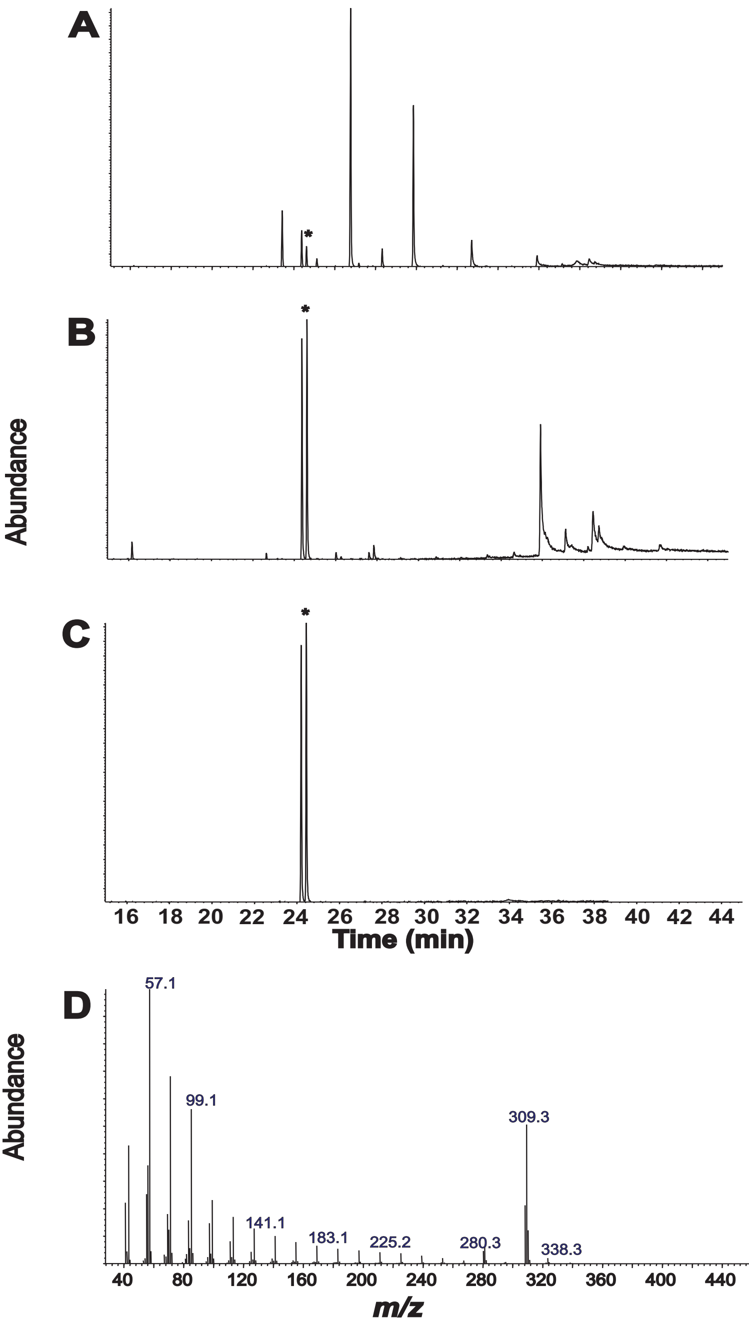


Figure S2.8: (A) TIC-chromatogram of alkanes fraction of CHC extract from female *I. minor* workers, (B) TIC-chromatogram of MBCH fraction of *I. minor* CHC extract. (C) TIC-chromatogram of 2-methyltricosane (achiral has no optical rotation) and 3-methyltricosane (*) from *I. minor* in a 45:55 ratio. (D) Mass spectrum of the isolated 3-methyltricosane from *I. minor*.

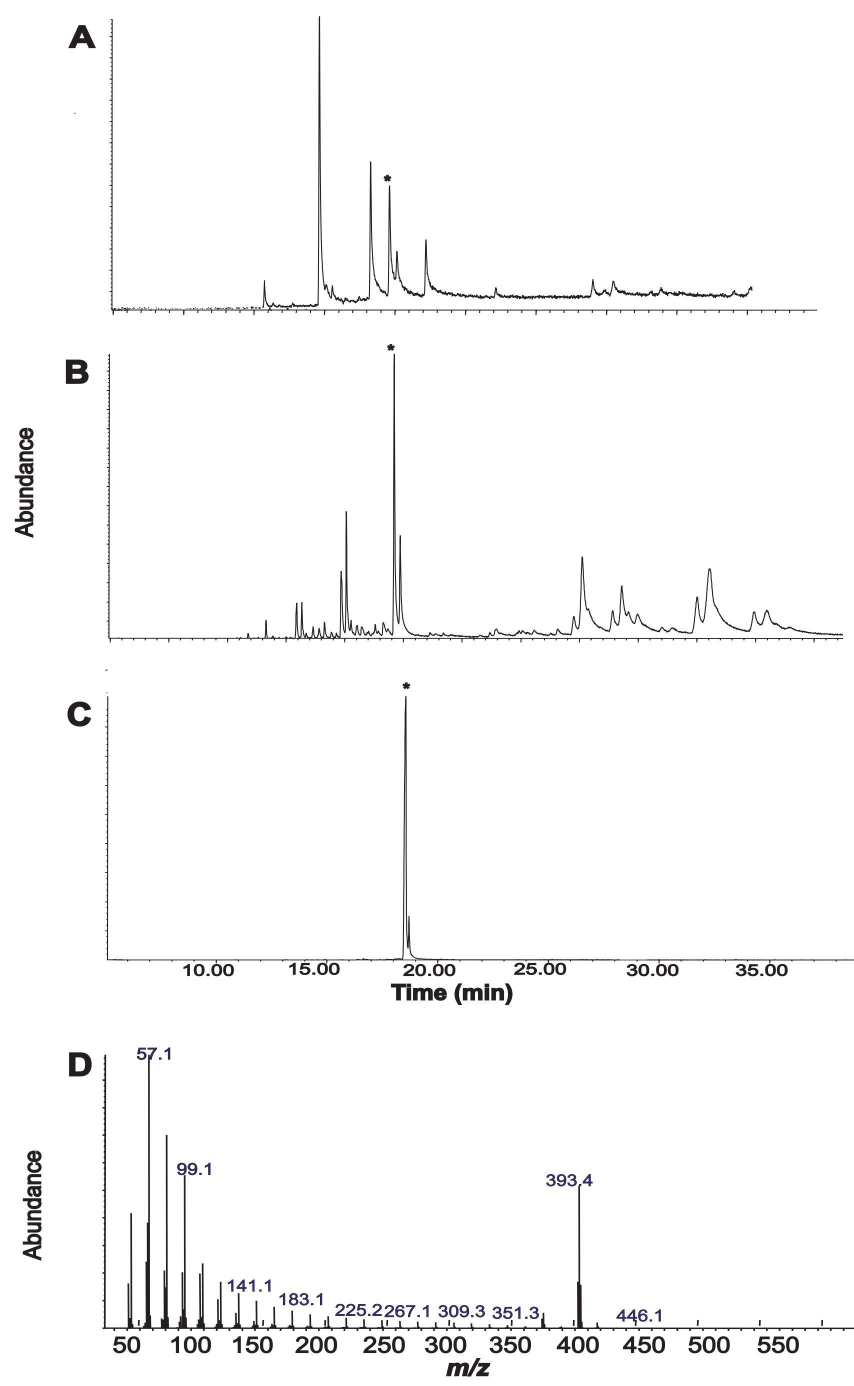


Figure S2.9: (A) TIC-chromatogram of alkanes fraction of the male *T. neocalifornicus* CHC extract, (B) TIC-chromatogram of MBCH fraction *T. neocalifornicus* CHC extract, (C) TIC-chromatogram of 3-methylnonacosane (*) with 2,7-dimethylnonacosane as a minor impurity. (D) Mass spectrum of the isolated 3-methylnonacosane from *T. neocalifornicus.*

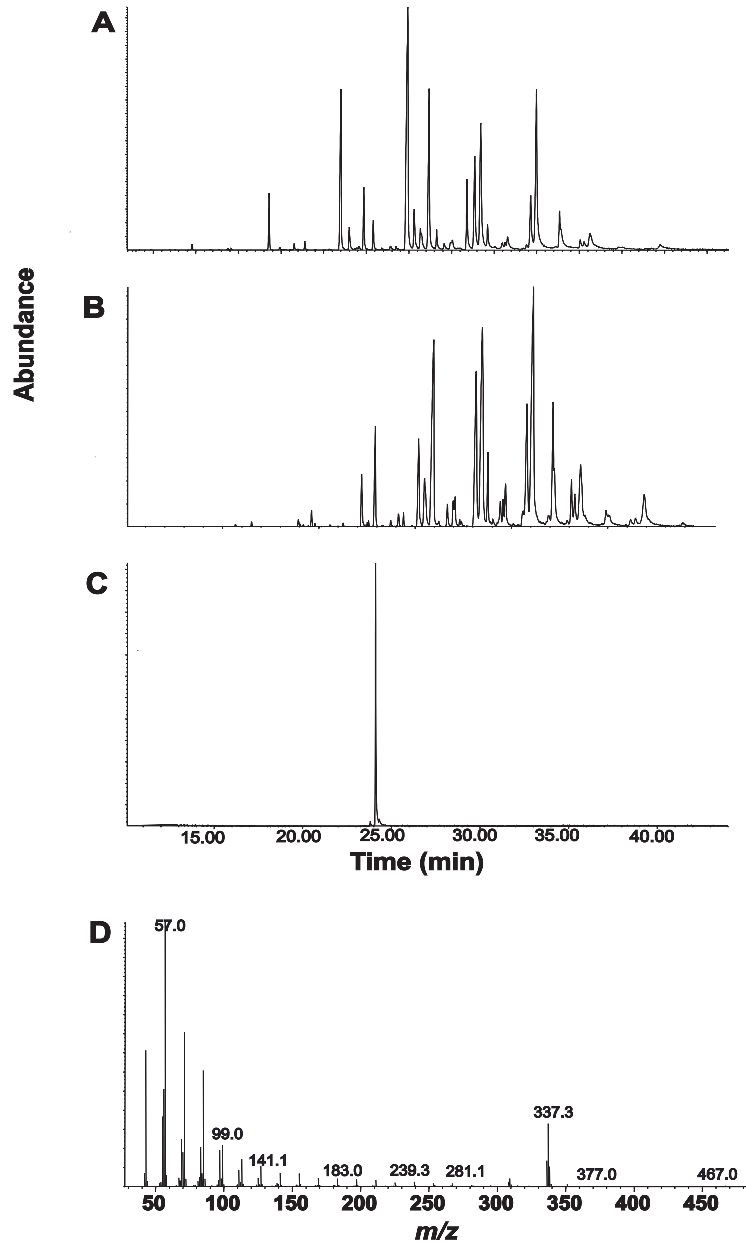


Figure S2.10: (A) TIC-chromatogram of alkanes fraction of female *B. gemmulatus* CHC extract, (B) TIC-chromatogram of MBCH fraction of *B. gemmulatus* CHC extract, (C) TIC-chromatogram of the isolated 3-methylpentacosane (*) from *B. gemmulatus*. (D) Mass spectrum of the isolated 3-methylpentacosane from *B. gemmulatus*

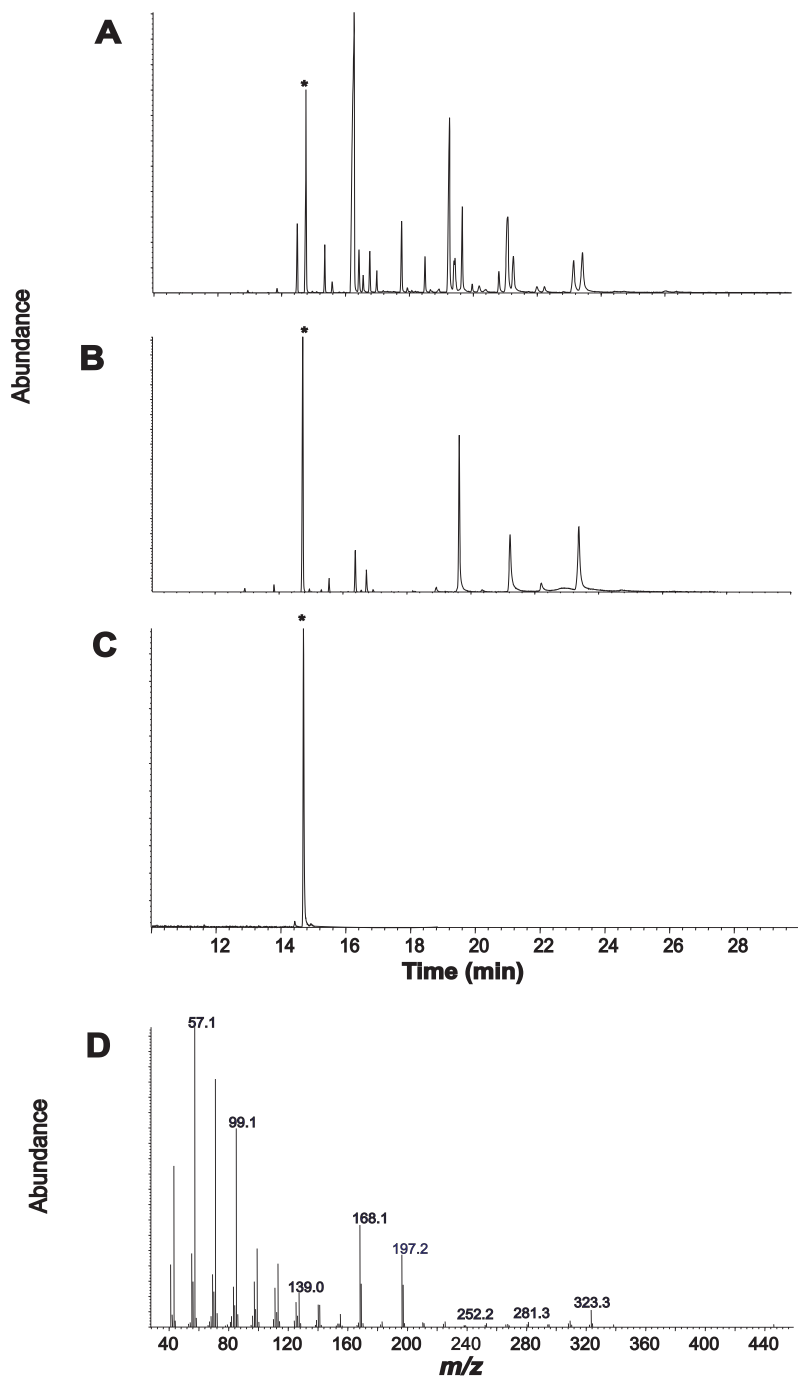


Figure S2.11: (A) TIC-chromatogram of the alkanes fraction of the female *T. molitor* CHC extract, (B) TIC-chromatogram of the MBCH fraction of the *T. molitor* CHC extract. (C) TIC-chromatogram of pure 11-methyltricosane (*) isolated from the *T. molitor* CHC extract. (D) Mass spectrum of the isolated 11-methyltricosane

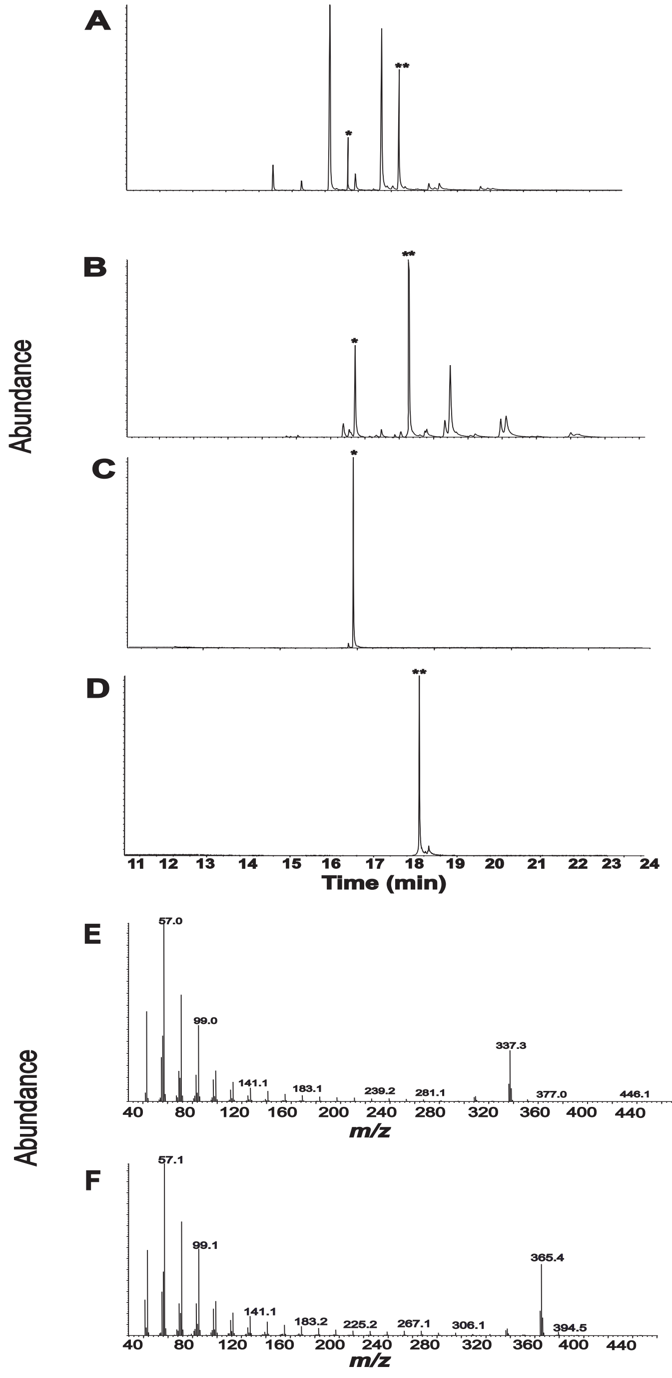


Figure S2.12: (A) TIC-chromatogram of alkanes fraction of *Z. morio* larvae CHC extract, (B) TIC-chromatogram of MBCH fraction of *Z. morio* CHC extract. (C) TIC-chromatogram of isolated pure 3-methylpentacosane(*) from *Z. morio* larvae, (D) TIC-chromatogram of isolated 3-methylheptacosane (**) from *Z. morio* larvae. (E and F) Mass spectra of the isolated 3-methylpentacosane (*) and 3-methylheptacosane (**) from *Z. morio* larvae respectively.

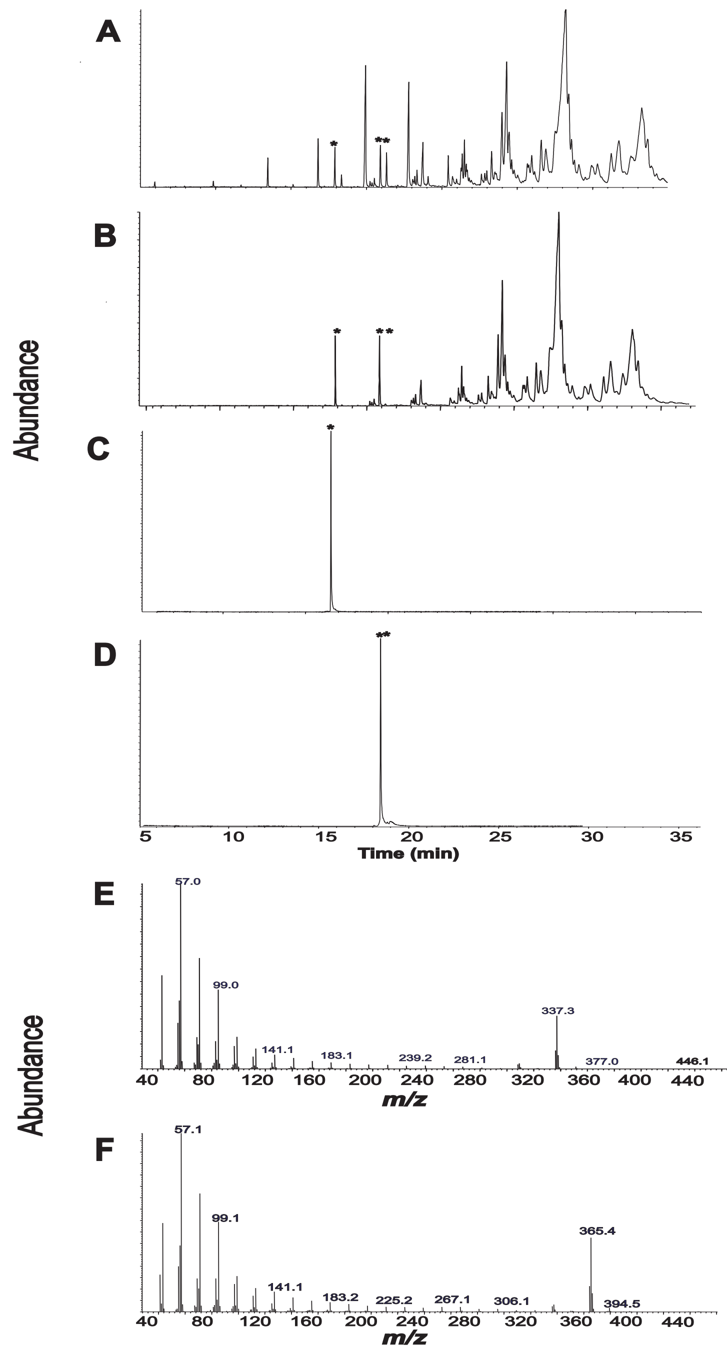


Figure S2.13: (A) TIC-chromatogram of the alkanes fraction of the *L. humile* worker CHC extract, (B) TIC-chromatogram of the MBCH fraction of the *L. humile* worker CHC extract, (C) TIC-chromatogram of the isolated pure 3-methylpentacosane (*) from *L. humile* workers, (D) TIC-chromatogram of the isolated 3-methylheptacosane (**) from *L. humile* workers. (E and F) Mass spectra of the isolated 3-methylpentacosane and 3-methylheptacosane from *L. humile* workers respectively.

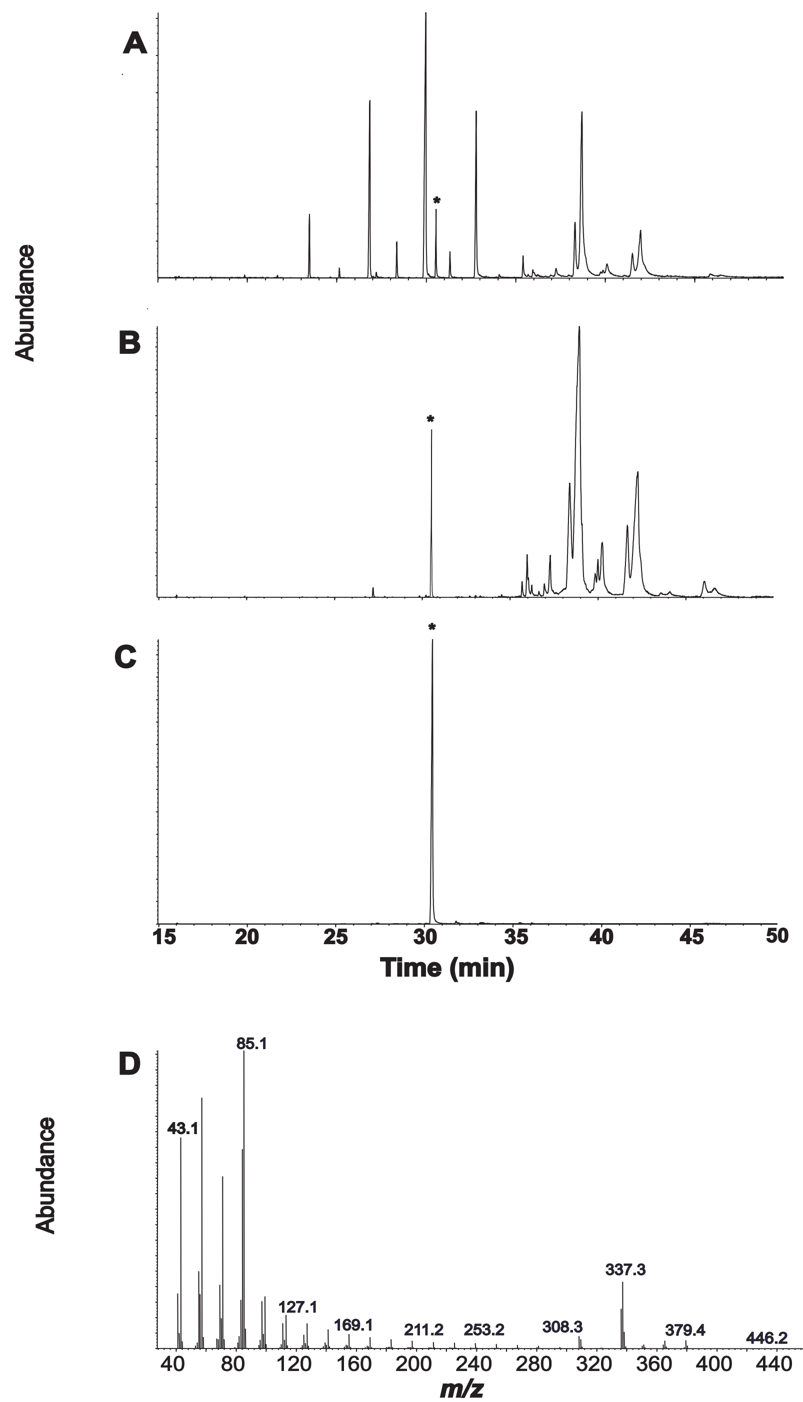


Figure S2.14: (A) TIC-chromatogram of alkanes fraction of the male *H. eglanterina* CHC extract, (B) TIC-chromatogram of the MBCH fraction of male *H. eglanterina* CHC extract. (C) TIC-chromatogram of pure 5-methylheptacosane (*) isolated from male *H.eglanterina*. (D) Mass spectrum of the isolated 5-methylheptacosane (*).

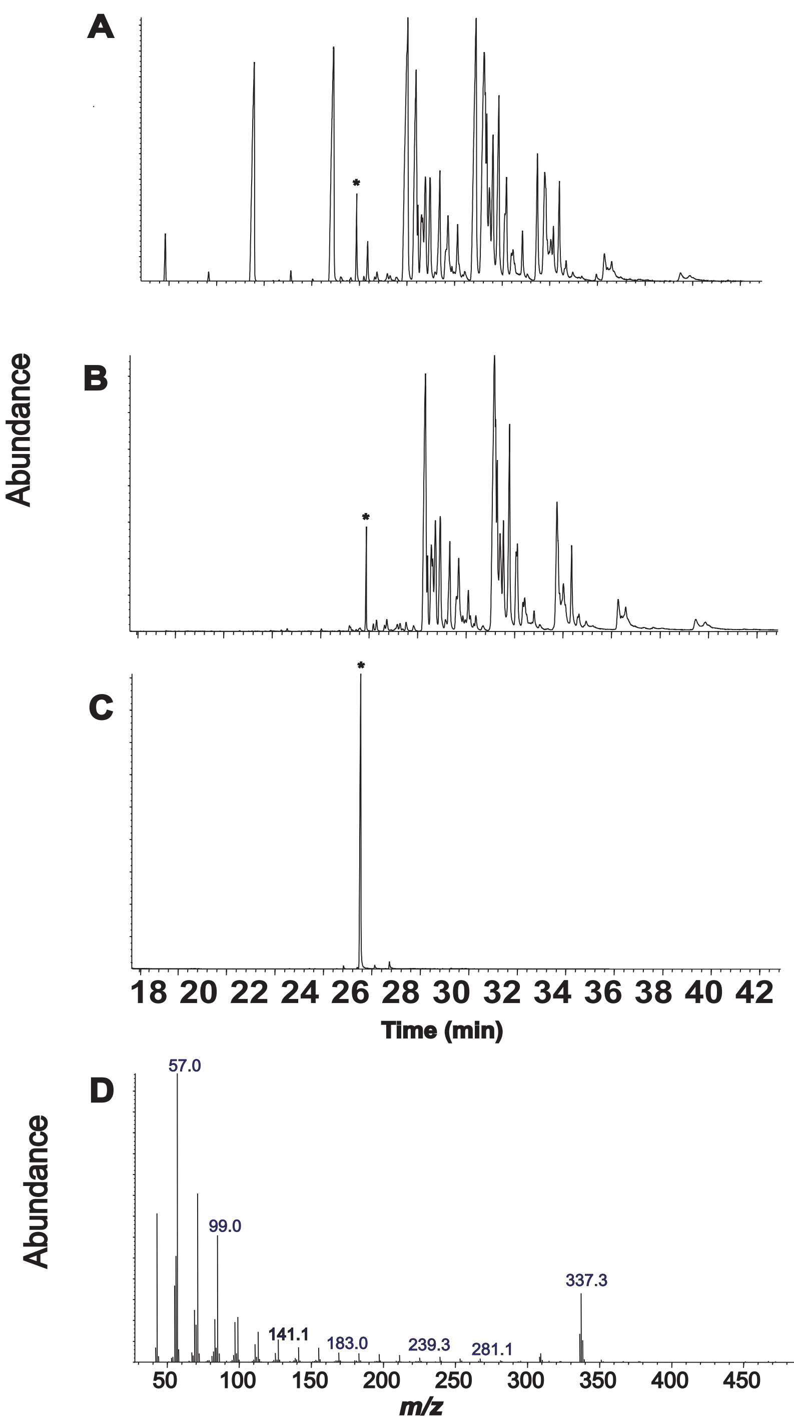


Figure S2.15: (A) TIC-chromatogram of alkanes fraction of *M. domestica* CHC extract. (B) TIC-chromatogram of MBCH fraction of *M. domestica* CHC extract. (C) TIC-chromatogram of Isolated 3-methylpentacosane (*) from *M. domestica*. (D) Mass spectrum of isolated 3-methylpentacosane from *M. domestica*

Chapter 3. Enantioselective Synthesis of Insect Methyl-Branched Hydrocarbons

3.1. Introduction

The waxy layer coating an insect's cuticle consists primarily of a mixture of straight-chain alkanes, methyl-branched hydrocarbons, and olefins with chain lengths from less than 20 to more than 50 carbons, admixed with lesser amounts of more polar compounds such as wax esters, long-chain alcohols, and carboxylic acids. The primary role of the cuticular wax layer is to protect the insect from desiccation, but in many insects, a subset of the components of the cuticular hydrocarbons have secondary roles as semiochemicals.[1] For example, methyl-branched hydrocarbons have been identified as contact sex pheromones in several species, allowing for species and sexual recognition between possible mates.[2-5] The majority of insect-produced methyl-branched hydrocarbons have the potential to be chiral due to the presence of a stereogenic center at any branch point other than carbon 2, or the central carbon of an odd-numbered carbon chain due to symmetry. Despite the fact that these methyl-branched hydrocarbons are ubiquitous components of insect cuticular lipids, no studies have attempted to determine whether they are biosynthesized in high enantiomeric purity. Whereas a few studies have tested whether insects can discriminate between enantiomers of methyl-branched hydrocarbons,[6-9] differing only in the lengths of the two hydrocarbon chains on either side of the chiral center, the enantiomer(s) that the insects are actually producing have not been determined. The latter is in large part due to the difficulty in determining the absolute configurations of methyl-branched hydrocarbons, which have small to minute

optical rotations, and no functional groups to provide points of contact with polar groups on chromatographic chiral stationary phases to assist in chiral recognition. The problem is exacerbated by the fact that the compounds are available in very limited quantities, with insects often producing microgram or lesser amounts of a particular hydrocarbon as part of a complex mixture of hydrocarbons. Furthermore, to our knowledge, there have been no reports of resolution of these compounds on cyclodextrin-based GC chiral stationary phases, in part due to the relatively high temperatures required for gas chromatography of such large and nonvolatile compounds. As a result, few studies have been performed to determine the effects of stereochemistry on the bioactivity of these compounds,[10] despite the fact that for many other types of insect pheromones, bioactivity is often directly correlated with chirality, with one enantiomer being active, and the other being benign or frequently, antagonistic.[11]

Previously published syntheses of monomethyl-branched hydrocarbons primarily have been based on the use of commercially available chiral synthons such as propylene oxide,[6] methyl 3-hydroxy-2-methylpropanoate,[8] citronellol or citronellal,[11,12] 2-methylbutanol,[13] aromadendrene,[14] or pulegone.[15] These starting materials are commercially available but often are relatively expensive, the overall yields have generally been rather low, and in at least some cases, the syntheses have been lengthly.
As a more economical and expeditious route, utilization of Evans' chiral auxiliaries to induce asymmetry at the methyl-branched stereogenic center seemed to offer a reasonable and highly flexible alternative (Scheme 3.1). Removal of the chiral auxiliary would produce a chiral alcohol intermediate **6**, which, after conversion of the alcohol to a

leaving group, could then be alkylated by a variety of methods to prepare chiral methyl-branched hydrocarbons in high enantiomeric purity (Scheme 3.1).

As a test of the methodology, we selected the homologous hydrocarbons 7-methylheptacosane **1** and 7-methylpentacosane **2** (Scheme 1), which had been identified as major components of the contact sex pheromone produced by females of the red-headed ash borer beetle, *Neoclytus acuminatus acuminatus*.[4] Synthetic (±)-**1** induced partial copulation responses by male *N. acuminatus*, and (±)-**2** synergized responses to (±)-**1**. However, the responses obtained to the blend of synthetic (±)-**1** and (±)-**2** were not as strong as those to a female beetle, suggesting that the correct absolute stereochemistry of the naturally occurring **1** and **2** might be important. To date, for reasons described above, it has not been possible to determine the absolute stereochemistry of these two components by analytical means. Herein we report the asymmetric synthesis of the enantiomers of **1** and **2** in 62-68% overall yields over 6 steps from intermediate **6**, prepared by the acylation and subsequent methylation of (*R*)- and (*S*)-4-isopropyloxazolidin-2-one chiral auxiliaries **3** respectively, followed by cleavage of the auxiliaries with simultaneous reduction to the alcohol.

Scheme 3.1. Retrosynthetic analysis of (*S*)-7-methylheptacosane **1** and (*S*)-7-methylpentacosane **2**.

3.2. Results and Discussion

Our synthesis began with the chiral auxiliary, (*R*)-4-isopropyloxazolidin-2-one **3**, which was prepared in 1 step from (*D*)-valinol following a known protocol.[16] Oxazolidinone (*R*)-**3** was deprotonated with *n*-BuLi in THF, then acylated with octanoyl chloride to afford the oxazolidinone imide **4** in 98% yield.[17] Deprotonation at the α-position of **4** with NaHMDS in THF selectively formed the (*Z*)-enolate, which was subsequently alkylated with methyl iodide to afford ((*R*)-2-methyloctanoyl)oxazolidinone **5** in 94% yield (d.r., > 99:1, as determined by GC analysis and ^{1}H NMR).[18] Reduction of

5 with $LiBH_4$ in Et_2O afforded the chiral alcohol intermediate (*R*)-**6** in 92% yield (e.e.> 98% as determined by chiral GC analysis), along with **3** (65-80% recovery) which could be recycled.[19] Alcohol **6** was then converted quantitatively to the alkyl triflate **7** by treatment of **6** with triflic anhydride and pyridine in CH_2Cl_2.[20] Triflate **7** was immediately alkynylated with nonadecynyl lithium in THF, prepared by deprotonation of 1-nonadecyne with *n*-BuLi in THF at -78 ºc, to form (*R*)-7-methylheptacos-9-yne **8** in 82% yield.[21] Alkynylation was used rather than direct alkylation by, for example, copper catalyzed reaction with a long-chain Grignard reagent because in our hands, such reactions proved capricious, often producing low yields and byproducts resulting from nucleophilic displacement of the triflate with the halide from the Grignard reagent rather than the desired alkylation. Following alkynylation, (*R*)-7-methylheptacosane **1** was obtained as a crystalline solid in 98% yield by 5% Rh on carbon catalyzed hydrogenation of (*R*)-**8** in hexanes.[22] Recrystallization of crude crystalline (*R*)-**1** from hexane/acetone (1:5) gave pure (*R*)-**1** in an overall yield of 68% from (*R*)-**3**. Although it was not possible to ascertain the chiral purity directly, it must be a minimum of >98% ee, reflecting the enantiomeric purity of alcohol intermediate (*R*)-**6** because none of the later steps could result in loss of stereochemical integrity. In addition, it is likely that the enantiomeric purity was even higher than that of (*R*)-**6** because recrystallization of the final product should have removed most if not all of any enantiomeric impurity present.

In the same manner (*S*)-4-isopropyloxazolidin-2-one (*S*)-**3**, prepared from (*L*)-valinol, afforded 98% pure (*S*)-7-methylheptacosane **1** in 65% overall yield from (*S*)-**3** in 6 steps (Scheme 3.2).

O NH (R)-3 a O O N 4

b O O N 5 c

OR e
6 R=H
d 7 R=Tf

$H_3C(H_2C)_{16}$ $(CH_2)_4CH_3$ f
8

$H_3C(H_2C)_{18}$ $(CH_2)_4CH_3$
(R)-1

Similarly

O NH (S)-3

$H_3C(H_2C)_{18}$ $(CH_2)_4CH_3$
(S)-1

Scheme 3.2. Synthesis of (*R*)- and (*S*)-7-methylheptacosane **1**. Reagents: (a) i. *n*-BuLi, THF; ii. $CH_3(CH_2)_6COCl$ (98%); (b) i. NaHMDS, THF; ii. MeI (94%); (c) $LiBH_4$, Et_2O (93%); (d) Tf_2O, pyridine, CH_2Cl_2 (quantitative for **7**); (e) lithium nonadecylide, THF (80%); (f) H_2 (1 atm), Rh/C, hexanes (96-98%).

(*R*)-7-methylpentacosane **2** was prepared in similar fashion from the shared chiral intermediate (*R*)-2-methyloctyltriflate **7** by alkynylation with heptadecynyl lithium, to form (*R*)-7-methylpentacos-9-yne **9** in 82% yield. After reduction and recrystallization as described above for **1**, (*R*)-7-methylpentadecane **2** was obtained in 63% overall yield from (*R*)-**3**. In a similar manner, (*S*)-2-methyloctanol **6** afforded (*S*)-7-methylpentacosane **2** in 62% overall yield from (*S*)-**3** in 6 steps (Scheme 3.3)

$H_3C(H_2C)_4$ OR —b→ $H_3C(H_2C)_{14}$ $(CH_2)_4CH_3$ **9**

a: **6** R=H → **7** R=Tf

—c→ $H_3C(H_2C)_{16}$ $(CH_2)_4CH_3$ (*R*)-**2**

Similarly

$H_3C(H_2C)_4$ OH **6** → $H_3C(H_2C)_{16}$ $(CH_2)_4CH_3$ (*S*)-**2**

Scheme 3.3. Synthesis of (*R*)- and (*S*)-7-methylpentacosane **2**. Reagents: (a) Tf_2O, pyridine, CH_2Cl_2 (quantitative for **7**); (b) lithium heptadecylide, THF (76%); (c) H_2 (1 atm), Rh/C, hexanes (97-99%).

Overall, this route to chiral methyl-branched hydrocarbons has a number of advantages. First, the route is short and highly efficient, proceeding in over 60% yield over 6 steps. Second, the route is amenable to making methyl-branched hydrocarbons of any desired length with methyl branches in any desired position, by using the appropriate acyl chloride and alkyne coupling partners, all of which are either commercially available or simply prepared (see appendix section **3.7** for library of chiral methyl-branched hydrocarbons synthesized analogously to the described compounds). Third, for small scale reactions or single use, the chiral auxiliaries **3** are commercially available, albeit expensive. For larger scale reactions or multiple parallel reactions, they can be readily prepared in multigram quantities from inexpensive starting materials, and the chiral auxiliaries can be recovered and reused. Fourth, the method should be readily adaptable

for the synthesis of more highly branched hydrocarbons by iterative use of the asymmetric induction step and coupling of the resulting methyl-branched intermediates.

3.3 Attempted Synthesis of Chiral Methyl-branched Hydrocarbons Utilizing Chiral Synthons and Grignard Coupling Reactions

The synthesis of chiral methyl-branched hydrocarbons was initially attempted utilizing methyl 3-hydroxy-2-methylpropanoate chiral synthons as starting material with methods developed by Gries et al. [8] (Scheme 3.4). Methyl (*R*)-(+)-3-hydroxy-2-methylpropanoate **10** was first protected by reaction with *tert*-butyldimethylsilyl chloride and triethylamine in dichloromethane with dimethylaminopyridine (DMAP) catalyst to form methyl (*R*)-3-((*tert*-butyldimethylsilyl)oxy)-2-methylpropanoate **11**. Reduction of the ester functionality with lithium borohydride in diethyl ether gave (*S*)-3-((*tert*-butyldimethylsilyl)oxy)-2-methylpropan-1-ol **12**. However, subsequent dilithium tetrachlorocuprate (Li_2CuCl_4) catalyzed alkylations of the triflate of **12** with various alkylmagnesium bromide reagents resulted in the formation of Wurtz coupling products and nucleophilic displacement of the triflate with bromide anion in addition to the desired products (Scheme 3.5). Further attempts at using Li_2CuCl_4-catalyzed Grignard cross coupling reactions with other sulfonate ester leaving groups (e.g. tosylate and mesylate) also resulted in low yields and production of both Wurtz coupling and bromide displacement products.

Scheme 3.4 Attempted synthetic scheme for chiral methyl-branched hydrocarbons. Reagents: (a) TBDMSCl, DMAP, CH_2Cl_2; (b) $LiBH_4$, Et_2O, -10 ºC; (c) Tf_2O, pyridine, CH_2Cl_2, -10 ºC; (d) Li_2CuCl_4 (cat.), R^1-MgBr, Et_2O, -40 ºC; (e) TBAF, THF, rt; (f) Tf_2O, pyridine, CH_2Cl_2, -10 ºC; (g) Li_2CuCl_4 (cat.), R^2-MgBr, Et_2O, -40 ºC.

Scheme 3.5 Attempted Grignard cross coupling reactions forming Wurtz coupling and bromide displacement products.

Recent work on efficient Grignard coupling reactions by Cahiez and coworkers suggested that use of *N*-methyl-2-pyrrolidinone (NMP) as a co-solvent, as well as constant slow addition of the Grignard reagent to the electrophile, can drastically improve yields in these types of cross-coupling reactions.[23,24] With this in mind, the Li_2CuCl_4-catalyzed Grignard reactions were repeated using various electrophiles, addition rates, solvents, and additives (Table 3.1). Trials of Li_2CuCl_4-catalyzed Grignard reactions with alkyl halide electrophiles using the Cahiez conditions resulted in good isolated yields of the desired cross-coupling products (entries 1 and 2, Table 3.1). However when alkyl

sulfonates were employed as electrophiles under the same reaction conditions, the addition of the NMP co-solvent resulted in low yields, and appeared to inhibit the formation of the desired cross-coupling products (entries 4 and 6, Table 3.1).

An observation made while reading the publications of both Cahiez[23,24] and Zhang[8] was that both research groups employed relatively concentrated Grignard reagents (2.0 M) in their reactions, which may have been a factor in their good yields. As a result of this observation, Li_2CuCl_4-catalyzed Grignard reactions utilizing varied concentrations of Grignard reagents were performed (Table 3.1). The reactions executed with more dilute Grignard reagents (<1.0 M) generally resulted in low overall yields, which was especially true for reactions with alkyl sulfonate electrophiles (entries 3-7, Table 3.1). Conversely, reactions performed with more concentrated Grignard reagents (>1.5M) produced the desired alkylation products in good overall yields regardless of electrophile type (entries 8-9, Table 3.1). Solvent effects were also observed to affect the cross-coupling reactions, but also appeared to be dependent on the electrophile type. Thus, the cross-coupling reactions performed with alkyl halide electrophiles in THF showed no negative solvent effects and proceeded smoothly with high isolated yields (entries 1-2, Table 3.1), whereas the analogous reactions performed with alkyl sulfonate electrophiles in THF resulted in the formation of Wurtz homo-coupling products and bromide displacement of the sulfonate moiety on the electrophile (entries 3-7, Table 3.1). However, identical reactions performed in diethyl ether resulted in moderate to high yields depending on the concentration of the Grignard reagent (Entries 7-9, Table 3.1).

Entry	Electrophile	Grignard	Solvent	Additive	Addition Method	GC Conversion	% Yield
1	Br	n-C_5MgCl (0.5M)	THF	NMP	Dropwise	95%	80%
2	Br	n-C_{11}MgBr (1M)	THF	none	Syringe pump	90%	82%
3	OTf	TBSO-$n$$C_{11}$-MgBr (0.5M)	THF	none	Syringe pump	30%	20%
4	OTf	TBSO-nC11-MgBr (0.5M)	THF	NMP	Syringe pump	10%	N/A
5	OMs	TBSO-nC11-MgBr (0.5M)	THF	none	Syringe pump	35%	N/A
6	OMs	TBSO-nC11-MgBr (0.5M)	THF	NMP	Syringe pump	<10%	N/A
7	OTf	TBSO-nC11-MgBr (0.5M)	Dry Ether	none	Syringe pump	65%	50%
8	OTf	TBSO-nC11-MgBr (2M)	Dry Ether	none	Syringe pump	90%	85%
9	OTf	TBSO-nC11-MgBr (2M)	Dry Ether	none	Syringe pump	92%	85%

Table 3.1. Copper catalyzed Grignard cross-coupling reactions with varied conditions, reagent concentrations, addition rates, solvents, and additives.

Although the conditions for a high-yielding Grignard coupling reaction were elucidated, there were still problems associated with side products from the reaction that led to the abandonment of this synthetic route in favor of the Evans' chiral auxiliary route. Specifically, when utilizing a long-chain alkyl Grignard to form the desired methyl-branched hydrocarbon, a long-chain *n*-hydrocarbon is formed as a side product

from the quenched excess Grignard reagent, which is difficult and/or tedious to remove from the desired product. The presence of minor synthetic byproducts can have a negative effect on the behavioral responses of insects, and may even completely inhibit the biological activity of a synthesized semiochemical. Therefore, the synthetic route employing Evan's chiral auxiliaries was the preferred route for the synthesis of chiral MBCHs, due to its efficiency, reliability, flexibility, the ease of purification and characterization of the intermediates, and its production of any desired methyl-branched product in high chemical and enantiomeric purity.

3.4. Conclusion

The concise total syntheses of the enantiomers of 7-methylheptacosane **1** and 7-methylpentacosane **2** were completed in 6 steps and 62-68% overall yield using Evans' chiral auxiliaries to induce the asymmetry in the stereogenic center. This concise and flexible synthetic route was utilized to prepare a large library of chiral methyl-branched hydrocarbons (Appendix section **3.7**). The ready availability of chiral methyl-branched hydrocarbons through straightforward reaction sequences should make this class of biologically active molecules much more accessible for biological testing. With synthetic hydrocarbons of known absolute configuration and high enantiomeric purities in hand, it will be possible to assess the abilities of insects to recognize and discriminate between long-chain hydrocarbons differing only in the absolute configuration of a methyl branch.

3.5. Experimental

3.5.1. General

All solvents were Optima grade (Fisher Scientific, Pittsburgh, PA). Tetrahydrofuran (THF) was distilled from sodium/benzophenone under argon atmosphere. ^{1}H and ^{13}C NMR spectra were recorded with a Varian INOVA-400 (400 and 100.5 MHz respectively) spectrometer (Palo Alto, CA), as $CDCl_3$ solutions. ^{1}H NMR chemical shifts are expressed in ppm relative to residual $CHCl_3$ (7.27 ppm) and ^{13}C NMR chemical shifts are reported relative to $CDCl_3$ (77.16 ppm). IR spectra were obtained with a Perkin Elmer Spectrum One FT-IR spectrometer (Bridgeport, CT) using the universal ATR sampling accessory. Unless otherwise stated, solvent extracts of reaction mixtures were dried over anhydrous Na_2SO_4 and concentrated by rotary evaporation under reduced pressure. Crude products were purified by vacuum flash chromatography or column flash chromatography on silica gel (230-400 mesh; Fisher Scientific). Yields refer to isolated yields of chromatographically pure products. Mass spectra were obtained with a Hewlett-Packard (HP) 5890 GC (Avondale, PA) interfaced to an HP 5970 mass selective detector, in EI mode (70 eV) with helium carrier gas. The GC was equipped with an HP5-MS column (25 m, 0.20 mm i.d. 0.33µm film). Reactions with air- or water-sensitive reagents were carried out in oven-dried glassware under an argon atmosphere. Specific rotations were obtained on a Rudolph Autopol IV digital polarimeter (Hackettstown, NJ) as $CHCl_3$, EtOH, or hexanes solutions, and five sequential measurements of each chiral intermediate was acquired and averaged to obtain the reported specific rotations.

3.5.2. (*R*)-4-isopropyl-3-octanoyloxazolidin-2-one (*R*)-4

To a cold (-78 ºC), stirred solution of (*R*)-4-isopropyloxazolidin-2-one **3** (2.5 g, 19.6 mmol) in dry THF (80 mL) was added *n*-BuLi (2.89 M, 7.12 mL, 20.6 mmol) dropwise over 15 min. The reaction was stirred at -78 ºC for 30 min. Octanoyl chloride (3.67 mL, 21.5 mmol) was then added dropwise to the reaction mixture. The resulting mixture was stirred at -78 ºC for 20 min and then warmed to -10 ºC for 2 h. The mixture was quenched with 1 M aqueous K_2CO_3 (100 mL) and warmed to room temperature. The crude products were extracted with hexanes (2 × 100 mL), and the combined hexane extracts were washed with water and brine, dried, and concentrated. The residue was purified by flash chromatography on silica gel (50g). Elution with hexane/EtOAc (9:1) afforded 4.93 g (19.3 mmol, 98%) of (*R*)-**4** as a colorless oil, ν_{max} (neat): 2958 (m), 2927 (s), 2854 (m), 1777 (s), 1699 (s), 1487 (w), 1465 (m), 1384 (s), 1301 (m), 1269 (w), 1232 (m), 1204 (s), 1120 (w), 1091 (w), 1059 (m), 1020 (m), 971 (w), 773 (m), 723 (w), 708 (w); ∂_H ($CDCl_3$): 0.78 (3H, m), 0.82 (6H, d, *J* = 6.2 Hz), 1.18 (6H, m), 1.55 (2H, m), 2.28 (1H, m), 2.74 (1H, pseudoquintet, *J* = 6.2 Hz), 2.89 (1H, pseudoquintet, *J* = 6.4 Hz), 4.11 (1H, dd, *J*= 16 Hz, 4 Hz), 4.18 (1H, pseudotriplet, *J* = 8.4 Hz), 4.34 (1H, m); ∂_C ($CDCl_3$): 14.0, 14.9, 18.0, 23.0, 24.9, 28.2, 29.3, 31.9, 35.8, 58.5, 63.5, 154, 173.8; GC-MS [Column: DB-5MS, 5% phenylmethylsiloxane, 30 m × 0.25 mm id; carrier gas, He; temp: 50-280 ºC (+10 ºC/min)]: t_R: 19.43 min (100%); MS of **4** (70 eV, EI); *m*/*z*: 255 (1, M^+), 212 (2), 184 (5), 171 (8), 142 (1), 127 (17), 109 (3), 85 (9), 71 (8), 57 (100), 41 (47).

3.5.3. (*S*)-4-isopropyl-3-octanoyloxazolidin-2-one (*S*)-4

In the same manner as described above, 2.5 g (19.6 mmol) of (*S*)-**3** gave 4.88 g (97%) of (*S*)-4-isopropyl-3-octanoyloxazolidin-2-one (*S*)-**4** as a colorless oil. Its spectral data were identical to those of (*R*)-**4**.

3.5.4. (*R*)-4-isopropyl-3-((*R*)-2-methyloctanoyl)oxazolidin-2-one (*R*)-5

To a cold (-78 ºC) stirred solution of (*R*)-**4** (4.5 g, 17.5 mmol) in dry THF (70 mL), was added NaHMDS (2.0 M, 9.63 mL, 19.3 mmol) dropwise over 10 min. The reaction was stirred at -78 ºC for 1 h. Iodomethane (4.34 mL, 70 mmol) was then added dropwise over 20 min and the resulting mixture was stirred at -78 ºC for 2 h. The reaction was warmed to 0 ºC and quenched with saturated aqueous NH_4Cl solution (50 mL), then extracted with hexane (2 × 100 mL). The organic layers were pooled and washed successively with 1M HCl (2 × 100 mL), saturated aqueous $NaHCO_3$ (2 × 100 mL), and brine, then dried and concentrated. The residue was chromatographed on silica gel (50g). Elution with hexane/EtOAc (9:1) afforded 4.46 g (16.5 mmol, 94 %) of (*R*)-**5** as a colorless oil, ν_{max} (neat): 2961 (s), 2932 (s), 2874 (m), 1774 (s), 1697 (s), 1487 (w), 1460 (m), 1383 (s), 1300 (m), 1232 (s), 1198 (s), 1120 (w), 1090 (w), 1057 (w), 990 (w), 955 (w), 773 (w), 758 (w), 726 (w), 701 (w); ∂_H ($CDCl_3$): 0.80 (3H, m), 0.84 (6H, d, *J* = 8.0 Hz), 1.12 (3H, d, *J* = 7.8 Hz), 1.21 (6H, m), 1.52 (1H, m), 1.65 (1H, m), 2.28 (1H, m), 3.65 (1H, sextet, *J* =6.2 Hz), 4.12 (1H, dd, *J* = 16 Hz, 4 Hz), 4.20 (1H, pseudotriplet, *J* = 8.4 Hz), 4.38 (1H, pseudoquintet, *J* = 4.0 Hz); ∂_C ($CDCl_3$): 14.2, 15.0, 18.0, 22.9, 27.8, 28.5, 29.0, 29.3, 32.0, 33.5, 38.0, 58.5, 63.8, 154.0, 177.5; GC-MS [Column: DB-5MS,

5% phenylmethylsiloxane, 30 m × 0.25 mm id; carrier gas, He; temp: 100-280 ºC (+10 ºC/min)]: t_R: 13.27 min (100%); MS of **5** (70 eV, EI); *m/z*: 269 (1, M^+), 198 (3), 185 (5), 141 (7), 130 (12), 112 (35), 86 (55), 57 (100), 41 (73).

3.5.5. (*S*)-4-isopropyl-3-((*S*)-2-methyloctanoyl)oxazolidin-2-one (*S*)-5

In the same manner as described above, 4.5 g (17.5 mmol) of (*S*)-**4** gave 4.38 g (92.5%) of (*S*)-4-isopropyl-3-((*S*)-2-methyloctanoyl)oxazolidin-2-one (*S*)-**5** as a colorless oil. Its spectral data were identical to those of (*R*)-**5**.

3.5.6. (*R*)-2-methyloctan-1-ol (*R*)-6

To a cold (0 ºC) stirred solution of (*R*)-**5** (3.2 g, 11.8 mmol) in Et_2O (50 mL) was added MeOH (957 µL, 23.6 mmol) followed by solid $LiBH_4$ (515 mg, 23.6 mmol). The reaction was stirred at 0 ºC for 2 h, then quenched with saturated aqueous NH_4Cl (30 mL). The layers were separated and the aqueous layer was extracted with Et_2O (2 × 50 mL). The organic layers were pooled and washed successively with Na_2CO_3 solution and brine, dried, and concentrated to give 3.5 g of a cloudy yellow oil. The crude product was chromatographed on silica gel (60 g). Elution with hexane/EtOAc (9:1) afforded 1.57 g (10.87 mmol, 92% yield, >98 % ee) of (*R*)-**6** as a colorless oil. Further elution with hexane/EtOAc (2:8) afforded 1.2 g of (*R*)-**3** as white crystals, which was reused in subsequent reactions. (*R*)-2-methyloctan-1-ol **6** showed the following properties: $[\alpha]_D^{25}$ = +13.16 (*c* = 1.5, EtOH); ν_{max} (neat): 3336 (br m), 2956 (s), 2923 (s), 2855 (s), 1465 (m), 1378 (w), 1032 (s), 938 (w), 908 (w), 842 (w) 723 (w); ∂_H ($CDCl_3$): 0.89 (6H, m), 1.21

(10H, m), 1.54 (1H, m), 1.90 (1H, s, OH), 3.38 (1H, pseudotriplet, *J* = 7.6 Hz), 3.44 (1H, dd, *J* = 12.0 Hz, 5.1 Hz); ∂_C ($CDCl_3$): 14.0, 16.8, 23.1, 27.0, 29.8, 32.1, 33.8, 36.0, 68.5; GC-MS [Column: DB-17MS, 17% phenylmethylsiloxane, 30 m × 0.25 mm id; carrier gas, He; temp: 50-280 ºC (+10 ºC/min)]: t_R: 7.20 min (96.5%); MS of **6** (70 eV, EI); *m/z*: 143 (1, M^+-1), 126 (5), 111 (5), 97 (21), 84 (25), 71 (65), 57 (100), 43 (57). The ee was determined by GC analysis using a β-DEX225 column [30 m × 0.25 mm id × 0.25 µm film; carrier gas, He; temp: 50-220 ºC (held at 50 ºC for 30 min, then + 5 ºC/ min)]: t_{major}: 43.95 min (100 %).

3.5.7. (*S*)-2-methyloctan-1-ol (*S*)-6

In the same manner as described above 3.0 g (11.1 mmol) of (*S*)-**5** gave 1.48 g (93% yield, 97.9% ee) of (*S*)-2-methyloctan-1-ol **6** as a colorless oil. $[\alpha]_D^{25}$ = -13.05 (*c* = 1.52, EtOH); Its spectra were identical to those of (*R*)-**6**. The ee was determined by GC analysis using a β-DEX225 column [same conditions as those of (*R*)-**6**]: t_{major} : 44.00 (98.9 %), t_{minor}: 43.94 min (1.07 %).

3.5.8. (*R*)-2-methyloctan-1-yl triflate (*R*)-7

To a cold (-10 ºC) stirred solution of (*R*)-2-methyloctan-1-ol **7** (500 mg, 3.47 mmol) in dry CH_2Cl_2 (15 mL) was added sequentially pyridine (269 µL, 3.47 mmol) and triflic anhydride (710 µL, 4.16 mmol). The reaction was stirred at -10 ºC for 1.5 h and then diluted with hexanes (60 mL) and stirred for 30 min. The resulting mixture was then filtered through a plug of silica gel (30 g), and the filter cake was washed with

hexanes/CH_2Cl_2 (4:1). The filtrate was concentrated in vacuo to give 970 mg (quantitative) of (*R*)-**7** as a colorless oil. This was used immediately in the next step without further purification or characterization.

3.5.9. (*S*)-2-methyloctan-1-yl triflate (*S*)-7

In the same manner as described above 500 mg (3.47 mmol) of (*S*)-2-methyloctan-1-ol **7** gave 963 mg (quantitative) of (*S*)-2-methyloctan-1-yl triflate **7** as a colorless oil, which was used immediately in the next step without further purification or characterization.

3.5.10. (*R*)-7-methylheptacos-9-yne (*R*)-8

In a three necked flask, 1-nonadecyne (1.0 g, 3.82 mmol) was dissolved in dry THF (15 mL) and the solution was cooled to -78 ºC. A hexane solution of *n*-BuLi (2.89 M, 1.32 mL, 3.82 mmol) was added dropwise, and the resulting mixture was stirred at -78 ºC for 10 min then at -10 ºC for 30 min. After cooling again to -78 ºC, (*R*)-**7** (958 mg, 3.47 mmol) dissolved in dry THF (3 mL) was added dropwise. The mixture was allowed to warm to -10 ºC and stirred for 5 h. The resulting mixture was then quenched with saturated aqueous NH_4Cl, and extracted with hexane. The hexane solution was washed with water, aqueous $NaHCO_3$, and brine, dried, and concentrated. The residue was flash chromatographed on C_{18} bonded silica gel (50 g). Elution with MeOH gave 1.11 g (2.85 mmol, 82%) of (*R*)-**8** as a clear oil. $[\alpha]_D^{22} = -0.43$ (c = 3.47, CH_2Cl_2); ν_{max} (neat): 2954 (m), 2921 (s), 2852 (s), 1464 (m), 1377 (w), 1251 (w), 1056 (w), 843 (w), 721 (w); ∂_H

($CDCl_3$): 0.84 (6H, m), 0.89 (3H, d, *J*=6.4 Hz), 1.1-1.4 (38H, br s), 1.48 (1H, dd, *J*= 12.0 Hz, 4.7 Hz), 1.55 (1H, m), 1.98 (1H, d, *J*=12 Hz), 2.17 (2H, m); ∂_C ($CDCl_3$): 14.32, 18.98, 19.74, 20.9, 26.39, 27.27, 29.05, 29.38, 29.58, 29.79, 29.91, 32.12, 33.07, 36.26, 79.56, 81.2; GC-MS [Column: DB-17MS, 17% phenylmethylsiloxane, 30 m × 0.25 mm id; carrier gas, He; temp: 100-280 ºC (+10 ºC/min)]: t_R: 18.78 min (97.32 %); MS of **8** (70 eV, EI); *m/z*: 390 (5, M^+), 361 (2), 333 (10), 305 (3), 280 (1), 263 (3), 236 (2), 208 (3), 179 (2), 151 (25), 126 (18), 109 (57), 81 (100), 57 (74), 41 (63); HRMS (EI) calcd for $C_{28}H_{54}$ (M^+): 390.4226. Found: 390.4234.

3.5.11. (*S*)-7-methylheptacos-9-yne (*S*)-8

In the same manner as described above 963 mg (3.48 mmol) of (*S*)-**7** gave 1.08 mg (79.9 %) of (*S*)-7-methylheptacos-9-yne **8** as a colorless oil, $[\alpha]_D^{22}$ = +0.37 (*c* = 3.42, CH_2Cl_2). Its spectra were identical to those of (*R*)-**8**. HRMS (EI) calcd for $C_{28}H_{54}$ (M^+): 390.4226. Found: 390.4229.

3.5.12. (*R*)-7-methylpentacos-9-yne (*R*)-9

In the same manner as described above, (*R*)- and (*S*)-**9** were prepared from triflates (*R*)- and (*S*)-**7,** substituting 1-heptadecyne for 1-nonadecyne. Purification by reverse phase flash chromatography as described above gave (*R*)-**9** in 75% yield as a colorless oil. $[\alpha]_D^{22}$ = -0.42 (*c* = 3.51, CH_2Cl_2); ν_{max} (neat): 2955 (m), 2921 (s), 2852 (s), 1464 (m), 1377 (w), 1342 (w), 1251 (w), 1056 (w), 843 (w), 721 (w); ∂_H ($CDCl_3$): 0.84 (6H, m), 0.89 (3H, d, *J*=6.4 Hz), 1.1-1.55 (38H, br m), 1.98 (1H, d, *J*=12 Hz), 2.17 (2H,

m); ∂_C ($CDCl_3$): 14.32, 18.98, 19.74, 20.9, 26.39, 27.27, 29.05, 29.38, 29.58, 29.79, 29.91, 32.12, 33.07, 36.26, 79.15, 81.33; GC-MS [same conditions as those for (*R*)-**7**]: t_R: 17.29 min (98.55 %); MS of **9** (70 eV, EI); *m/z*: 362 (5, M^+), 347 (2), 320 (2), 305 (10), 277 (3), 250 (2), 235 (3), 208 (3), 193 (2), 166 (5), 151 (24), 124 (18), 109 (65), 81 (100), 57 (88), 41 (64); HRMS (EI) calcd for $C_{26}H_{50}$ (M^+): 362.3907. Found: 362.3918.

3.5.13. (*S*)-7-methylpentacos-9-yne (*S*)-9

In the same manner as described above 887 mg (3.21 mmol) of (*S*)-**7** gave 884 mg (76%) of (*S*)-7-methylpentacos-9-yne **9** as a colorless oil, $[\alpha]_D^{22}$ = +0.43 (*c* = 3.62, CH_2Cl_2). Its spectra were identical to those of (*R*)-**9**. HRMS (EI) calcd for $C_{26}H_{54}$ (M^+): 362.3907. Found: 362.3919.

3.5.14. (*R*)-7-methylheptacosane (*R*)-1

To a solution of (*R*)-**8** (970 mg, 2.48 mmol) in hexanes (15 mL) was added 5% Rh-C (97 mg, 10 % wt) and powdered Na_2CO_3 (781.7 mg, 7.38 mmol). The reaction was stirred vigorously under an atmosphere of H_2 for 12 h, then filtered through a pad of silica gel (10 g) and concentrated. The resulting solid was recrystallized from acetone/hexane (5:1) to afford 968 mg (2.45 mmol, 98.9%) of (*R*)-**1** as white crystals, mp 33.5-34.5 ºC; $[\alpha]_D^{21}$ = -0.247 ± 0.013 (*c* = 3.75, CH_2Cl_2). ν_{max} (neat): 2955 (m), 2920 (s), 2852 (s), 1465 (m), 1377 (w), 1301 (w), 721 (m); ∂_H ($CDCl_3$): 0.84-0.9 (9H, br m), 1.0-1.2 (2H, m), 1.21-1.44 (46H, br s), 1.51 (1H, s); ∂_C ($CDCl_3$): 14.31, 19.43, 22.90, 23.54, 27.29, 29.553, 29.91, 30.26, 32.13, 32.95, 36.99, 37.32; GC-MS [same conditions as for

(*R*)-**8**]: t_R: 17.29 min (99.37%); MS of **1** (70 eV, EI); *m/z*: 394 (1, M^+), 379 (5), 351 (3), 325 (2), 309 (25), 280 (10), 253 (3), 225 (4), 197 (5), 169 (7), 141 (9), 112 (55), 85 (42), 71 (98), 57 (100), 43 (50); HRMS (EI) calcd for $C_{28}H_{58}$ (M^+): 394.4539. Found: 394.4533.

3.5.15. (*S*)-7-methylheptacosane (*S*)-1

In the same manner as described above 870 mg (2.23 mmol) of (*S*)-**8** gave 858 mg (97.5%) of (*S*)-7-methylheptacosane **1** as a crystalline solid, mp 32-33 ºC; $[\alpha]_D^{21}$ = +0.236 ± 0.021 (c = 3.48, CH_2Cl_2). Its spectra were identical to those of (*R*)-**1**. HRMS (EI) calcd for $C_{28}H_{58}$ (M^+): 394.4539. Found: 394.4547.

3.5.16. (*R*)-7-methylpentacosane (*R*)-2

In the same manner as described above, 930 mg (2.56 mmol) of (*R*)-**9** gave 924 mg (2.53 mmol, 99.1%) of (*R*)-**2** as white crystals, mp 27-28.5 ºC; $[\alpha]_D^{22}$ = -0.253 ± 0.009 (c = 3.56, CH_2Cl_2). ν_{max} (neat): 2955 (m), 2920 (s), 2850 (s), 1465 (m), 1377 (w), 1301 (w), 1222 (w), 721 (m); ∂_H ($CDCl_3$): 0.84-0.9 (9H, br m), 1.0-1.2 (2H, m), 1.21-1.44 (42H, br s), 1.51 (1H, s); ∂_C ($CDCl_3$): 14.31, 19.91, 22.90, 27.27, 29.55, 29.91, 30.23, 32.13, 32.95, 37.32; GC-MS [same conditions as for (*R*)-**8**]: t_R: 16.55 min (99.94%); MS of **2** (70 eV, EI); *m/z*: 366 (1, M^+), 351 (7), 323 (3), 308 (2), 281 (28), 252 (10), 226 (2), 211 (4), 183 (5), 155 (8), 127 (12), 112 (62), 85 (42), 71 (98), 57 (100), 42 (60); HRMS (EI) calcd for $C_{26}H_{54}$ (M^+): 366.4220. Found: 366.4218.

3.5.17. (*S*)-7-methylpentacosane (*S*)-2

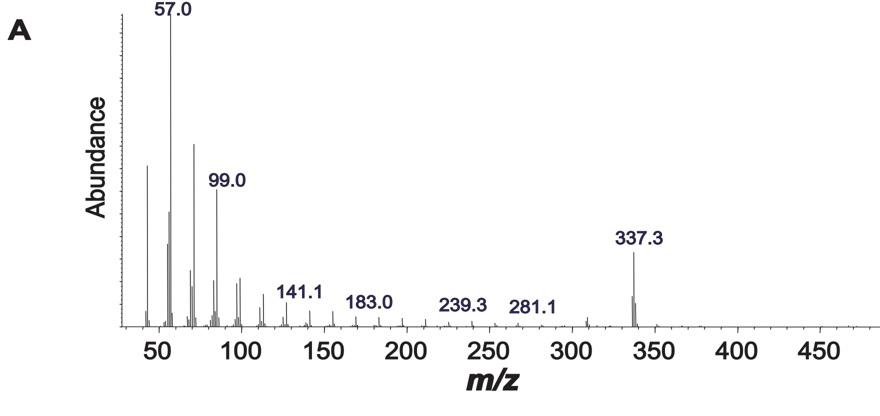
In the same manner as described above 855 mg (2.36 mmol) of (*S*)-**9** gave 847 mg (98.4%) of (*S*)-7-methylpentatacosane **1** as a crystalline solid, mp 28-29 ºC; $[\alpha]_D^{22}$ = +0.241 ± 0.016 (*c* = 3.71, CH_2Cl_2). Its spectra were identical to those of (*R*)-**2**. HRMS (EI) calcd for $C_{26}H_{54}$ (M^+): 366.4220. Found: 366.4234.

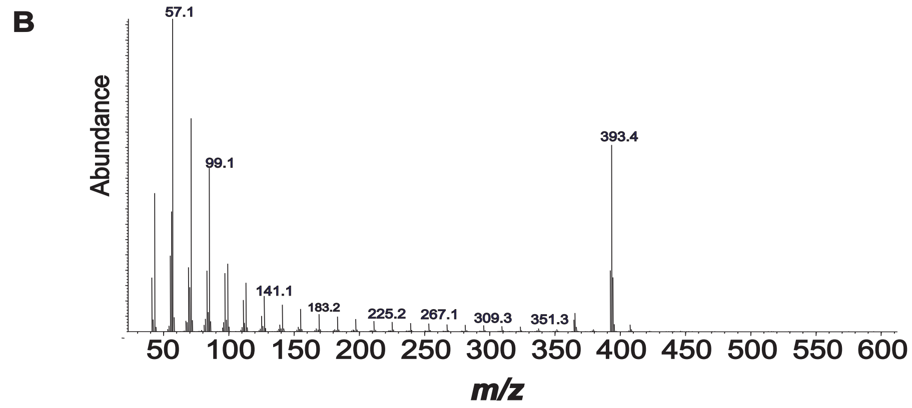
3.6. Appendix

A library of chiral methyl-branched hydrocarbons was synthesized using the Evan's alkylation methodology described in sections **3.1-3.2** of this chapter. The following table shows the products synthesized, the acid chloride and alkynyl lithium coupling partners utilized in the formation of the products, specific rotations of the final products, and GC-MS spectral information. The synthesis of the 3-methyl-branched hydrocarbon standards can be seen in chapter 4 of this dissertation.

Product	Acid Chloride/Chiral Synthon	Terminal Alkyne	EI-MS Diagnostic Ions	Specific Rotation $[\alpha]_D^{25}$
(*R*)-3-methylpentacosane	(*R)*-2-methylbutanol	1-heneicosyne	351 (M^+-15), 337, 309, 57	-3.30 ± 0.01 (c=3.35, $CHCl_3$)
(*S*)-3-methylpentacosane	(*S*)-2-methylbutanol	1-heneicosyne	351 (M^+-15), 337, 309, 57	+3.28 ± 0.03 (c=2.5, $CHCl_3$)
(*R*)-3-methylnonacosane	(*R)*-2-methylbutanol	N/A	407 (M^+-15), 393, 365, 57	-3.13 ± 0.03 (c=1.5, $CHCl_3$)
(*S*)-3-methylnonacosane	(*S*)-2-methylbutanol	N/A	407 (M^+-15), 393, 365, 57	+3.11 ± 0.03 (c=2.1, $CHCl_3$)
(*R*)-3-methylhentriacontane	(*R)*-2-methylbutanol	N/A	435 (M^+-15), 421, 393, 57	-3.05 ± 0.01 (c=2.5, $CHCl_3$)
(*S*)-3-methylhentriacontane	(*S*)-2-methylbutanol	N/A	435 (M^+-15), 421, 393, 57	+3.01 ± 0.05 (c=2.1, $CHCl_3$)
(*R*)-5-methylpentacosane	hexanoyl chloride	1-nonadecyne	351 (M^+-15), 309, 281, 85	-0.85 ± 0.01 (c=2.0, CHCl3)
(*S*)-5-methylpentacosane	hexanoyl chloride	1-nonadecyne	351 (M^+-15), 309, 281, 85	+0.82 ± 0.03 (c=2.0, CHCl3)
(*R*)-5-methylheptacosane	hexanoyl chloride	1-heneicosyne	379 (M^+-15), 337, 309, 85	-0.77 ± 0.01 (c=1.33, CHCl3)
(*S*)-5-methylheptacosane	hexanoyl chloride	1-heneicosyne	379 (M^+-15), 337, 309, 85	+0.73 ± 0.01 (c=1.35, CHCl3)
(*R*)-5-methylnonacosane	hexanoyl chloride	1-tricosyne	407 (M^+-15), 365, 337, 85	-0.69 ± 0.03 (c=2.5, CHCl3)
(*S*)-5-methylnonacosane	hexanoyl chloride	1-tricosyne	407 (M^+-15), 365, 337, 85	+0.70 ± 0.03 (c=2.3, CHCl3)
(*R*)-7-methylpentacosane	octanoyl chloride	1-heptadecyne	351 (M^+-15), 282, 252, 112	-0.253 ± 0.01 (*c* = 3.56, CH_2Cl_2)
(*S*)-7-methylpentacosane	octanoyl chloride	1-heptadecyne	351 (M^+-15), 282, 252, 112	+0.241 ± 0.01 (*c* = 3.71, CH_2Cl_2).
(*R*)-7-methylheptacosane	octanoyl chloride	1-nonadecyne	394 (M+), 308, 282, 112	-0.247 ± 0.013 (c = 3.75, CH_2Cl_2)
(*S*)-7-methylheptacosane	octanoyl chloride	1-nonadecyne	394 (M+), 308, 282, 112	+0.236 ± 0.021 (*c* = 3.48, CH_2Cl_2)
(*R*)-7-methylnonacosane	octanoyl chloride	1-heneicosyne	422 (M+), 338, 308, 112	+0.238 ± 0.01 (*c* = 2.51, CH_2Cl_2).
(*S*)-7-methylnonacosane	octanoyl chloride	1-heneicosyne	422 (M+), 338, 308, 112	+0.241 ± 0.01 (*c* = 2.90, CH_2Cl_2).
(*R*)-9-methylpentacosane	decanoyl chloride	1-pentadecyne	351 (M^+-15), 252, 224, 140	-0.181 ± 0.01 (*c* = 3.0, $CHCl_3$).
(*S*)-9-methylpentacosane	decanoyl chloride	1-pentadecyne	351 (M^+-15), 252, 224, 140	+0.185 ± 0.01 (*c* = 3.1, $CHCl_3$).
(*R*)-9-methylnonacosane	decanoyl chloride	1-nonadecyne	422 (M+), 308, 280, 140	-0.168 ± 0.01 (*c* = 3.5, $CHCl_3$).
(*S*)-9-methylnonacosane	decanoyl chloride	1-nonadecyne	422 (M+), 308, 280, 140	+0.170 ± 0.01 (*c* = 3.5, $CHCl_3$).
(*R*)-11-methylheptacosane	dodecanoyl chloride	1-tridecyne	379 (M^+), 252, 168	-0.102 ± 0.03 (*c* = 3.0, $CHCl_3$).
(*S*)-11-methylheptacosane	dodecanoyl chloride	1-tridecyne	379 (M^+), 252, 168	+0.100 ± 0.01 (*c* = 3.5, $CHCl_3$).
(*R*)-11-methylnonacosane	dodecanoyl chloride	1-pentadecyne	407 (M^+), 280, 168	-0.095 ± 0.03 (*c* = 3.0, $CHCl_3$).
(*S*)-11-methylnonacosane	dodecanoyl chloride	1-pentadecyne	407 (M^+), 280, 168	+0.101 ± 0.03 (*c* = 3.6, $CHCl_3$).
(R)-13-methylheptacosane	tetradecanoyl chloride	1-tridecyne	379 (M^+-15), 224, 196	-0.051 ± 0.03 (*c* = 4.5, $CHCl_3$).
(*S*)-13-methylheptacosane	tetradecanoyl chloride	1-tridecyne	379 (M^+-15), 224, 196	+0.055 ± 0.03 (*c* = 4.5, $CHCl_3$).

Table 3.2. Synthesized chiral methyl-branched hydrocarbons and relevant spectral information.





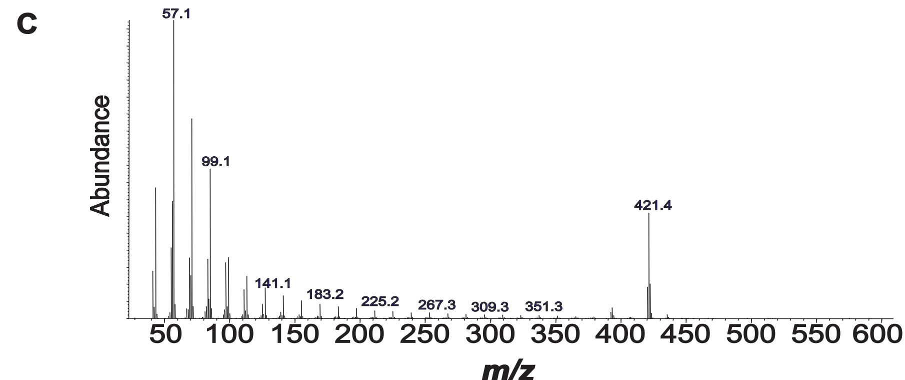


Figure 3.1. Mass spectra of chiral synthetic 3-methylalkanes: (A) mass spectrum of (*R*)- and (*S*)-3-methylpentacosane; (B) mass spectrum of (*R*)- and (*S*)-3-methylnonacosane; (C) mass spectrum of (*R*)- and (*S*)-3-methylhentriacontane.

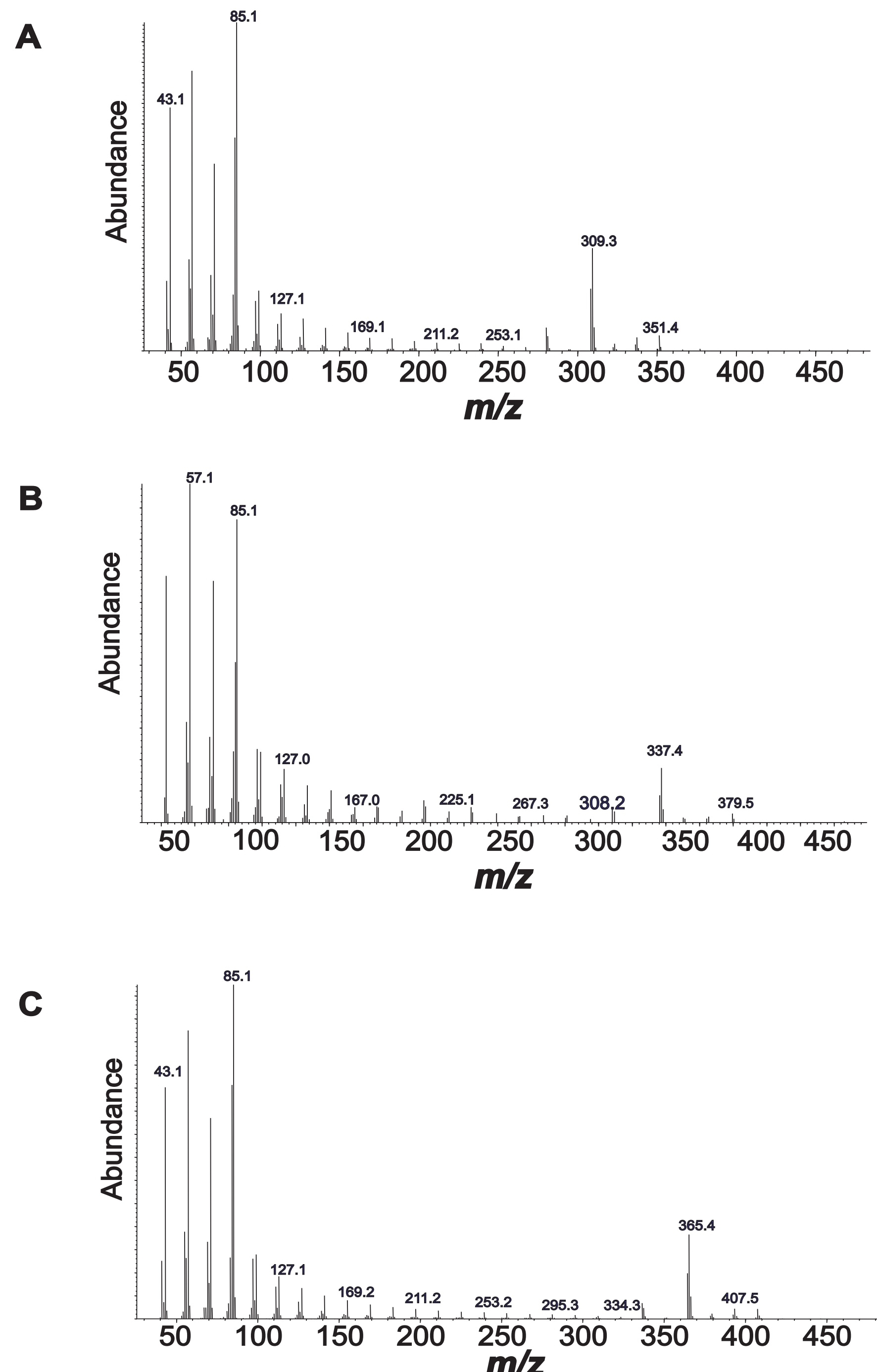
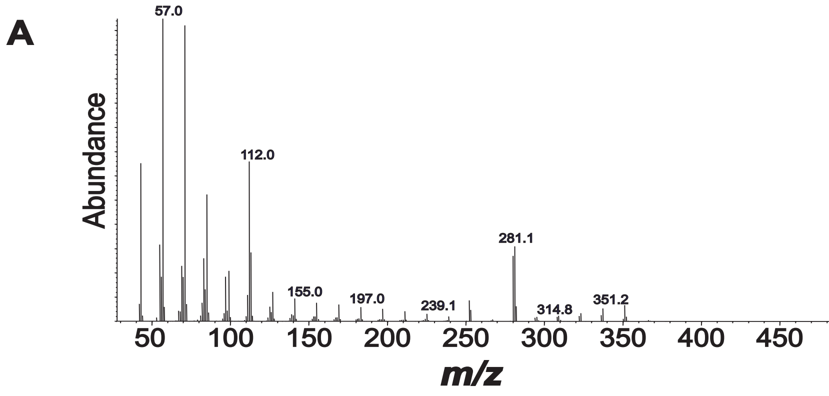
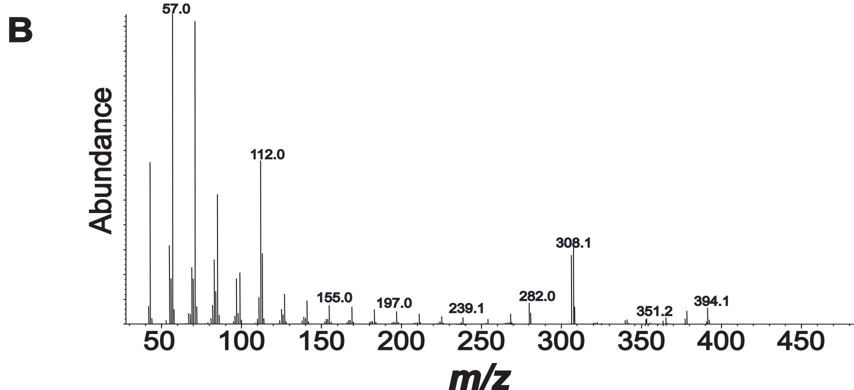


Figure 3.2. Mass spectra of chiral synthetic 5-methylalkanes: (A) mass spectrum of (*R*)- and (*S*)-5-methylpentacosane; (B) mass spectrum of (*R*)- and (*S*)-5-methylheptacosane; (C) mass spectrum of (*R*)- and (*S*)-5-methylnonacosane.





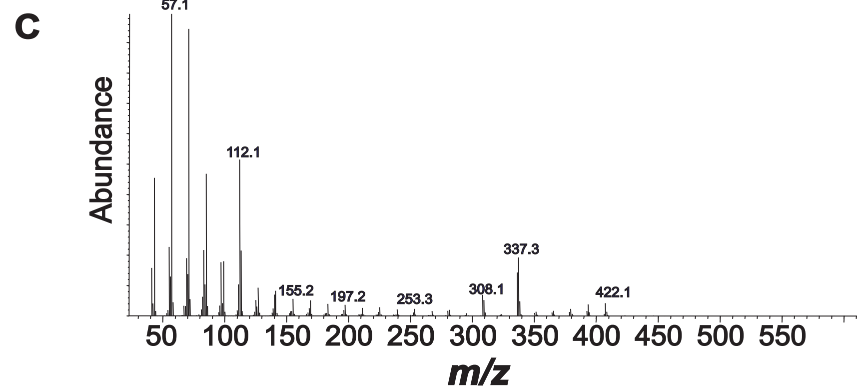
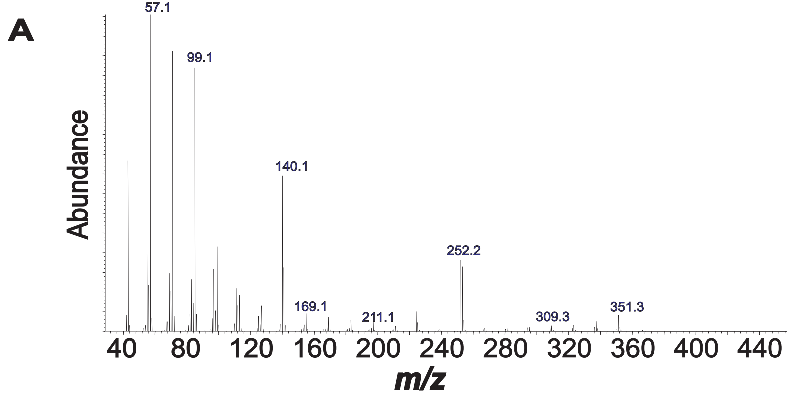


Figure 3.3. Mass spectra of chiral synthetic 7-methylalkanes: (A) mass spectrum of (*R*)- and (*S*)-7-methylpentacosane; (B) mass spectrum of (*R*)- and (*S*)-7-methylheptacosane; (C) mass spectrum of (*R*)- and (*S*)-7-methylnonacosane.



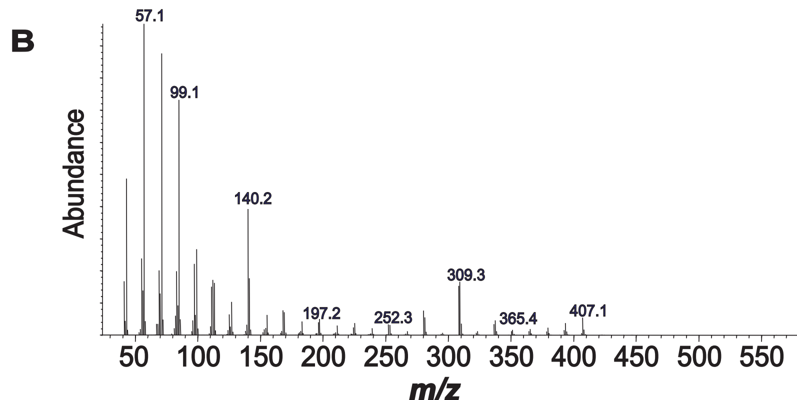
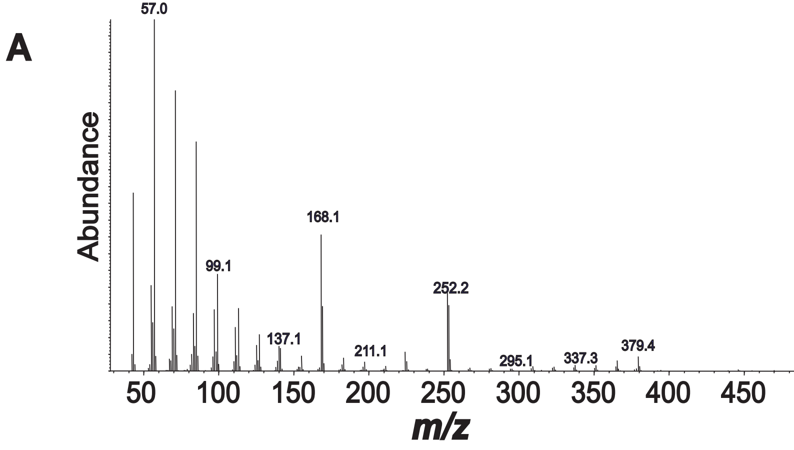


Figure 3.4. Mass spectra of chiral synthetic 9-methylalkanes: (A) mass spectrum of (*R*)- and (*S*)-9-methylpentacosane; (B) mass spectrum of (*R*)- and (*S*)-9-methylnonacosane.



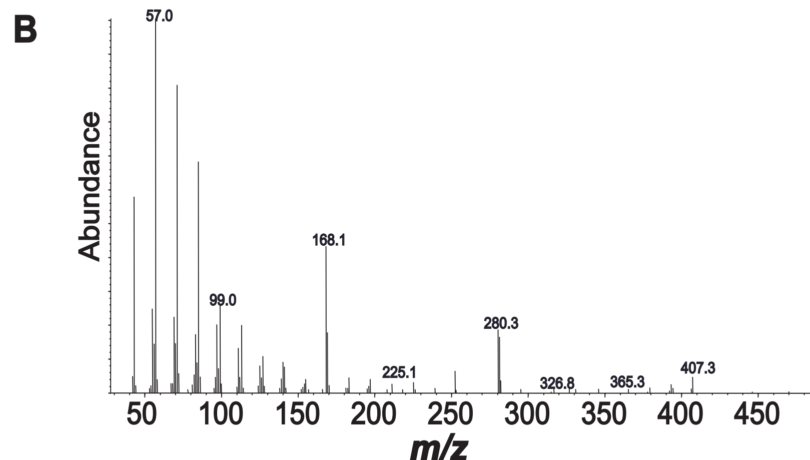


Figure 3.5. Mass spectra of chiral synthetic 11-methylalkanes: (A) mass spectrum of (*R*)- and (*S*)-11-methylheptacosane; (B) mass spectrum of (*R*)- and (*S*)-11-methylnonacosane.

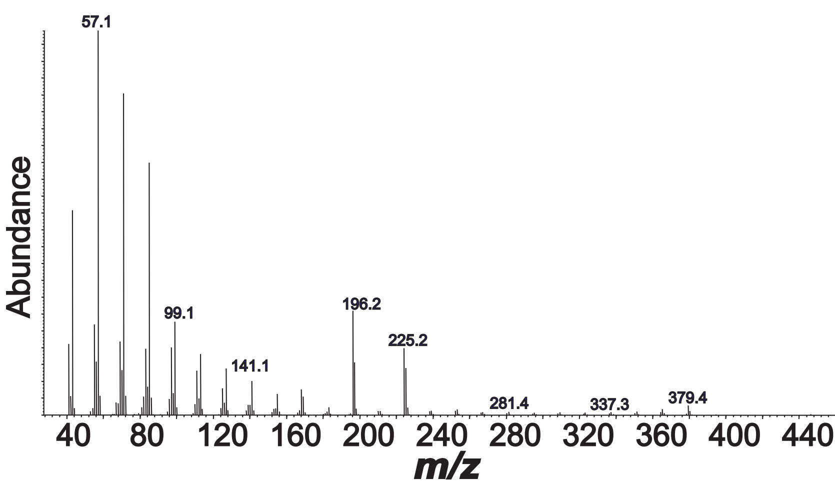


Figure 3.6. Mass spectrum of (*R*)- and (*S*)-13-methylheptacosane.

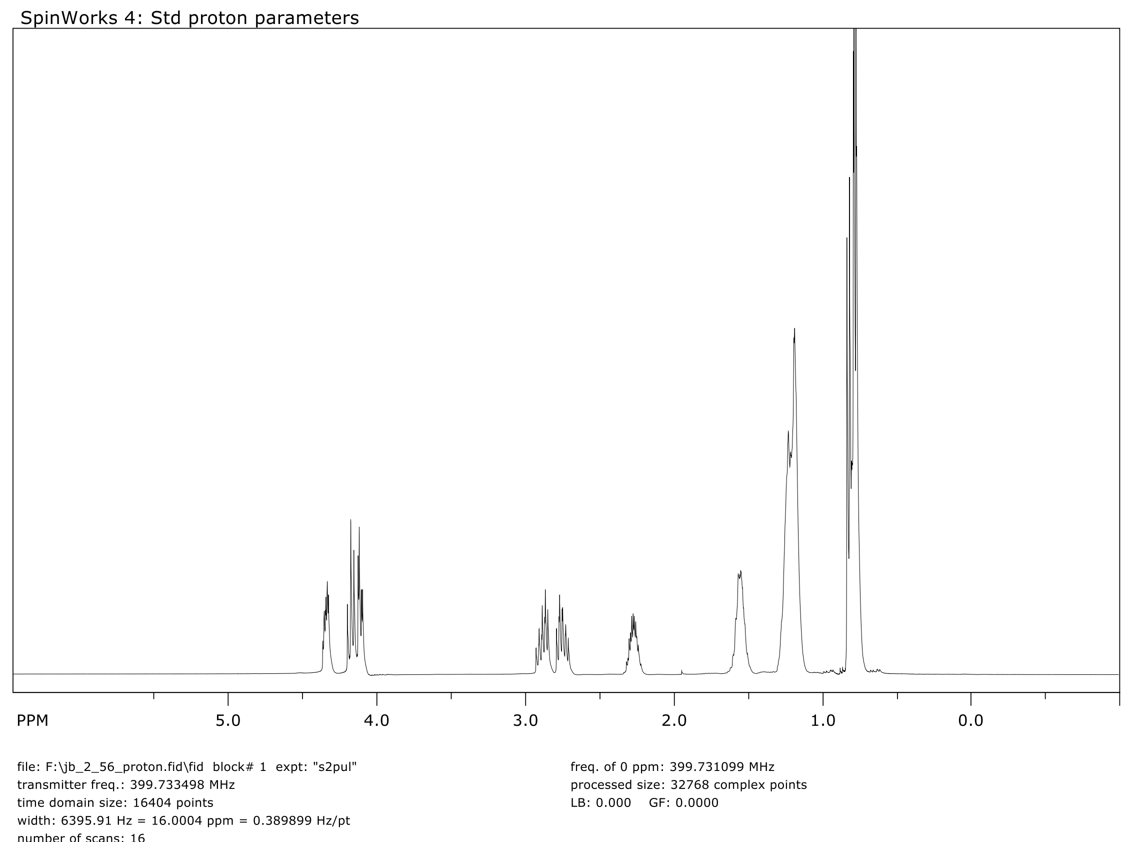


Figure 3.7. Proton NMR of (*R*)-4-isopropyl-3-octanoyloxazolidin-2-one, (*R*)-**4**

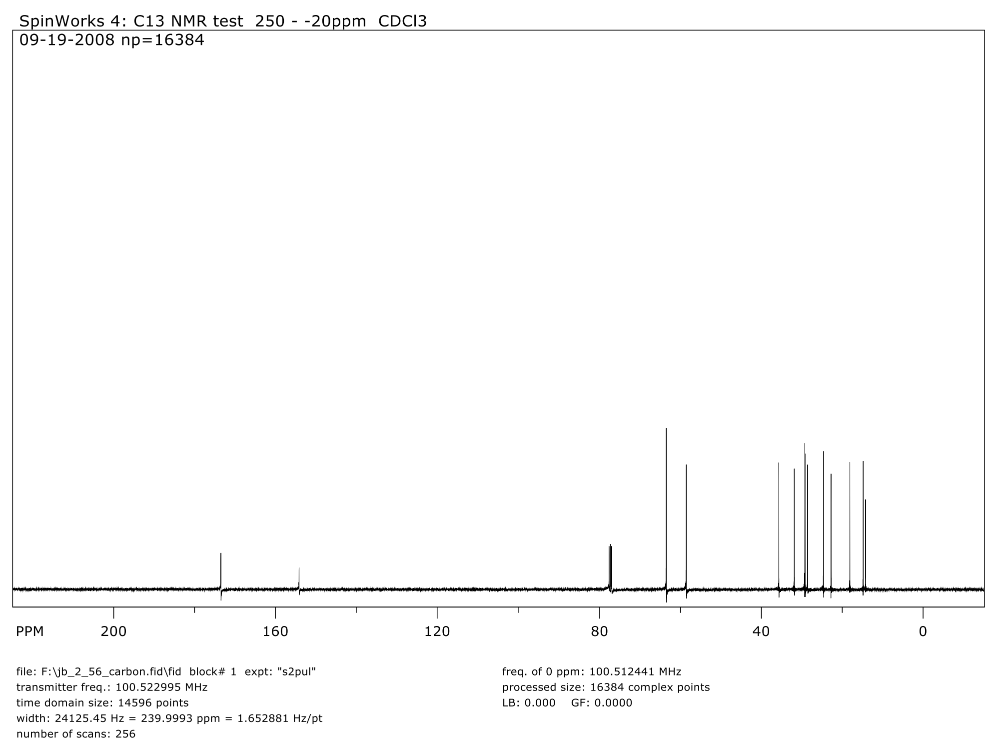


Figure 3.8. Carbon-13 NMR of (*R*)-4-isopropyl-3-octanoyloxazolidin-2-one, (*R*)-**4.**

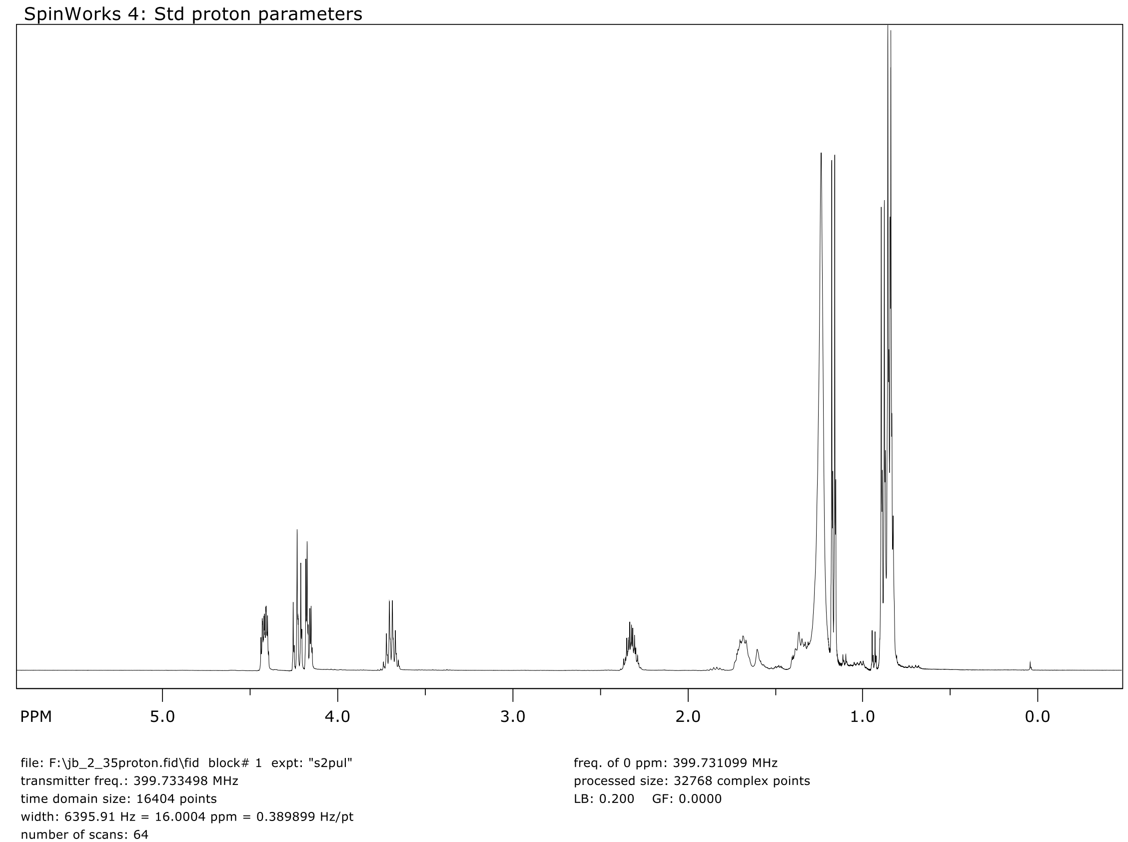


Figure 3.9. Proton NMR of (*R*)-4-isopropyl-3-((*R*)-2-methyloctanoyl)oxazolidin-2-one, (*R*)-**5.**

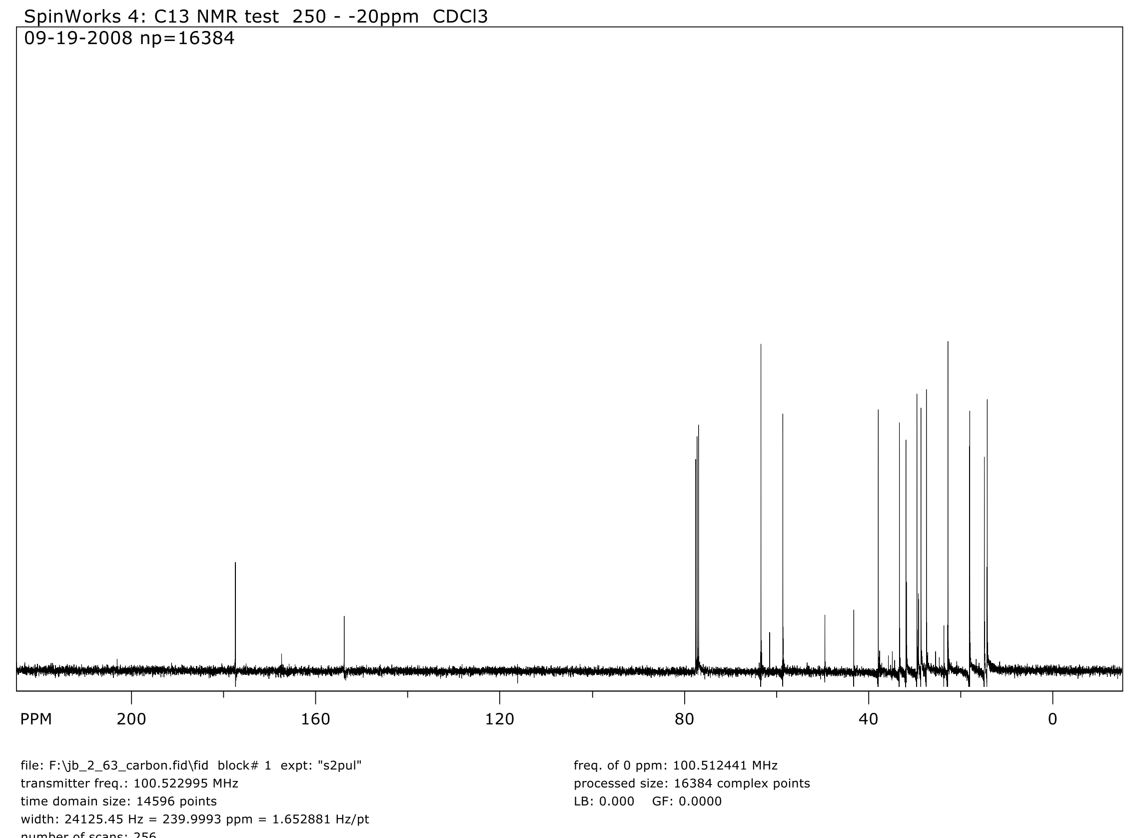


Figure 3.10. Carbon-13 NMR of (*R*)-4-isopropyl-3-((*R*)-2-methyloctanoyl)oxazolidin-2-one, (*R*)-**5.**

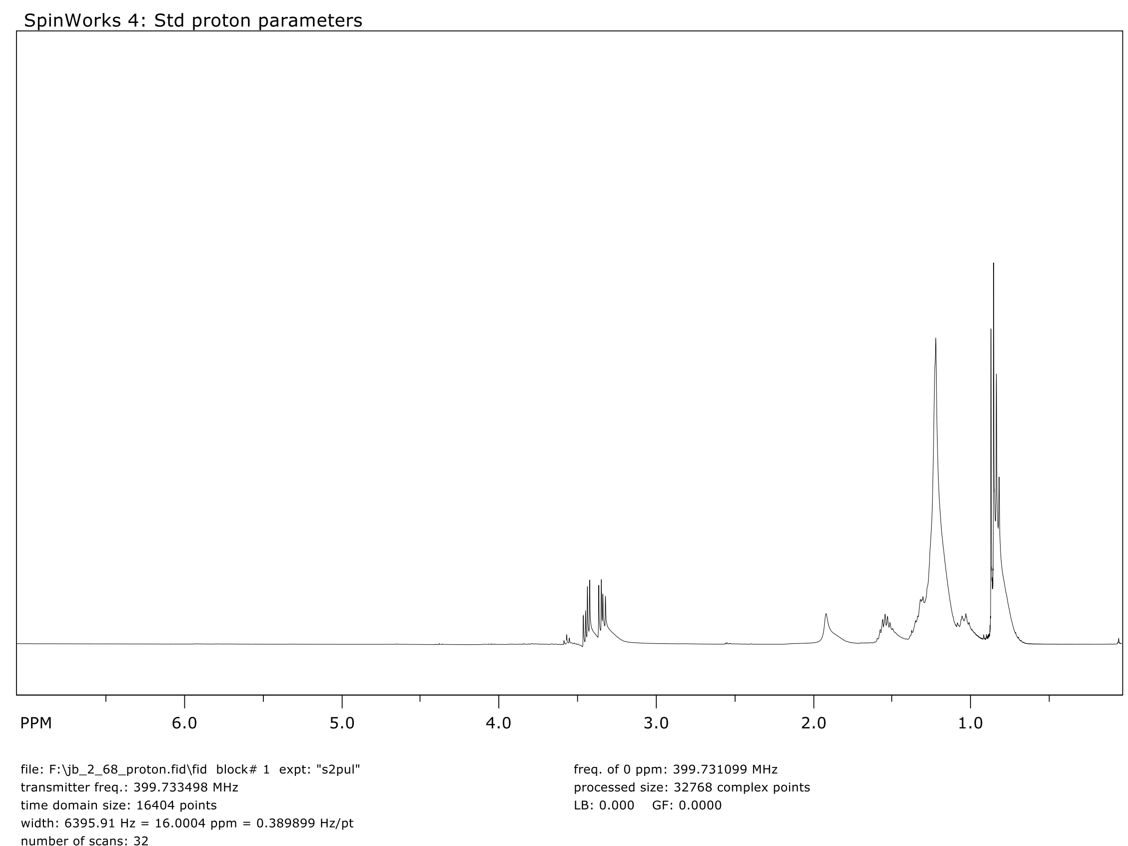


Figure 3.11. Proton NMR of (*R*)-2-methyloctan-1-ol, (*R*)-**6**.

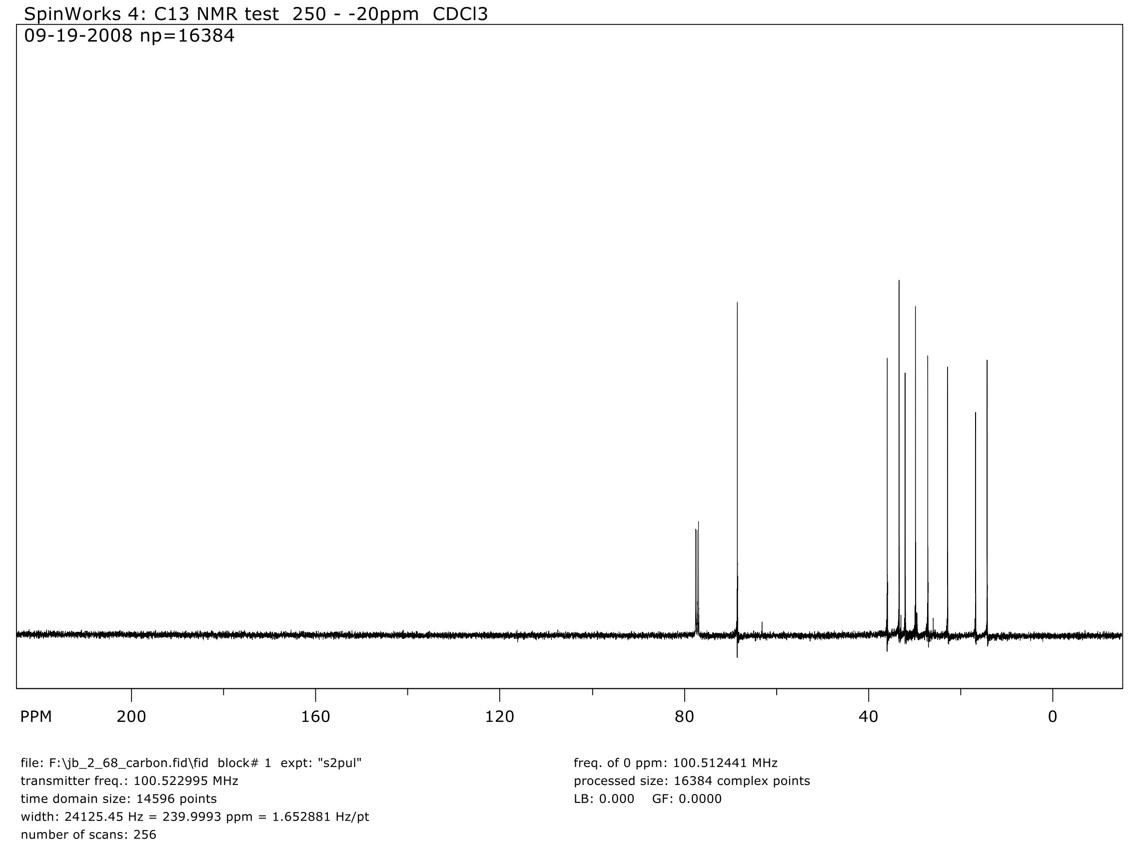


Figure 3.12. Carbon-13 NMR of (*R*)-2-methyloctan-1-ol, (*R*)-**6**.

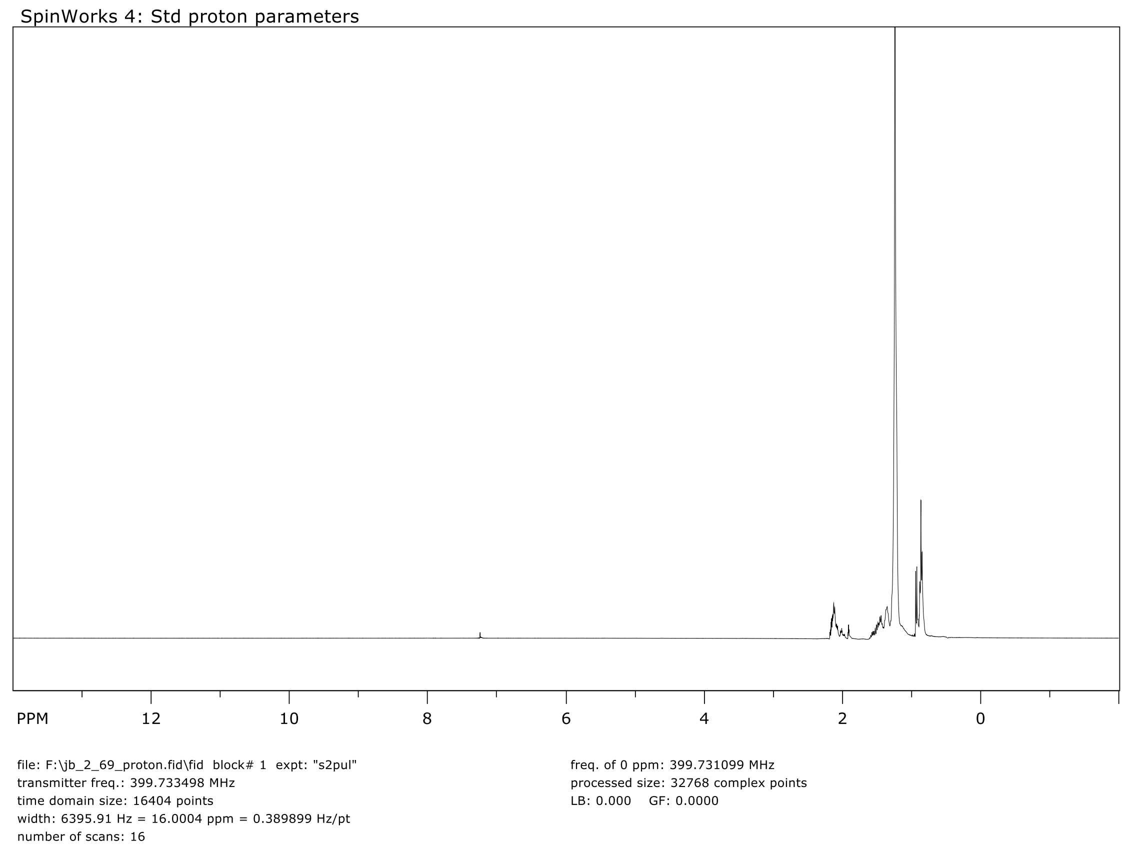


Figure 3.13. Proton NMR of (*R*)-7-methylheptacos-9-yne, (*R*)-**8**.

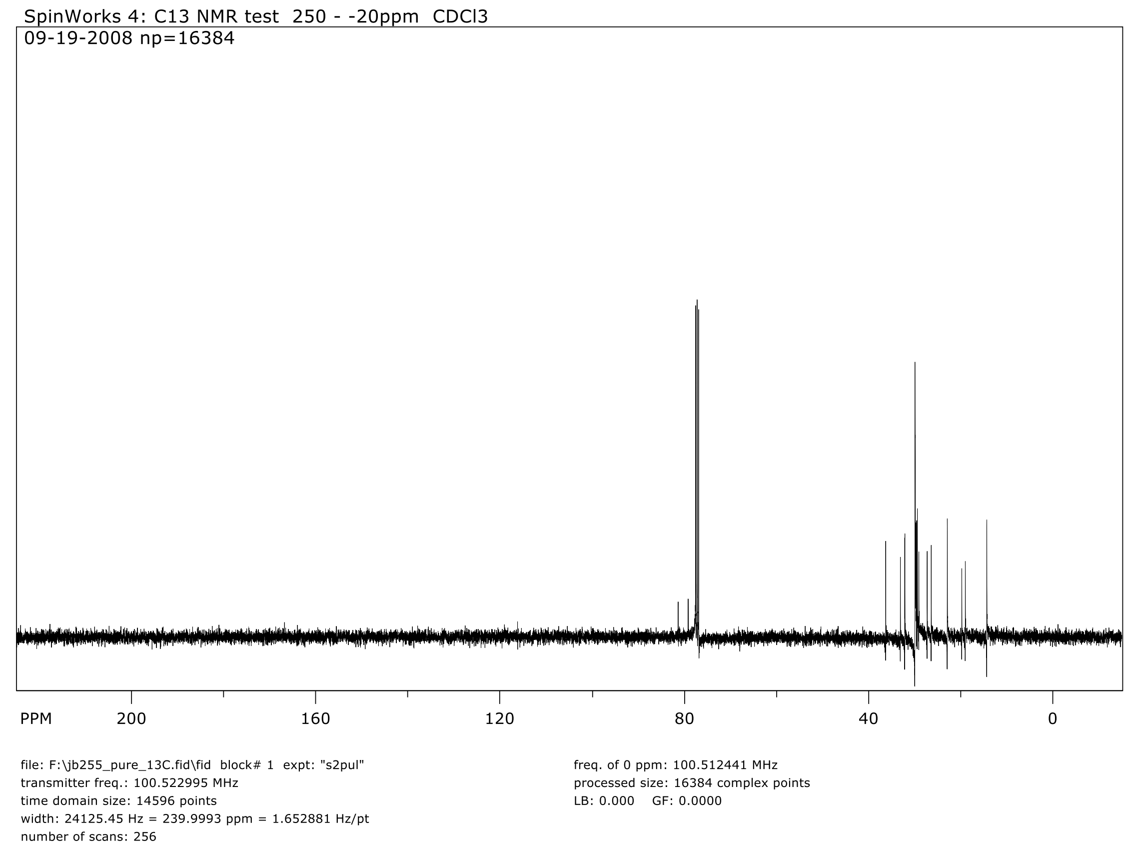


Figure 3.14. Carbon-13 NMR of (*R*)-7-methylheptacos-9-yne, (*R*)-**8**.

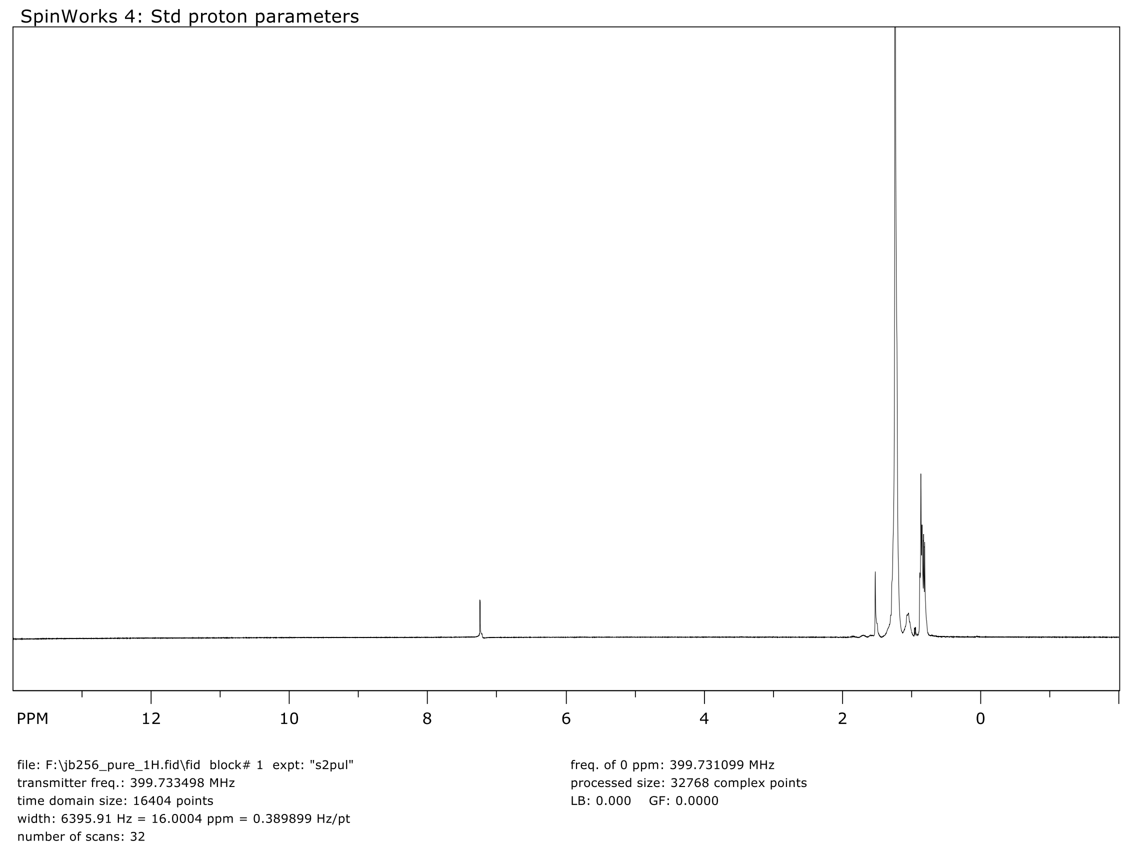


Figure 3.14. Proton NMR of (*R*)-7-methylheptacosane, (*R*)-**1**.

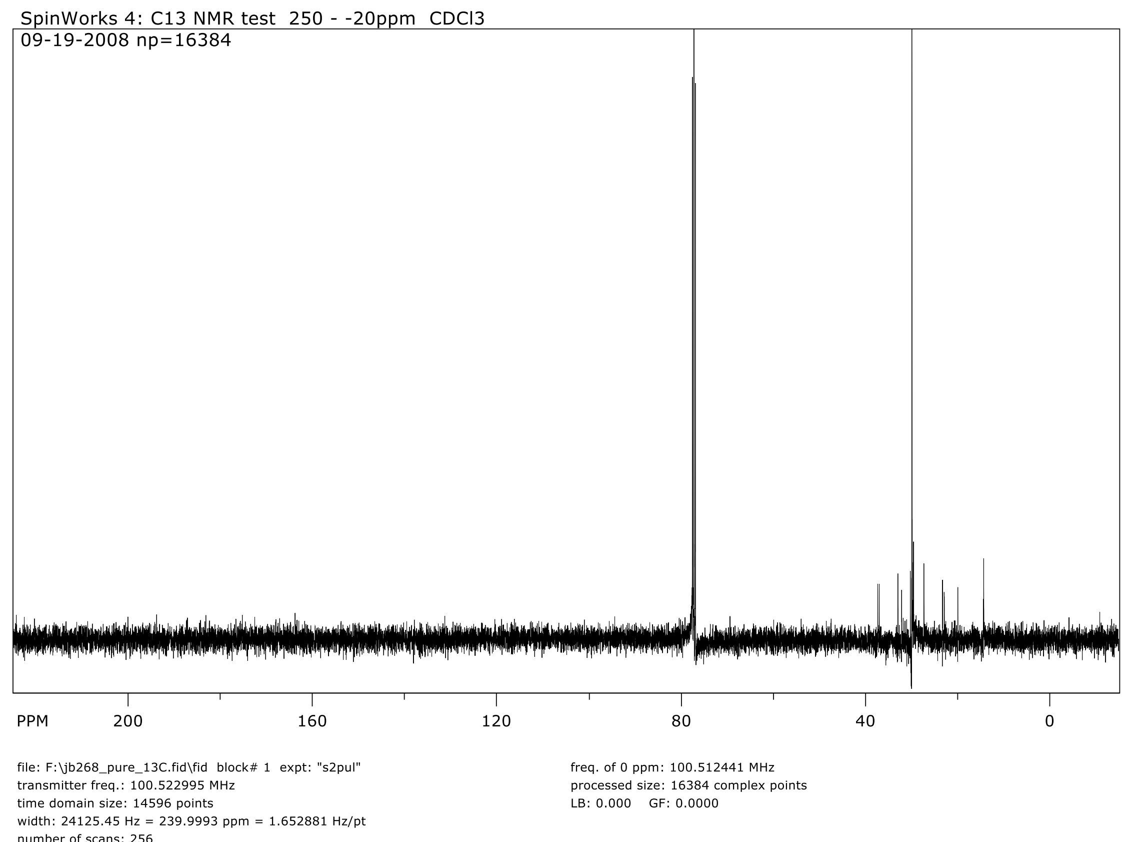


Figure 3.16. Carbon-13 NMR of (*R*)-7-methylheptacosane, (*R*)-**1**.

3.8. References

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Part II. Applications of Chiral Methyl-branched Hydrocarbons

Chapter 4. Elucidating Structure-Bioactivity Relationships of Methyl-Branched Alkanes in the Contact Sex Pheromone of the Parasitic Wasp *Lariophagus distinguendus*

4.1. Introduction

Insects utilize chemical signals and cues in all aspects of their life histories and ecologies, and thus possess an innate ability to detect and discriminate different chemicals and associate them with the correct biological context. It has been well established that insects employ volatile substances for long-range communication, but more recently it has become clear that many insects also utilize nonvolatile compounds as short-range or contact pheromones.[1] These compounds are components of the protective layer of cuticular lipids covering the insect exoskeleton. This lipid layer consists primarily of a complex blend of n-alkanes, methyl-branched alkanes, and alkenes, typically with chain lengths of about 21-37 carbons (referred to as cuticular hydrocarbons, CHCs), as well as more polar compounds such as long-chain fatty acids, alcohols, aldehydes, wax esters, and triacylglycerides.[2-5] CHCs function primarily as a hydrophobic barrier preventing desiccation,[6] but components of this protective layer are also utilized in insect communication[1]. Solitary insects use CHCs to recognize conspecifics and to determine gender, and thus identify potential mates.[1,7,8] CHCs are also employed as fertility signals, to mark territories, and exploited as kairomones .[7,9] In social insects, CHCs are directly involved in nestmate recognition, formation and maintenance of social castes, and determination of the health and fecundity of the reproductive caste.[1]

The CHC profiles of insects can range from relatively simple mixtures of only a few compounds to complex blends of more than 100 substances.[10,11] However, little is known about how insects perceive and process the information that is encoded in the cuticular lipids.[12-15] It is assumed that insects use only a small subset of the cuticular compounds as semiochemicals[16-20] with the majority of CHC components being considered to have little or no communicative function.[10,11,21] The biological activity of methylalkanes and alkenes is directly correlated with their chain lengths and the positions of methyl branch points and double bonds, respectively.[11,13,17-19,22] This suggests that methylalkanes and alkenes are better suited for use as signal molecules than straight-chain alkanes because they possess additional structural features that provide for discrimination using criteria other than chain length alone.[10,21,23] Methylalkanes often occur on the insect cuticle as series of homologs, with methyl branch points at the same position in chains of variable length.[11] It is still unclear whether insects are able to discriminate such homologs or if they "generalize" them. In the latter case, methylalkanes differing only in chain length might convey the same amount of information and therefore be used as "synonyms."[11,13,19] This would make CHC profiles functionally far less complex than one would expect from the mere number of compounds.[11,24] Further potential information might be encoded in the stereochemistry of methylalkanes[3,25,26] and the relative proportions in which they occur in the CHC profiles of insects.[10,12] However, despite the substantial body of literature on the semiochemical functions of CHCs, many details on the relationships between structural features and bioactivity remain to be elucidated.

Lariophagus distinguendus Förster (Hymenoptera: Pteromalidae) is an idiobiont ectoparasitoid that parasitizes the larvae and pupae of several species of beetles that infest stored products.[27,28] Females produce a contact sex pheromone which is secreted onto their cuticles. Males are arrested by this pheromone and respond by performing stereotypical courtship behaviors that include high-frequency wing-fanning.[29,30] Interestingly, the pupae of both sexes as well as newly emerged males apparently produce the same pheromone blend as females, but young males deactivate the pheromone within 32 hours after emergence. This deactivation is accompanied by the loss of 3-methylheptacosane (3-MeC27) and some minor CHCs.[31,32] The mechanism behind the disappearance of 3-MeC27 from the maturing male cuticle is not yet known, but it has been shown that males killed before the pheromone deactivation period retain the attractive hydrocarbon blend indefinitely.[31] Reapplication of synthetic 3-MeC27 onto the cuticle of aged males fully reinstated the pheromonal activity, so that they were courted by sexually mature males.[3] Thus, 3-MeC27 appears to be a key component of the *L. distinguendus* contact sex pheromone. However, experiments using fractionated bioactive lipid extracts revealed that 3-MeC27 only elicited a response when it was presented in combination with a chemical background of the other CHCs and triacylglycerides that also occur on the cuticle of *L. distinguendus* wasps.[3] The results mentioned above have shown that the disappearance of a single compound from a bioactive CHC profile can terminate the wing-fanning response of *L. distinguendus* males. It is not known, however, whether a bioactive CHC profile can also be disturbed by adding individual compounds, as has been shown in the context of nestmate recognition in social insects.[33]

In this study, we investigated the structure-bioactivity relationships of methyl-branched CHCs in *L. distinguendus*. In particular, we tested whether the responses of males to 3-MeC27 were specific with respect to chain length, position of the methyl branch, and absolute configuration. In addition, we tested the hypothesis that the response elicited by bioactive CHC profiles, such as those of females and newly emerged males, can be disrupted by the addition of synthetic methylalkanes and n-alkanes to those cuticular profiles.

4.2. Materials and Methods

4.2.1. Insects

Lariophagus distinguendus wasps were reared on late instar larvae and prepupae of the granary weevil *Sitophilus granarius* (Curculionidae) at 25 °C and 40–50% relative humidity under a photoperiod of 12 h:12 h light:dark.[28] Male wasps used as responders in bioassays were isolated shortly after emergence and kept separately for two days under the described rearing conditions. Two types of dead wasps were used as dummies to study the effects of added synthetic alkanes on the pheromonal activity of the wasps' CHC profiles. Type one dummies were males that had been isolated for 4 d and were subsequently freeze-killed (referred to as 4-d-old males). These males no longer elicited pheromonally-induced wing-fanning responses from courting males[31] and were used in experiment one (see below). Type two dummies were males and females which were freeze-killed immediately after emergence (referred to as 0-d-old males/females). These dummies elicited intense wing-fanning behavior in responding males[31] and were utilized

in experiment two. All dummies were stored at -23° C, and were defrosted immediately prior to bioassays.

4.2.2. Synthesis of the enantiomers of 3-methylhentriacontane and 3-methylnonacosane

The synthesis of the enantiomers of 3-methylhentriacontane and 3-methylnonacosane utilized a combination of both the alkynylations and Grignard cross coupling reactions described in chapter 3. Both methods were utilized as chain extension methods to avoid the use of long-chain alkyl Grignard and alkynyl lithium alkylation reagents. The use of these long-chain alkylation agents inevitably resulted in the contamination of the chiral methyl-branched hydrocarbon products with long-chain alkyl side products that were difficult or impossible to remove by recrystallization, or tedious, time-consuming, and expensive (in terms of solvent and chromatography media) to remove by reverse phase chromatography. The synthesis of (*R*)-3-methylhentriacontane began with the enzymatic resolution of (±)-2-methylbutanol with *Pseudomonas fluorescence* lipase and vinyl acetate in dry CH_2Cl_2.[40] This kinetic resolution enantioselectively esterifies (*S*)-(-)-2-methylalkanols to form the corresponding (*S*)-(-)-2-methylalkyl acetate while leaving the (*R*)-(+)-2-methylalkanols largely unreacted in good enantiomeric excess (Scheme 4.1). (*R*)-(+)-2-Methylbutan-1-ol **1** was isolated from the mixture by column chromatography and the enantiomeric purity was measured by gas chromatography on a β-Dex225 chiral stationary phase column (Figure 4.1). (*R*)-(+)-2-Methylbutan-1-ol was then treated with triflic anhydride and pyridine to form (*R*)-(+)-2-methylbutan-1-yl triflate **2**, which subsequently underwent a Li_2CuCl_4-catalyzed Grignard cross coupling reaction with (11-

((*tert*-butyldimethylsilyl)oxy)undecyl) magnesium bromide to form *tert*-butyldimethyl-((13-methylpentadecyl)oxy)silane **3** in 78% yield (Scheme 4.2).

P. fluorescens Lipase, vinyl acetate

CH_2Cl_2, 25 ºC, 24-36 h

Scheme 4.1. Enzymatic resolution of (±)-2-methylalkan-1-ol with *Pseudomonas fluorescens* lipase.

The formation of the desired chiral methyl-branched hydrocarbon product required deprotection of **3**, then triflation of the corresponding alcohol to form a leaving group, and alkynylation of the resulting triflate. Chong-Armstrong et al. have reported that efficient alkynylation of alkyl halides occurs under reflux conditions in THF with terminal alkynyllithiums.[45] In an effort to avoid time-consuming deprotection and purification steps, the direct bromination of **3** was performed by treatment of the silyl ether with Ph_3PBr_2 in dichloromethane at -10 ºC to form alkyl bromide **4** (91% yield), with Ph_3PBr_2 being prepared *in situ* by addition of Br_2 to Ph_3P in dichloromethane at -78 ºC.[36] Dilution of the reaction mixture with hexanes (3x reaction volume) results in the precipitation of triphenylphosphine oxide, which can be easily removed by filtration. The resulting TBDMSiOH byproduct can also be easily removed from the desired alkyl bromide by Kugelrohr distillation. Alkynylation of **4** with 1-hexadecynyllithium in refluxing THF resulted in the formation of 29-methylhentriacont-15-yne **5** (81 % yield), which was subsequently reduced to the desired methyl alkane (*R*)-**7** *via* a Rh/C catalyzed

hydrogenation (53% overall yield). (*R*)-3-Methylnonacosane (*R*)-**8** was obtained in similar fashion by substitution of 1-hexadecynyllithium with 1-tetradecynyllithium.

Scheme 4.2. Synthesis of (*R*)-3-methylnonacosane [(*R*)-**8**], (*R*)-3-methylhentriacontane [(*R*)-**7**], (*S*)-3-methylnonacosane [(*S*)-**8**], and (*S*)-3-methylhentriacontane [(*S*)-**7**]. Reagents: **(a)** Tf_2O, pyridine, CH_2Cl_2 (quantitative); **(b)** (11-(*tert*-butyldimethylsilyloxy)undecyl)magnesium bromide, Li_2CuCl_4, Et_2O (78 %); **(c)** Ph_3PBr_2, CH_2Cl_2 (91.5 %); **(d)** tetradecynyllithium for **6**, hexadecynyllithium for **5**, THF (82 % and 85 %, respectively); **(e)** 5 % Rh/C, H_2, hexane (91.5 %, 53% overall yield).

(*S*)-3-methylhentriacontane **7** and (*S*)-3-methylnonacosane **8** were obtained in similar fashion by substitution of (*R*)-(-)-2-methylbutan-1-ol for (*S*)-(+)-2-methylbutan-1-ol. Unlike (*R*)-(-)-2-methylbutan-1-ol, which was obtained by kinetic enzymatic

resolution of racemic 2-methylbutanol, (*S*)-(+)-2-methylbutan-1-ol is commercially available and so was purchased (Alfa Aesar).

4.2.3. Synthesis of (*R*)-5-Methylheptacosane (*R*)-15 and (*S*)-5-Methylheptacosane (*S*)-15 (Scheme 4.3)

(*R*)-5-methylpentacosane **15** was obtained utilizing the same synthetic procedure for the enantiomers of 7-methylheptacosane described in chapter 3. The synthesis began with deprotonation of the chiral auxiliary, (*R*)-4-isopropyloxazolidin-2-one **9**, with *n*-BuLi at -78 ºC, which was subsequently acylated with hexanoyl chloride to form (*R*)-3-hexanoyl-4-isopropyloxazolidin-2-one **10** in 96% yield.[41] The chiral auxiliary **9** was prepared in 1 step by addition of (*D*)-valinol to diethyl carbonate under reflux conditions.[42] Asymmetric alkylation of **10** was then completed by α-deprotonation of the oxazolidinone imide with NaHMDS in THF at -78 ºC. The resulting (Z)-enolate intermediate was subsequently alkylated with methyl iodide to afford ((*R*)-2-methylhexanoyl)oxazolidinone **11** in 94% yield (d.r., > 99:1, as determined by GC analysis and ^{1}H NMR).[43] Reduction of **11** with $LiBH_4$ in Et_2O resulted in preparation of the chiral alcohol intermediate (*R*)-**12** in 90% yield (e.e. > 97% as determined by chiral GC analysis), along with **9** (75% recovery) which was reused in subsequent Evans' alkylation reactions.[44] Alcohol **12** was then converted quantitatively to the alkyl triflate **13** by sequential treatment of **12** with triflic anhydride and pyridine in CH_2Cl_2.[45] Triflate **12** was immediately alkynylated with heneicosynyl lithium in THF, prepared by deprotonation of 1-heneicosyne with *n*-BuLi in THF at -78 ºC, to form (*R*)-5-methylheptacos-7-yne **14** in 80% yield.[45] 1-Heneicosyne was prepared in 3 steps from 1-

eicosanol using the methodology developed by Corey and Fuchs for the preparation of terminal alkynes.[46] Following alkynylation, (*R*)-5-methylheptacosane **15** was obtained as a crystalline solid in 98% yield by 5% Rhodium on carbon catalyzed hydrogenation of (*R*)-**14** in hexanes.[47] The crude crystalline (*R*)-**15** was recrystallized from 100 % acetone to gave pure (*R*)-**15** in an overall yield of 68% from (*R*)-**3**. Although it was not possible to ascertain the chiral purity directly, it must be a minimum of >98% e.e., reflecting the enantiomeric purity of alcohol intermediate (*R*)-**6** because none of the later steps could result in loss of stereochemical integrity (Scheme 4.3).

In the same manner (*S*)-4-isopropyloxazolidin-2-one (*S*)-**9**, prepared from (*L*)-valinol, afforded 98% pure (*S*)-5-methylheptacosane **15** in 65% overall yield in 6 steps (Scheme 4.3).

4.2.5. Synthesis of (*R*)- and (*S*)-7-Methylheptacosane

The enantiomers of 7-methylheptacosane were synthesized as previously described.[47]

4.2.6. Synthesis of *(R)*-and *(S)*-3-Methylpentacosane, and *(R)*- and *(S)-3-* methylheptacosane

The enantiomers of 3-methylpentacosane and 3-methylheptacosane were synthesized as previously described.[48]

Scheme 4.3. Synthesis of (*R*)- and (*S*)-5-methylheptacosane (**15**). Reagents: **(a)** i. *n*-BuLi, THF; ii. $CH_3(CH_2)_4COCl$ (96 %); **(b)** i. NaHMDS, THF; ii. MeI (94 %); **(c)** $LiBH_4$, Et_2O (92 %); **(d)** Tf_2O, pyridine, CH_2Cl_2 (quantitative for **7**); **(e)** heneicosynyllithium, THF (76 – 80 %); **(f)** H_2 (1 atm), 5 % Rh/C, hexane (96 – 97 %).

4.2.7. Bioassays

General Procedures for Bioassays

Bioassays were performed in a round test arena (diameter: 10 mm; height: 3 mm) lined with filter paper. Individual unmated newly-emerged male and female *Lariophagus distinguendus* cadavers (Experiment 2) and 4-day-old unattractive male dummies

(Experiment 1) were presented to 48-hour-old live males in the test arena for 5 min intervals and the wing fanning behavioral response of males was recorded and interpreted as a direct pheromonal response to the treatment(s). The length of the wing fanning response was recorded and compared to the response time of males to solvent treated controls. For both experiments, each 48-hour-old male *L. distinguendus* was exposed to a treated cadaver and a control dummy, however the exposure to treatment group or control was randomized to prevent learning responses.[31,32]

Aliquots of 1 µl containing 150 ng of synthetic compounds (treatment) or the pure solvent (dichloromethane, control) were applied evenly to the cuticle of individual dummies with a 5 µl syringe (Hamilton, Bonaduz, Switzerland). After the solvent had evaporated for 2 min, treated dummies were transferred to the test arena and the total duration of wing-fanning of a test male was recorded during the subsequent 5 min using a stereo microscope and The Observer XT 9.0 scientific software (Noldus Information Technology, Wageningen, The Netherlands). Each male was tested twice, first with a control dummy and subsequently with a treated dummy. Test males that did not perform wing-fanning behavior towards the dummy in a given bioassay were additionally exposed to a 0-d-old female dummy as a positive control to make sure that they were responsive. Data from those few males (< 1% of all tested males) that did not respond to this positive control were discarded. All experiments were conducted with a sample size of 20 replicates (N=20). After every replicate, the test arena was thoroughly cleaned with ethanol.

Experiment 1: Restoration of Pheromonal Activity to 4-d-Old Male Dummies

This experiment was performed to determine if other structurally related methylalkanes, differing in chain length or methyl-branch position, could mimic the pheromonal activity of 3-MeC27 when added to the cuticle of 4-d-old male dummies. For this purpose, the following enantiomerically pure methylalkanes (synthesized as described above) were applied at doses of 150 ng each to the cuticle of 4-d-old male dummies: (*R*)- and (*S*)-enantiomers respectively of 3-MeC25, 3-MeC29, 3-MeC31 (correct position of the methyl branch, differing chain length), and 5-MeC27 and 7-MeC27 (correct chain length, differing position of the methyl branch). (*R*)- and (*S*)-3-MeC27 also were tested as positive controls. The dose of 150 ng for all compounds was chosen because it is the approximate amount of 3-MeC27 found on the cuticle of female wasps [31]. All compounds tested in this experiment are minor components of the *L. distinguendus* CHC profile.[31] The absolute configuration of the natural products is unknown.

Experiment 2: Interruption of Pheromonal Activity in 0-d-Old Male and Female Dummies

The disappearance of 3-MeC27 from the cuticle of aging males results in the deactivation of the contact pheromone response. Therefore we tested whether specific changes to the bioactive CHC profiles of newly emerged male and female dummies, such as the addition of isomers and homologs of 3-MeC27, could inhibit or interrupt the responses of courting males. For this purpose, the following methylalkanes were applied individually at doses of 150 ng to 0-d-old male or female dummies: (*R*)- and (*S*)-

enantiomers of 3-MeC29, 3-MeC31, 5-MeC27, and 7-MeC27. Additionally, we tested whether the addition of straight-chain alkanes (150 ng n-C27, n-C29, or n-C31) or an excess of the key component 3-MeC27 (150 ng of the (*R*)- or (*S*)-enantiomer) added to the cuticle of otherwise attractive 0-d-old male dummies, affected the wing-fanning behavior of test males. All n-alkanes tested in this experiment are minor components of the *L. distinguendus* CHC profile.[31]

4.2.4. Statistical Analysis

Data did not meet the assumptions for parametric statistical analysis. Therefore, non-parametric Wilcoxon signed rank tests were used for the comparison of the duration of wing-fanning exhibited by responding males towards different treatments (addition of a given synthetic alkane) and the corresponding solvent controls. For statistical calculations, the software R, version 2.15.1 was used.[49]

4.3. Results

Experiment 1: Restoration of Pheromonal Activity in 4-d-Old Male Dummies

The addition of either (*R*)- or (*S*)-3-MeC27 to unattractive 4-d-old male dummies restored the wing-fanning behavioral responses elicited from test males, with responding males wing-fanning for significantly longer periods in the presence of pheromone-treated dummies than in the presence of solvent-treated controls (Figure 4.1). None of the other compounds when applied at doses of 150 ng to 4-d-old male dummies affected the wing-fanning behavior of test males (Figure 4.1). Thus, males specifically detected and

responded to the key component 3-MeC27, but did not distinguish between the enantiomers when they were applied to 4-d-old dummies.

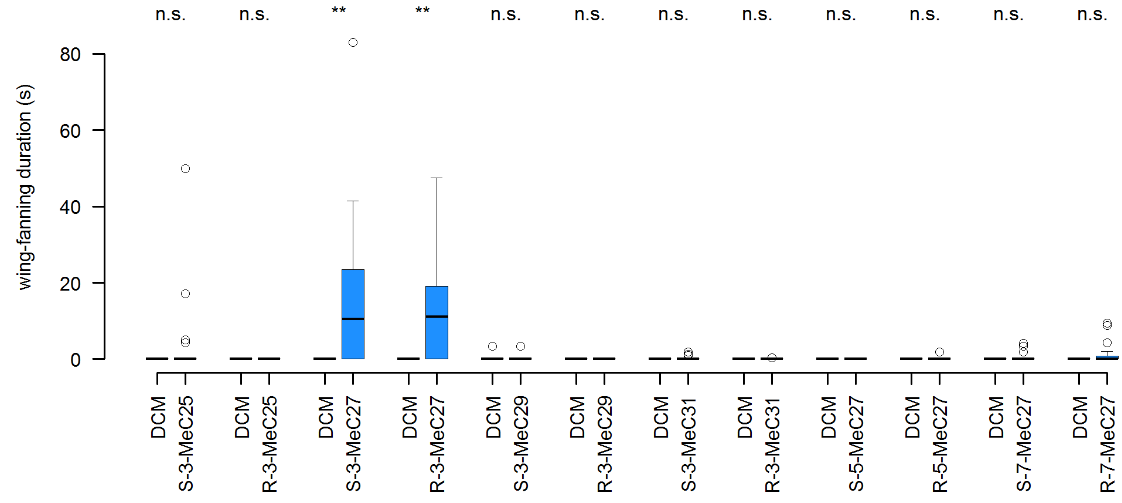

Figure 4.1. Restoration of pheromonal activity in 4-d-old male dummies (experiment 1). Wing-fanning duration during a 5-min observation period performed by *L. distinguendus* responder males towards 4-d-old male dummies treated with dichloromethane (DCM, control = white) and with 150 ng of different methyl-branched alkanes in dichloromethane, respectively (blue). Box-and-whisker plots show median (horizontal line), 25 – 75 percent quartiles (box), maximum/minimum range (whiskers) and outliers (° > 1.5 x above box height). Asterisks indicate significant differences between a methylalkane treatment and the corresponding DCM control (P > 0.05 = non-significant (n.s.), P < 0.01 = **, Wilcoxon signed rank test; N=20).

Experiment 2: Interruption of Pheromonal Activity in 0-d-Old Male and Female Dummies

The application of 150 ng of any of the tested straight-chain or methyl-branched alkanes other than (*R*)- or (*S*)-3-MeC27 onto bioactive, wing-fanning-inducing 0-d-old male dummies resulted in a significant decrease in the duration of wing-fanning by test males (Figure 4.2a). Similar results were found for the methylalkanes when added to 0-

d-old female dummies, except for (*S*)-7-MeC27, for which the decrease in wing-fanning duration was not statistically significant when compared to the solvent control (Figure 4.2b).

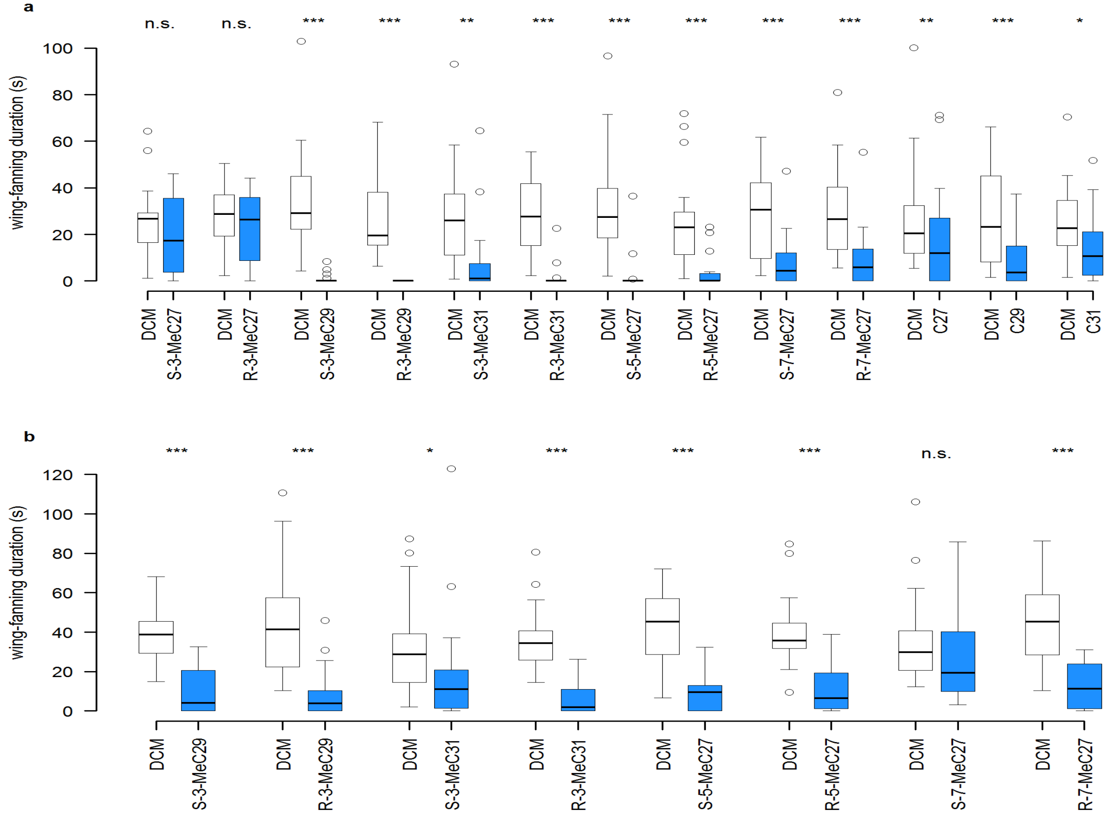

Figure 4.2. Interruption of pheromone bioactivity in 0-d-old male and female dummies (experiment 2). Duration of wing-fanning by *L. distinguendus* responder males is shown for a 5-min observation period towards 0-d-old **(a)** male and **(b)** female dummies treated either with dichloromethane (DCM, control = white) or 150 ng of methyl-branched and straight-chain alkanes, respectively (blue). Box-and-whisker plots show median (horizontal line), 25-75 percent quartiles (box), maximum/minimum range (whiskers) and outliers (° > 1.5 x above or below box height). Asterisks indicate significant differences ($P > 0.05$ = non-significant (n.s.), $P < 0.05$ = *, $P < 0.01$ = **, $P < 0.001$ = ***) between alkane treatment and the corresponding DCM control (Wilcoxon signed rank test; $N = 20$).

4.4. Discussion

The results of the present study in combination with previous work on *L. distinguendus*[3,31,50] shed new light on the role of CHCs as contact sex pheromones by showing that the CHC profile is perceived as a whole by males of this species. This is in contrast to the CHC-based contact sex pheromones of some other insects, in which individual methylalkanes have been shown to elicit behavioral responses.[18-20,51-54] In *L. distinguendus,* both the removal as well as the addition of individual components to bioactive CHC profiles disrupted the behavioral response of the receiver. Under natural conditions, conspecific males stop responding to aging males as the major contact sex pheromone component, 3-MeC27, disappears from their cuticle. The evolution of this process has presumably been driven by the fitness costs imposed on young males by the courtship activities of conspecific males[50] and has been the prerequisite for the sex-specific conveyance of information. The deactivation of the pheromone in older males was shown to be reversible experimentally by the addition of synthetic 3-MeC27. Furthermore, the response was very specific to 3-MeC27 because when equal amounts of structurally related methylalkanes with differing chain lengths or methyl branch positions were applied to dummies, they did not restore the wing-fanning response. Thus, *L. distinguendus* males respond very specifically to 3-MeC27 and can discriminate variations in chain length of two carbons, and variations in methyl branch position of two or more positions. These results suggest that a missing key component in the CHC profile cannot be replaced by a structurally related analogue, and emphasizes the critical role of 3-MeC27 in the *L. distinguendus* contact sex pheromone. In contrast, the leaf beetle

Gastrophsa atrocyanea has been shown to tolerate slight variations in the chain lengths and methyl branch points of methylalkanes in its contact pheromone without loss of bioactivity.[18] Similarly, in the longhorned beetle *Neoclytus acuminatus acuminatus,* three methylalkanes (7-MeC25, 7-MeC27 and 9-MeC27) differing in chain length or position of the methyl branch have been identified as the female contact sex pheromone. Each compound was active alone, but the combination of all three was required to elicit the full behavioral response from males.

The designation of 3-MeC27 as a key component of the contact sex pheromone of *L. distinguendus* is corroborated by the fact that treatment of attractive 0-d-old male dummies with an unnaturally high dose of synthetic 3-MeC27 resulted in no significant change in the wing-fanning responses elicited from courting males. In contrast, the application of any of the other synthetic methylalkanes onto 0-d-old male dummies resulted in a significant decrease in the wing-fanning response. The same was true when 0-d-old female dummies were treated with synthetic methylalkanes other than 3-MeC27, with the exception of those treated with (*S*)-7-MeC27, which had no significant effect on the bioactivity of 0-d-old female dummies as compared to the solvent treated controls (P=0.08). The reason for this anomaly is unclear, particularly as both enantiomers of 7-MeC27 significantly disrupted responses from males when applied to 0-d-old male dummies. All compounds interrupting the pheromone response in *L. distinguendus* males when added to bioactive CHC profiles are minor components of the natural CHC profile of this species.[31] This suggests that it is not the appearance of a novel foreign compound

but a shift in the ratios of familiar compounds which causes the loss of pheromone activity.

Disturbance of CHC profiles by the addition of synthetic compounds also has been demonstrated in the context of nestmate recognition in social insects. Addition of specific alkanes to the CHC profile of individual workers increased aggressive behavior by nestmates in some species of ants (reviewed by van Zweden & d'Ettore)[33]. Some studies provided evidence that methyl-branched alkanes might be more important in this respect than straight chain alkanes.[10,12,15,21,55] The results of the present study show that the addition of both straight-chain and methyl-branched alkanes can disturb bioactive CHC profiles in the context of sexual communication (Figure 4.1.2). In the context of nestmate recognition, it should be much easier to render a nestmate unacceptable by experimental manipulation of its CHC profile than the reverse, i.e., rendering a non-nestmate acceptable. That is, if a nestmate recognizes a non-nestmate as foreign by perceiving the species-specific CHCs in ratios differing from the known colony blend, only the exact correction of the imbalance could render it acceptable. In contrast, many different compounds added to the cuticle of a nestmate could make it unacceptable. Transferring these considerations to the present study might explain why *L. distinguendus* responded only to 3-MeC27 in the pheromone restoration experiment (experiment 1), whereas in the pheromone interruption experiment (experiment 2) many different compounds disrupted the pheromonal response equally well.

The striking parallels between the role of CHCs in nestmate recognition of social insects and sexual communication in *L. distinguendus* suggest that there might also be

analogies in the sensory organs used to detect these compounds. It has been suggested that CHCs used as sex pheromone components are perceived by gustatory sensilla. These sensilla have been predicted to be unsuitable for the perception of complex CHC profiles because they typically are innervated by only a small number of receptor neurons.[15] This idea was corroborated by the identification of a single sensillum type in the ant *Camponutus japonicus* which is innervated by multiple olfactory receptor neurons and capable of discriminating complex CHC profiles originating from nestmates and non-nestmates, respectively.[14] Given the results of the present study suggesting that *L. distinguendus* wasps, like the ants, perceive CHC profiles as a whole, it will be interesting to determine whether similar specialized sensilla also are present on the antennae of this species.

Apart from 7-MeC27 tested on 0-d-old female dummies, *L. distinguendus* males did not discriminate between the enantiomers of synthetic methylalkanes. However, in a previous study,[3] males preferred (*S*)-3-MeC27 over (*R*)-3-MeC27 when presented in a different context, i.e., when applied to filter paper together with a chemical background of the other CHCs and triacylglycerides. This preference was not seen in the present study when the enantiomers were tested with three-dimensional 4-d-old male dummies.[3] These results suggest that chemically-based sex recognition in *L. distinguendus* is supported by visual and/or tactile stimuli, as previously shown for the pteromalid wasps *Nasonia vitripennis* and *Dibrachys cavus*.[56,57] Thus, the absolute configuration of 3-MeC27 occurring on the cuticle of *L. distinguendus* wasps could not be concluded unambiguously from the behavior of males as has been done, for example, for the

enantiomers of 5-MeC27 with the egg parasitoid *Oenocyrtus kuvanae* (Encyrtidae).[25] Thus, the absolute configuration of 3-MeC27, or the ratio of enantiomers in *L. distinguendus* remains to be established by analytical methods. However, before this can be accomplished, methods need to be developed for resolving the enantiomers of methylalkanes, or of determining their absolute configuration on microgram to nanogram scale.[58,59]

4.5. Experimental

General Methods and Information for Synthesis

All solvents were Optima grade (Fisher Scientific, Pittsburgh, PA, USA). Tetrahydrofuran (THF) was distilled from sodium/benzophenone under argon. ^{1}H and 13C NMR spectra were recorded with a Varian INOVA-400 (400 and 100.5 MHz, respectively) spectrometer (Palo Alto, CA, USA), as $CDCl_3$ solutions. ^{1}H NMR chemical shifts are expressed in ppm relative to residual CHCl3 (7.27 ppm) and ^{13}C NMR chemical shifts are reported relative to CDCl3 (77.16 ppm). Solvent extracts of reaction mixtures were dried over anhydrous Na2SO4 and concentrated by rotary evaporation under reduced pressure. Crude products were purified by vacuum flash chromatography or column flash chromatography on silica gel (230-400 mesh; Fisher Scientific). Yields refer to isolated yields of chromatographically pure products. Mass spectra were obtained with a Hewlett- Packard (HP) 6890 GC (Avondale, PA) interfaced to an HP 5973 mass selective detector, in EI mode (70 eV) with helium as carrier gas. The GC was equipped with a DB17-MS column (25 m × 0.20 mm i.d., 0.33 μm film). Reactions with air- or

water-sensitive reagents were carried out in oven-dried glassware under argon. Specific rotations were obtained on a Rudolph Autopol IV digital polarimeter (Hackettstown, NJ) as CH_2Cl_2, EtOH, or $CHCl_3$ solutions, and five sequential measurements of each chiral intermediate was acquired and averaged to obtain the reported specific rotations.

4.5.1. (*R*)-2-Methylbutan-1-ol (*R*)-1

To a solution of (±)-2-methylbutan-1-ol (12.4 mL, 114 mmol) in dry dichloromethane (220 mL) was added vinyl acetate (42 mL, 454 mmol) The mixture was stirred 5 min, then *Pseudomonas fluorescens* lipase (980 mg, 300 units/mmol of substrate, Aldrich Chemical Co.) was added in one portion. The resulting mixture was stirred for 30 h and the enantiomeric excess of (*R*)-2-methylbutan-1-ol was monitored via chiral stationary phase GC (see Figure 4.1). The crude product was chromatographed on silica gel (60 g). Elution with hexane/EtOAc (9:1) afforded 1.88 g of pure (*R*)-**1**. (*R*)-2-methylbutan-1-ol **1** had the following properties: $[\boldsymbol{\alpha}]_D^{25}$= +13.46 (*c* = 2.5, EtOH); $\boldsymbol{\nu}_{max}$ (neat): 3336 (br m), 2956 (s), 2923 (s), 2855 (s), 1465 (m), 1378 (w), 1032 (s), 938 (w), 908 (w), 842 (w) 723 (w); ^{1}H NMR, ∂_H ($CDCl_3$): 0.89 (6H, m), 1.17 (1H, m), 1.18 (1H, m), 1.87 (1H, broad s, OH), 3.45 (1H, dd, *J*= 11.7 Hz, 4.8 Hz), 3.47 (1H, dd, *J*= 10.5 Hz, 5.3 Hz); ^{13}C NMR, ∂_C ($CDCl_3$): 14.0, 16.8, 27.0, 33.8, 68.5; GC-MS [Column: DB-5MS, 5% phenylmethylsiloxane, 30 m × 0.25 mm id; carrier gas, He; temp: 40-280 ºC (+ 5 ºC/min)]: t_R: 4.51 min (96.5%); MS of **6** (70 eV, EI); *m/z*: 87 (1, M^+-1), 70 (35), 56 (100), 41 (70). The enantiomeric excess (ee) was determined by GC analysis using a **β**-DEX225

column [30 m × 0.25 mm id × 0.25 μm film; carrier gas, He; temp: 35-220 ºC (held at 35 ºC for 30 min, then + 5 ºC/ min)]: t_{major}: 43.95 min (100 %).

4.5.2. (*R*)-2-Methyl-1-butan-1-yl triflate (*R*)-2

To a cold (-10 ºC) stirred solution of (*R*)-2-methylbutan-1-ol (*R*)-**1** (1.44 g, 16.3 mmol) in dry CH_2Cl_2 (80 mL) was added sequentially pyridine (1.31 mL, 16.3 mmol) and triflic anhydride (3.34 mL, 19.56 mmol). The reaction was stirred at -10 ºC for 1 h and then diluted with pentanes (160 mL) and stirred for 30 min. The resulting mixture was filtered through a plug of silica gel (30 g), and the filter cake was washed with hexanes/CH_2Cl_2 (4:1). The filtrate was concentrated in vacuo to give 3.59 g (quantitative) of (*R*)-**2** as a colorless oil, which was used immediately in the next step without further purification or characterization.

4.5.3. (*S*)-2-Methyl-1-butan-1-yl triflate (*S*)-2

In the same manner as above (*S*)-2-methylbutan-1-ol (*S*)-**1** (1.45 g, 16.5 mmol; Alfa Aesar) gave 3.63 g (quantitative) of (*S*)-2-methylbutan-1-yl triflate (*S*)-**2** as a colorless oil, which was used immediately in the next step without further purification or characterization.

4.5.4. (*R*)-*tert*-Butyldimethyl((13-methylpentadecyl)oxy)silane (*R*)-3

To a cold (-40 ^{o}C) solution of (*R*)-2-methylbutan-1-yl triflate **2** (3.59 g, 16.3 mmol) in Et_2O (60 mL) was added dropwise Li_2CuCl_4 (0.394 M, 2 mL, 0.788 mmol, 5

mol % catalyst), and the reaction was stirred 10 min. (11-((*tert*-Butyldimethylsilyl)oxy)undecyl)-magnesium bromide (2.0 M, 7.5 mL, 15 mmol) was then added to the reaction mixture over 15 min by syringe pump. The mixture was stirred for 2 h at -40 ^{o}C until the Grignard was fully consumed, then warmed to room temperature, and quenched with saturated aqueous NH_4Cl (40 mL). The layers were separated and the aqueous layer was extracted with hexanes (2 × 75 mL). The resulting organic layers were combined and washed with saturated NH_4Cl (2 × 100 mL) and brine (2 × 100 mL), dried over Na_2SO_4, and concentrated in vacuo. The crude product was chromatographed on silica gel (60 g), eluting with hexane/EtOAc (9:1) to yield 4.39 g (82%) of pure (*R*)-**3** as a colorless oil. (*R*)-*tert*-Butyldimethyl((13-methylpentadecyl)oxy)silane, (*R*)-**3,** showed the following properties: $[\boldsymbol{\alpha}]_D^{25}$= -3.87 ± 0.013 (*c* = 2.70, CH_2Cl_2); ^{1}H NMR, ∂_H ($CDCl_3$): 0.21 (6H, s), 0.85 (3H, d, *J*=11.4 Hz), 0.89 (3H, pseudotriplet, *J*=7.6 Hz), 0.98 (9H, s), 1.25 (20H, br m), 1.48 (2H, m), 1.52 (1H, quint, *J*=4.3 Hz), 3.6 (2H, td, *J*=7.6 Hz, 0.9 Hz); ^{13}C NMR, ∂_C (CDCl3): -1.90, 11.5, 22.0, 26.7, 28.2, 29.3, 29.7, 30.2, 30.5, 31.3, 33.0, 36.5, 38.0, 63.0; GC-MS [Column: DB-5MS, 5% phenylmethylsiloxane, 30 m × 0.25 mm id; carrier gas, He; temp: 100-280 ºC (+10 ºC/min)]: t_R: 16.24 min (98.5%); MS of **3** (70 eV, EI); *m/z*: 299 (41, M^+-57), 171 (1), 143 (5), 129 (2), 111 (6), 97 (17), 89 (21), 75 (100), 57(42), 41(42).

4.5.5. (*S*)- *tert*-Butyldimethyl((13-methylpentadecyl)oxy)silane 3

In the same manner as above (*S*)-2-methyl-1-butanyl triflate (3.5 g, 15.9 mmol) gave 4.10 g (76 %) of pure (*S*)-**3** as a colorless oil. $[\boldsymbol{\alpha}]_D^{25}$= +3.95 ± 0.03 (*c* = 2.70, CH_2Cl_2)

4.5.6. (*R*)-13-Methyl-1-bromopentadecane (*R*)-4

To a cold (-10 ºC) solution of PPh_3 (7.38 g, 27.63 mmol) in dry CH_2Cl_2 (100 mL) was added Br_2 (1.4 mL, 27.6 mmol) dropwise with vigorous stirring. The reaction was slowly warmed to room temperature over 30 min and stirred another 30 min. (*R*)-*tert*-Butyldimethyl((13-methylpentadecyl)oxy)silane (*R*)-**3** (3.71 g, 11.05 mmol) was then slowly added to the reaction mixture and the resulting solution was stirred 1.5 h. The reaction mixture was diluted with hexanes (200 mL) and filtered through a plug of silica gel (15 g) eluting with hexanes. The resulting solution was concentrated in vacuo to afford 3.03 g (91.5 % yield) of (*R*)-13-methyl-1-bromopentadecane (*R*)-**4** as a colorless oil with the following properties: $[\boldsymbol{\alpha}]_D^{25}$= -3.35 ± 0.05 (*c* = 1.90, CH_2Cl_2), ^{1}H NMR, ∂_H ($CDCl_3$): 0.90 (3H, t, *J*= 7.6 Hz), 0.98 (3H, d, *J*=12 Hz), 1.31 (20H, broad m), 1.54 (2H, m), 1.65 (1H, m), 1.85 (2H, m), 3.46 (1H, pseudotriplet, J=7.4 Hz), 3.49 (1H, dd, *J*=11.5, 5.3 Hz); ^{13}C NMR, ∂_c ($CDCl_3$): 11.8, 20.5, 27.1, 28.5, 29.8, 30.0, 30.2, 33.5, 32.1, 33.7, 35.5, 37.8; GC-MS [Column: DB-5MS, 5% phenylmethylsiloxane, 30 m × 0.25 mm id; carrier gas, He; temp: 100-280 ^{o}C (+10 ºC/min)]: t_R: 14.14 min (99.5%); MS of **4** (70 eV, EI); *m*/*z*: 304 (1, M^+), 275 (3), 221 (1), 207 (1), 179 (1), 163 (1), 149 (1), 135 (1), 113 (3), 97 (8), 85 (12), 71 (28), 57 (100), 41 (58).

4.5.7. (*S*)-13-Methyl-1-bromopentadecane (*S*)-4

In the same manner as described above 3.0 g (11.1 mmol) of (*S*)-**3** gave 1.48 g (93% yield, 97.9% ee) of (*S*)-13-methyl-1-bromopentadecane as a colorless oil, $[\boldsymbol{\alpha}]_D^{25}$ = +3.41 ± 0.05 (*c* = 1.90, CH_2Cl_2). Its spectra were identical to those of (*R*)-**4**.

4.5.8. (*R*)-29-Methyl-15-hentriacontyne (*R*)-5

To a cold (-78 ºC) solution of 1-hexadecyne (682 mg, 3.07 mmol) in dry THF (15 mL), was added *n*-BuLi dropwise (2.89 M, 1.32 mL, 3.82 mmol) and the resulting mixture was stirred at -78 ºC for 10 min, then at -10 ºC for 30 min. After warming to 25 ºC, (*R*)-**4** (800 mg, 2.63 mmol) dissolved in dry THF (3 mL) was added dropwise. A reflux condenser was added to the reaction apparatus and the mixture was heated to a gentle reflux and stirred overnight. The resulting mixture was then cooled to 25 ºC, quenched with saturated aqueous NH_4Cl, and extracted with hexane. The organic layer was washed with water (2 × 50 mL), aqueous $NaHCO_3$ (2 × 50 mL), and brine (50 mL), dried, and concentrated. Unreacted 1-hexadecyne was removed by Kugelrohr distillation of the crude product (oven temp. 60 ºC, 0.1 mm Hg). The residue was chromatographed over silica gel (50 g). Elution with hexanes gave 890 mg (2.15 mmol, 82%) of (*R*)-**5** as a clear oil. $[\boldsymbol{\alpha}]_D^{25}$ = -3.11 (*c* = 3.47, CH_2Cl_2); $\mathbf{v}_{max}$ (neat): 2954 (m), 2921 (s), 2852 (s), 1464 (m), 1377 (w), 1251 (w), 1056 (w), 843 (w), 721 (w); ^{1}H NMR, ∂_H ($CDCl_3$): 0.86 (6H, m), 0.91 (3H, d, *J*=6.4 Hz), 1.1-1.4 (46H, br s), 1.48 (1H, dd, *J*= 12.0 Hz, 4.7 Hz), 1.55 (1H, m), 1.98 (1H, d, *J*=12 Hz), 2.15 (2H, m); ^{13}C NMR, ∂_C ($CDCl_3$): 14.32, 18.99, 19.74, 20.9, 26.39, 27.27, 28.61, 29.05, 29.38, 29.58, 29.79, 29.91, 32.12, 33.07, 36.26,

79.56, 81.2; GC-MS [Column: DB-5MS, 5% phenylmethylsiloxane, 30 m × 0.25 mm id; carrier gas, He; temp: 100-280 ºC (+10 ºC/min)]: t_R: 33.52 min (97.32 %); MS of **5** (70 eV, EI); *m/z*: 446 (1, M^+), 417 (16), 355 (2), 324 (1), 281 (1), 225 (3), 197 (2), 141 (3), 113 (2), 85 (11), 71 (25), 57 (100), 41 (88); HRMS (EI) calcd for $C_{32}H_{62}$ (M^+): 446.4852. Found: 446.4858.

4.5.9. (*S*)-29-Methyl-15-hentriacontyne (*S*)-5

In the same manner as described above 549 mg (2.34 mmol) of (*S*)-**4** gave 846 mg (80% yield) of (*S*)-29-methyl-15-hentriacontyne **5** as a colorless oil. $[\alpha]_D^{25}$ = +3.15 ± 0.05 (*c* = 2.10, CH_2Cl_2); Its spectra were identical to those of (*R*)-**5**. HRMS (EI) calcd for $C_{32}H_{62}$ (M^+): 446.4852. Found: 446.4847.

4.5.10. (*R*)-27-Methyl-13-nonacosyne (*R*)-6

(*R*)-29-Methyl-13-nonacosyne **6** was prepared in the same fashion as (*R*)-**5** from alkyl bromide (*R*)-**4** (530 mg, 2.33 mmol) by substitution of 1-tetradecyne in place of 1-hexadecyne. Purification by Kugelrohr distillation removed unreacted 1-tetradecyne, and subsequent chromatography of the residue over silica gel (50 g) with hexanes as eluent gave 736 mg of (*R*)-**6** in 78 % yield. $[\alpha]_D^{25}$= -3.13 (*c* = 3.00, CH_2Cl_2); ν_{max} (neat): 2954 (m), 2921 (s), 2852 (s), 1464 (m), 1377 (w), 1251 (w), 1056 (w), 843 (w), 721 (w); ^{1}H NMR, ∂_H ($CDCl_3$): 0.86 (6H, m), 0.91 (3H, d, *J*=6.4 Hz), 1.1-1.4 (42H, br s), 1.48 (1H, dd, *J*= 12.0 Hz, 4.7 Hz), 1.55 (1H, m), 1.98 (1H, d, *J*=12 Hz), 2.15 (2H, m); ^{13}C NMR, ∂_C ($CDCl_3$): 14.32, 18.99, 19.74, 20.9, 26.39, 27.27, 28.61, 29.05, 29.11, 29.58, 29.79, 29.91,

32.12, 33.07, 36.26, 79.56, 81.2; GC-MS [Column: DB-5MS, 5% phenylmethylsiloxane, 30 m × 0.25 mm id; carrier gas, He; temp: 100-280 ºC (+10 ºC/min)]: t_R: 29.82 min (97.32 %); MS of **6** (70 eV, EI); *m/z*: 418 (1, M^+), 389 (10), 355 (2), 324 (1), 281 (1), 225 (3), 197 (2), 141 (3), 113 (2), 85 (11), 71 (25), 57 (100), 41 (88); HRMS (EI) calcd for $C_{30}H_{58}$ (M^+): 418.4539. Found: 418.4544.

4.5.11. (*S*)-27-Methyl-13-nonacosyne (*S*)-6

In the same manner as described above 600 mg (2.55 mmol) of (*S*)-**4** gave 874 mg (82% yield) of (*S*)-29-methyl-15-hentriacontyne **6** as a colorless oil. $[\boldsymbol{\alpha}]_D^{25}$ = +3.18 ± 0.05 (*c* = 2.10, CH_2Cl_2). Its spectra were identical to those of (*R*)-**22**. HRMS (EI) calcd for $C_{30}H_{58}$ (M^+): 418.4539. Found: 418.4547.

4.5.12. (*R*)-3-Methylhentriacontane (*R*)-7

To a slurry of 5 % Rh/C (80 mg) and anhydrous Na_2CO_3 (700 mg, 5.2 mmol) in hexanes (10 ml) was added a solution of (*R*)-**5** (800 mg, 1.93 mmol) in hexanes (5 mL) and the resulting mixture was stirred for 10 h under a slight positive pressure of H_2. The mixture was filtered through a plug of silica gel and concentrated to afford 763 mg of crude crystalline (*R*)-3-methylnonacosane [38]. Recrystallization from hexane/acetone (1:5) gave 737 mg of pure (91% yield) (*R*)-**7** in 50 % overall yield in 5 steps, mp 34 ºC, $[\boldsymbol{\alpha}]_D^{25}$= -3.05± 0.01 (c = 2.50, CH_2Cl_2). ∂_H (CDCl3): 0.84 (3H, d, *J*= 6.3 Hz), 0.85 (3H, t, *J*= 6.7 Hz), 0.87 (3H, t, *J*= 6.5 Hz), 1.16-1.4 (53 H, broad m). ^{13}C NMR, ∂_c (ppm): 11.62, 14.32, 19.45, 22.91, 25.67, 27.36, 29.58, 29.72, 29.93, 30.25, 31.81, 32.16, 34.62, 36.88.;

GCMS [Column: DB-17MS, 17% phenylmethylsiloxane, 30 m × 0.25 mm id; carrier gas, He; temp: 100-280 ºC (+20 ºC/min)]: t_R: 14.12 min (100%); MS of **7** (70 eV, EI); 421 (54, M^+-29), 407 (1), 393 (6), 379 (1), 365 (1), 351 (1), 337 (1), 323 (1), 309 (2), 295 (2), 281 (2), 267 (2), 253 (2), 239 (3), 225 (3), 211 (2), 197 (3), 183 (3), 169 (6), 155 (7), 141 (10), 127 (12), 113 (17), 99 (23), 85 (55), 71 (70), 57 (100), 43 (45). HRMS (EI) calcd for $C_{32}H_{66}$ (M^+): 450.5165. Found: 450.5159.

4.5.13. (*S*)-3-Methylhentriacontane (*S*)-7

In the same manner as described above 800 mg (1.93 mmol) of (*S*)-**5** gave 746 mg (93% yield) of (*S*)-3-methylhentriacontane **(*S*)-7** in 53% overall yield. $[\alpha]_D^{25}$= +3.01 ± 0.05 (*c* = 2.10, CH_2Cl_2); mp = 36 ºC. Its spectra were identical to those of (*R*)-**23**. HRMS (EI) calcd for $C_{32}H_{66}$ (M^+): 450.5165. Found: 450.5169.

4.5.14. (*R*)-3-methylnonacosane 8

In the same manner as described above 700 mg (1.67 mmol) of (*R*)-**6** gave 670 mg (94% yield) of (*R*)-3-methylnonacosane (*R*)-**8** in 53% overall yield. $[\alpha]_D^{23}$= -3.13 ± 0.03 (*c* = 1.50, CH_2Cl_2); mp = 34 ºC; ^{1}H NMR, ∂_H (ppm): 0.84 (3H, d, *J*= 6.3 Hz), 0.85 (3H, t, *J*= 6.7 Hz), 0.87 (3H, t, *J*= 6.5 Hz), 1.16-1.4 (53 H, broad m). ^{13}C NMR, ∂_c (ppm): 11.62, 14.32, 19.45, 22.91, 25.67, 27.36, 29.58, 29.72, 29.93, 30.25, 31.81, 32.16, 34.62, 36.88. GCMS [Column: DB-17MS, 17% phenylmethylsiloxane, 30 m × 0.25 mm id; carrier gas, He; temp: 100-280 ºC (+20 ºC/min)]: t_R=12.62 min; MS of **8** (70 eV, EI); 422

(1, M^+), 407 (1), 393 (42), 379 (1), 365 (4), 351 (1), 337 (1), 323 (1), 309 (1), 295 (1), 281 (1), 267 (2), 253 (2), 239 (3), 225 (2), 211 (2), 197 (3), 183 (4), 169 (5), 155 (5), 141 (7), 127 (10), 113 (13), 99 (19), 85 (49), 71 (64), 57 (100), 43 (47). HRMS (EI) calcd for $C_{30}H_{62}$ (M^+): 422.4852. Found: 422.4850.

4.5.15. (*S*)-3-Methylnonacosane (*S*)-**8**

In the same manner as described above 700 mg (1.67 mmol) of (*S*)-**6** gave 653 mg (93% yield) of (*S*)-3-methylnonacosane (*S*)-**8** in 53% overall yield. $[\alpha]_D^{25}$ = +3.11 ± 0.02 (*c* = 2.10, CH_2Cl_2); mp = 36 ºC. Its spectra were identical to those of (*R*)-**8**. HRMS (EI) calcd for $C_{30}H_{62}$ (M^+):422.4852. Found: 422.4846.

4.5.16. (*R*)-3-Hexanoyl-4-oxazolidin-2-one (*R*)-**10**

(*R*)-4-Isopropyloxazolidin-2-one (*R*)-**9** (2.25 g, 17.7 mmol) was dissolved in dry THF (70 ml) and cooled to -78 ºC, *n*-BuLi (2.89 M in hexanes, 6.4 ml, 18.5 mmol) was added dropwise over 10 min and the reaction was stirred for 1 h. Hexanoyl chloride (2.82 ml, 19.5 mmol) was then added dropwise and the resulting mixture was stirred at -78 ºC for 20 min, then warmed to 0 ºC for 1.5 h. The reaction was quenched with 1 M aqueous K_2CO_3 (50 ml) and extracted with hexane. The hexane extract was washed with water and brine, dried and concentrated, and the residue was purified by column chromatography (EtOAc/hexanes, 1:9) to afford oxazolidinone amide (*R*)-**10** (3.91 g, 95.6 %) as a colorless oil. (*R*)-**10** showed the following properties: ∂_H ($CDCl_3$): 0.89 (9H, broad m), 1.21 (4H, m), 1.63 (2H, m), 2.35 (1H, m), 2.79 (1H, pseudoquintet, *J*=6.1 Hz),

2.91 (1H, pseudoquintet, *J*=6.2 Hz), 4.11 (1H, dd, *J*= 13 Hz, 3.5 Hz), 4.20 (1H, pseudotriplet, *J*=8.2 Hz), 4.40 (1H, m). ∂_C (CDCl3): 14.0, 14.9, 18.0, 23.0, 24.9, 28.2, 29.3, 31.9, 35.8, 58.5, 63.5, 154.3, 174.1; GC-MS [Column: DB-17MS, 17% phenylmethylsiloxane, 30 m × 0.25 mm id; carrier gas, He; temp: 50-280 ºC (+10 ºC/min)]: t_R: 16.28 min (100 %), MS of **10** (70 eV, EI): 227 (2, M^+), 198 (1), 184 (34), 171 (41), 156 (1), 142 (5), 130 (18), 114 (4), 99 (100), 85 (16), 71 (25), 55 (11), 43 (22).

4.5.17. (*S*)-3-Hexanoyl-4-oxazolidin-2-one (*S*)-**10**

In the same manner as described above 2.5 g (19.67 mmol) of (*S*)-**9** gave 4.20 g (93% yield) of (*S*)-3-hexanoyl-4-oxazolidin-2-one (*S*)-**10**. Its spectra were identical to those of (*R*)-**9**.

4.5.18. ((*R*)-2-Methylhexanoyl)oxazolidinone (*R*)-**11**

To a solution of (*R*)-**10** (3.0 g, 12.95 mmol) in dry THF at -78 ºC was added sodium hexamethyldisilazide (NaHMDS, 2.0 M in THF, 7.12 ml, 14.25 mmol) dropwise over 15 min. The reaction was stirred at -78 ºC for 1 h, then MeI (3.22 ml, 52 mmol) was added dropwise, and the resulting solution was stirred at -78 ºC for 2 h. The reaction was quenched with saturated aqueous NH_4Cl (75 ml) and extracted with hexane. The hexane extract was washed sequentially with 1 M HCl (2 x 100 mL), saturated $NaHCO_3$ (2 x 100 mL), and brine (100 mL), then dried and concentrated. The residue was purified by column chromatography over SiO_2 with EtOAc/Hexanes (1:9) as eluent, to afford ((*R*)-2-methylhexanoyl)oxazolidinone (*R*)-**11** (3.01 g, 94 % yield) as a colorless oil. ∂_H ($CDCl_3$):

0.89 (9H, broad m), 1.14 (3H, d, *J*=7.6 Hz), 1.21 (4H, m), 1.63 (1H, m), 2.35 (1H, m), 2.79 (1H, pseudoquintet, *J*=6.1 Hz), 2.91 (1H, pseudoquintet, *J*=6.2 Hz), 4.11 (1H, dd, *J*= 13 Hz, 3.5 Hz), 4.20 (1H, pseudotriplet, *J*=8.2 Hz), 4.40 (1H, m). ∂_c ($CDCl_3$): 14.0, 14.9, 18.0, 23.0, 24.9, 28.2, 29.3, 31.9, 35.8, 58.5, 64.1, 156.8, 175.0; GC-MS [Column: DB-17MS, 17% phenylmethylsiloxane, 30 m × 0.25 mm id; carrier gas, He; temp: 50-280 ºC (+10 ºC/min)]: t_R: 16.61 min (99 %), MS of (*R*)-**11** (70 eV, EI): 241 (6, M^+), 198 (17), 185 (23), 156 (4), 142 (3), 130 (100), 113 (55), 97 (5), 85 (73), 69 (23), 55 (13), 43 (27).

4.5.19. ((*S*)-2-Methylhexanoyl)oxazolidinone (*S*)-**11**

In the same manner as described above 3.0 g (12.95 mmol) of (*S*)-**10** gave 2.89 g (91% yield) of (*S*)-**11**. Its spectra were identical to those of (*R*)-**11**.

4.5.20. (*R*)-2-Methyl-1-hexanol (*R*)-**12.**

To a cold (0 ºC) solution of ((*R*)-2-methylhexanoyl)oxazolidinone (*R*)-**11** (3.0 g, 12.9 mmol) in Et_2O (80 ml) , was added dry MeOH (1.9 ml, 25.3 mmol) and the mixture was stirred for 5 min. $LiBH_4$ (562 mg, 25.8 mmol) was then added in one portion, and the reaction was allowed to stir at 0 ºC for 3 h, then quenched with saturated $NaHCO_3$ (60 ml), and extracted with Et_2O. The ether extract was washed with saturated aqueous NH_4Cl (1 x 50 mL), water (1 x 50 mL), and brine (2 x 50 mL). The organic layer was then dried with anhydrous Na_2SO_4 and concentrated in *vacuo* to afford a crude oil. The crude residue was subsequently purified by column chromatography (EtOAc/hexane 1:5)

to afford alcohol **12** (1.35 g, 90 %) as a colorless oil. (*R*)-2-methylhexanol showed the following properties: $[\alpha]_D^{25}$= +13.08 (*c* = 2.1, EtOH); ν_{max} (neat): 3336 (br m), 2956 (s), 2923 (s), 2855 (s), 1465 (m), 1378 (w), 1032 (s), 938 (w), 908 (w), 842 (w) 723 (w); ∂_H (CDCl3): 0.89 (6H, m), 1.05 (1H, m), 1.24-1.35 (5H, broad m), 1.59 (1H, m), 1.79 (OH, broad s), 3.39 (1H, dd, *J*=11.8 Hz, 5.8 Hz), 3.45 (1H, dd, *J*=12.3 Hz, 6.8 Hz). ∂_c (CDCl3): 14.1, 17.0, 23.5, 29.8, 33.0, 38.5, 68.5. [Column: DB-17MS, 17% phenylmethylsiloxane, 30 m × 0.25 mm id; carrier gas, He; temp: 40-280 ºC (+10 ºC/min)]: t_R: 5.98 min (98.7 %), MS of **12** (70 eV, EI): 115 (5, M^+-1), 98 (12), 84 (28), 70 (45), 56 (100), 42 (57).

4.5.21. (*S*)-2-Methyl-1-hexanol (*S*)-**12**

In the same manner as described above 3.0 g (12.95 mmol) of (*S*)-**11** gave 1.25 g (84% yield) of (*S*)-**12**. Its spectra were identical to those of (*R*)-**12**. $[\alpha]_D^{25}$= -13.17 (*c* = 2.5, EtOH).

4.5.22. (*R)*-2-Methylhexan-1-yl triflate (*R*)-**13.**

To a cold (-10 ºC) solution of Alcohol (*R*)-**12** (500 mg, 4.3 mmol) in CH_2Cl_2 (20 ml) was added sequentially pyridine (346 µl, 4.3 mmol) and triflic anhydride (880 µl, 5.16 mmol). The reaction was then stirred for 1 h at -10 ºC and then diluted with pentane (60 ml), warmed to rt, and filtered through a plug of silica gel. The filter cake was rinsed with 3:1 hexane:CH_2Cl_2 (2 x 75 mL). The filtrates were combined and concentrated *in* vacuo

to gave alkyl triflate (*R*)-**13** (1.07 g, quantitative) as a colorless oil, which was used immediately without further purification.

4.5.23. ((*S*)- 2-Methylhexan-1-yl triflate (*S*)-**13**

In the same manner as described above 500 mg (4.3 mmol) of (*S*)-**11** gave 1.05 g (quantitative) of (*S*)-**13**, which was used immediately without further purification or spectral identification.

4.5.24. (*R*)-5-Methylheptacos-7-yne (*R*)-**14.**

A solution of 1-heneicosyne (1.23 g, 4.2 mmol) in 10 ml of dry THF was cooled to -10 ºC, *n*-BuLi (2.89 M in hexanes, 1.46 ml, 4.22 mmol) was added dropwise over 10 min, and the reaction was stirred for 1 h. Alkyl triflate (*R*)-**13** (1.07 g, 4.3 mmol) in 5 ml THF was then added by syringe pump over 30 min, and the reaction was stirred at -10 ºC for 5 h. The reaction was quenched with water (20 ml) and extracted with hexane. The hexane extract was washed with brine, dried, concentrated, and the residue was purified by flash chromatography(hexane) to afford (*R*)-5-methylheptacos-7-yne (*R*)-**14** (1.35 g, 80 %) as a colorless oil. $[\boldsymbol{\alpha}]_D^{25}$= -0.89 (*c* = 1.50, $CHCl_3$); $\boldsymbol{\nu}_{max}$ (neat): 2954 (m), 2921 (s), 2852 (s), 1464 (m), 1377 (w), 1251 (w), 1056 (w), 843 (w), 721 (w); ∂_H (CDCl3): 0.89 (6H, m), 0.95 (3H, d, *J*=6.4 Hz), 1.2-1.5 (38H, br m), 1.53 (1H, m), 1.65 (1H, m), 1.98 (1H, d, *J*=12 Hz), 2.17 (2H, m); ∂_C ($CDCl_3$): 14.32, 18.98, 19.74, 20.9, 26.39, 27.27, 29.05, 29.38, 29.58, 29.79, 29.91, 32.12, 33.07, 36.26, 79.56, 81.2; GC-MS [Column: DB-17MS, 17% phenylmethylsiloxane, 30 m × 0.25 mm id; carrier gas, He; temp: 100-280 ºC (+10

ºC/min)]: t_R: 18.98 min (97.32 %); MS of **8** (70 eV, EI); *m/z*: 390 (5, M^+), 361 (2), 333 (35), 305 (5), 280 (1), 266 (2), 252 (5), 208 (3), 179 (2), 151 (25), 126 (18), 109 (35), 81 (100), 57 (89), 41 (55); HRMS (EI) calcd for $C_{28}H_{54}$ (M^+): 390.4233. Found: 390.4229.

4.5.25. ((*S*)-5-Methylheptacos-7-yne (*S*)-14

In the same manner as described above 1.05 g (4.27 mmol) of (*S*)-**13** gave 1.28 g (78%) of (*S*)-5-methylheptacos-7-yne (*S*)-**14**. Its spectral properties matched those of (*R*)-**14.** $[\alpha]_D^{25}$= +0.92 (*c* = 1.55, CHCl3).

4.5.26. (*R*)-5-Methylheptacosane (*R*)-15

(*R*)-5-methylheptacos-7-yne (*R*)-**14** (1.35 g, 3.44 mmol) was added to a slurry of 5% Rh/C (135 mg) and anhydrous Na_2CO_3 (1.09 g, 10.3 mmol) in hexane (15 ml). The reaction was stirred under a slight positive pressure of H_2 for 8 h, then filtered through a plug of silica gel to afford crude (*R*)-5-methylheptacosane. After concentration, the residue was dissolved in boiling acetone (10 ml) and the solution was cooled to -20 ºC. Filtration and vacuum drying yielded pure (*R*)-5-methylheptacosane (*R*)-**15** (1.29 g , 96 % yield) as white waxy crystals in 64 % overall yield in 6 steps. Mp = 32 ºC, $[\alpha]_D^{23}$= -0.770 (c = 1.33, $CHCl_3$). ^{1}H NMR ($CDCl_3$), ∂_H (ppm): 0.83 (3H, d, *J*= 6.3 Hz), 0.85 (3H, t, *J*= 6.7 Hz), 0.87 (3H, t, *J*= 6.5 Hz), 1.16-1.4 (49 H, broad m). ^{13}C NMR, ∂_c (ppm): 11.52, 14.14, 19.72, 22.61, 23.67, 27.09, 29.38, 29.70, 29.93, 30.35, 31.81, 32.16, 34.62, 36.73. MS (EI, 70 eV, *m/z*, relative abundance): 394 (M^+, 1), 365 (3), 337 (39), 308 (12),

295 (1), 281 (1), 253 (3), 225 (3), 197 (1), 183 (2), 169 (2), 155 (1), 141 (9), 112 (35), 85 (42), 71 (98), 57 (100), 43 (50).

4.5.27. (*S*)-5-Methylheptacosane (*S*)-**15**

(*S*)-5-methylheptacosane [(*S*)-**15**] (61 % yield, purity > 99 %) was prepared in analogous fashion by substitution of (*S*)-4-isopropyloxazolidin-2-one [(*S*)-**9**] for (*R*)-4-isopropyloxazolidin-2-one [(*R*)-**9**] in the first reaction, mp = 31.5 ºC, $[\boldsymbol{\alpha}]_D^{23}$= + 0.731 (c=1.35, CH_2Cl_2). Its spectroscopic data were analogous to those of (*R*)-**15**.

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Chapter 5: Concluding Remarks

Methyl-branched cuticular hydrocarbons (MBCHs) are ubiquitous components of the protective cuticular hydrocarbon (CHC) layer that covers the exoskeleton of most insect species. These simple, non volatile, branched alkanes were primarily thought to be involved in water balance and preventing dessication, but a number of them now are known to have important secondary roles as mediators of short-range intraspecific communication.[1,2] For example, in several species of solitary insects, MBCHs have been shown to be utilized as signals used in mate recognition, and for eliciting courtship behaviors between a conspecific pair.[3-7] This phenomenon can be clearly seen in the courtship and mating behaviors of the cerambycid beetle *Neoclytus acuminatus acuminatus*, in which males attempt to copulate with solvent-stripped female carcasses that have been treated with a synthetic blend of (±)-7-methylheptacosane, (±)-7-methylpentacosane, and (±)-9-methylheptacosane.[4] The communicative roles of MBCHs are even more evident in eusocial hymenopterans (wasps, bees, and ants), in which they act as chemical fingerprints allowing differentiation of nestmates from nonnestmates.[8,9] Methyl-branched cuticular hydrocarbons, specifically 3-methylalkanes, are also known to function as queen primer pheromones for several species of eusocial wasps, ants, and bees, inducing worker sterility by inhibiting ovarian development of the worker class.[10-12] Despite these important roles for methyl-branched alkanes in the chemical communication systems of insects, there has previously been little information on the importance of chirality, both structurally and functionally, for these insect-produced natural products. Although it has been assumed that the biosynthesis of methyl-branched

hydrocarbons is enantiospecific, there has been little hard evidence to support this hypothesis.[1]

The importance of chirality for volatile semiochemicals has been well established. As discussed in Chapter 1 of this dissertation, for some species of insects only one enantiomer of a volatile pheromone component elicits the correct behavioral response, whereas the opposite enantiomer can be benign, or can inhibit the effects of the active semiochemical.[13,14] There are also several cases where a specific ratio of the enantiomers of a pheromone is required for biological activity.[14]

For methyl-branched cuticular hydrocarbons used as contact pheromones, information regarding the chirality of these molecules has been difficult to obtain because of several related factors. First, the specific rotations of chiral methyl-branched hydrocarbons are quite small (< 3º, depending on location of the branch point) and would require amounts (hundreds of µg to several mg) much greater than an individual insect produces (100 ng to tens of µg) to measure by polarimetry. Second, insect methyl-branched hydrocarbons occur as components of a complex blend of saturated hydrocarbons, alkenes, and polar constituents. The saturated hydrocarbon fraction is inseparable with normal phase liquid chromatography, and insoluble in the aqueous solvent systems typically used with reverse phase chromatography. Furthermore, saturated alkanes and nonconjugated alkenes are undetectable by the detectors typically used with HPLC, such as UV-visible and diode-array detectors, due to the absence of functional groups or chromophores. Lastly, the synthesis of chiral methyl-branched standards has previously been unnecessarily lengthy, inefficient, and costly. Thus, the

large majority of the studies in which the biological activities of methyl-branched hydrocarbons have been tested have used racemic compounds.

One of the main goals of this dissertation project was to develop a procedure for isolating pure MBCHs from the CHC extracts of insects. This then enabled a second major goal, the determination of the enantiospecificity of insect MBCH biosynthesis. In Chapter 2, I demonstrated that the isolation of pure methyl-branched cuticular hydrocarbons from insect cuticular extracts is possible, using a combination of previously known CHC fractionation methods and a more modern analytical separation technique. Thus, CHC extracts were first fractionated with $AgNO_3$-impregnated silica gel chromatography into fractions containing saturated alkanes, alkenes, and more polar CHC components respectively.[15] The methyl-branched alkanes were subsequently isolated from the saturated alkanes by adsorption of the *n*-alkanes into 5 Å molecular sieves, using a branched hydrocarbon solvent.[16,17] The fraction containing methyl-branched alkanes was then separated by chain-length and, in some cases, by branch point by using reverse phase high performance liquid chromatography (RP-HPLC) and a completely non-aqueous solvent system of ethyl acetate and methanol. Using an essentially universal detector, an evaporative light scattering detector, solved the problem of detecting MBCHs as they eluted. The combination of these fractionation methods allowed the isolation of 36 methyl-branched compounds in high purity from 20 species of insects spanning 9 orders of the Insecta (Table 2.1, Chapter 2).

The ultimate goal of the MBCH isolations was to determine whether MBCHs are biosynthesized as one enantiomer, and whether the biosynthetic pathways are conserved

within the class Insecta. This was accomplished by measuring the specific rotations of the purified natural products using a Rudolph Autopol IV digital polarimeter with a 250 μL micro-cell. This instrument was sufficiently sensitive to allow the direct measurement of the specific rotations of the isolated MBCHs (Table 2.2, Chapter 2). Remarkably, the specific rotations of all 36 of the isolated MBCHs were negative (-), matching those of enantiopure synthesized (*R*)-MBCH standards. This suggests that the enzymatic reaction controlling the stereochemistry of these molecules is probably conserved within at least the 9 orders of Insecta studied. Furthermore, the reaction gives the (*R*)-enantiomers regardless of methyl branch position (3, 5, 7, or 9) or chain length.

It is known that insects utilize the fatty acid synthase (FAS) enzymatic pathway to biosynthesize the CHC components of their epicuticular hydrocarbon layers.[18] This biosynthetic pathway is very stereotyped, repeating the same four enzymatic steps with malonyl-ACP (acyl carrier protein), methylmalonyl-ACP, and acetyl-ACP substrates to build up the various components of insect CHCs (Discussed in detail in Chapters 1.2 and 2.3).[19] From a strictly organic chemistry perspective, the enzymatic domain that must control the stereochemistry of the methyl branch points is the enoyl-ACP reductase (ERT) domain of the FAS, which catalyzes the reduction of the 2-methyl-2-enoyl-ACP hydrocarbon precursors (Scheme 5.1).[19] The enoyl-ACP reductase domain of insect microsomal FAS has not yet been isolated, but it is likely to be similar to members of the eukaryotic medium-chain dehydrase/reductase (MDR) enzymatic family.[20] Enoyl reductase members of this family isolated from various fungal sources have highly conserved structural homology and NADPH binding sites,[20-23] suggesting that the

microsomal enoyl reductase domain may also be highly conserved in the Insecta. Furthermore, the conservation of the microsomal enoyl reductase domain would explain the lack of stereochemical diversity in the isolated insect MBCH compounds and suggest the cause of the stereospecificity seen in MBCH biosynthesis. However, it must be noted that if the stereochemistry of insect MBCHs is controlled by the ERT reduction, molecules which have the methyl branch point inserted early in the hydrocarbon chain would have the same spatial orientation as those inserted after the center of the hydrocarbon chain, but would be assigned the opposite stereochemical configurations ((*R*) vs (*S*)) due to nomenclature rules.

R S-ACP NADPH + H^+ **Enoyl-ACP Reductase** R SACP

Scheme 5.1. Postulated asymmetric reduction of Enoyl-ACP by the enoyl-ACP reductase domain of microsomal FAS.

A third major objective of this dissertation was to determine the structure-bioactivity relationships of methyl-branched hydrocarbons used as contact pheromones. To address this objective, a library of synthetic chiral MBCHs was synthesized. Previous syntheses of chiral MBCHs utilized expensive chiral synthons as building blocks and in most cases had low overall yields.[24] In Chapter 3, I described the development of a new efficient synthesis for chiral MBCHs, which utilizes an Evans' alkylation reaction to induce the asymmetry of the methyl branch point (Scheme 5.2). The synthetic route

involves first deprotonation of the chiral auxiliary, (*R*)-4-isopropyloxazolidin-2-one **1**, with *n*-Buli and subsequent acylation with any desired acid chloride to form the oxazolidinone imide **2**. Deprotonation of **2** with NaHMDS at -78 ºC selectively forms the (*Z*)-enolate, which is alkylated by iodomethane to form intermediate **3**. Reduction of **3** with $LiBH_4$ forms a chiral alcohol intermediate **4**, which after triflation can be alkynylated with various alkynyllithium reagents to form the generic chiral methyl-branched alkyne **6**. Rhodium on carbon catalyzed hydrogenation of **6** in hexanes then produced any desired chiral MBCH **7** in 58-63 % overall yield. This synthetic method has several advantages over previously published synthetic routes. First, it affords chiral methyl-branched hydrocarbons efficiently (6 steps, 58-63 % overall yield) in high chemical and stereochemical purity. Second, the chiral auxiliaries are recyclable and can be reused after the $LiBH_4$ cleavage step. Third, the route is very flexible, allowing synthesis of either enantiomer of any desired methyl-branched hydrocarbon, of any chain length and with the branch point in any position. Using this method, I was able to synthesize a library of 45 compounds for use in biological assays and for calibration of polarimetric instruments and detectors (Table 3.1).

Scheme 5.2. Generic synthesis route to (*R*)- and (*S*)-methylalkanes. Reagents: (a) i. *n*-BuLi, THF; ii. RCOCl (98%); (b) i. NaHMDS, THF; ii. MeI (94%); (c) $LiBH_4$, Et_2O (93%); (d) Tf_2O, pyridine, CH_2Cl_2 (quantitative for **7**); (e) alkynyllithium, THF (80%); (f) H_2 (1 atm), Rh/C, hexanes (96-98%).

In chapter 4, several synthetic chiral methyl-branched hydrocarbons were utilized to determine the structure-bioactivity relationship of the MBCH contact pheromone of the parasitic wasp *Lariophagus distinguendus.* This study was performed in collaboration with Professor Joachim Ruther and Stephan Kuhbandner, who carried out the biological assays. *Lariophagus distinguendus* is an idiobiont ectoparasitoid (i.e., a parasitoid that

paralyzes its host and then develops attached to the host's exoskeleton) of beetles that infest stored grain. Females and newly emerged males of this species produce a contact sex pheromone, 3-methylheptacosane, which arrests sexually mature males and induces a wing-fanning courtship display. Interestingly, 3-methylheptacosane disappears from the cuticular profiles of males 3-4 days postemergence, resulting in the cessation of the homosexual courtship responses.

In this study, we showed that the addition of either enantiomer of the active contact pheromone, 3-methylheptacosane, to the cuticle of mature male wasp cadavers restored the elicitation of homosexual courtship attempts. Other methyl-branched isomers of 3-methylpentacosane or structurally related homologs did not elicit any behavioral responses to treated *L. distinguendus* cadavers (Figure 4.1 in Chapter 4). In contrast, adding biologically relevant amounts of either enantiomer of the structurally related synthetic homologs and isomers of 3-methylheptacosane, as well as several *n*-alkanes, to the cuticle of attractive wasp cadavers resulted in a significant decrease in the wing-fanning responses of males, suggesting that changes to the ratios of naturally occurring, but non-bioactive, CHCs on the cuticle of attractive wasps can significantly decrease the biological activity (Figure 4.2 in Chapter 4.). The results of this study showed that male *L. distinguendus* perceive the complete CHC profiles of females and newly emerged males, and require the correct ratio of the naturally occurring compounds to elicit a pheromonal response. This result is unlike the contact pheromone response of most other insect species, in which courtship displays and copulation can be initiated as long as key components of the contact sex pheromone blend are present.[3-6,25]

The structure-bioactivity relationship studies of *L. distinguendus* contact pheromone also clarified that exposure to either enantiomer of 3-methylheptacosane could induce the wing-fanning courtship response displayed by mature male wasps. This was unexpected due of the prevalence of (*R*)-methyl-branched alkanes in Insecta (see chapter 2), but there are at least two possible explanations for this lack of enantiomeric preference. The first is that newly emerged male and female *L. distinguendus* produce opposite enantiomers of 3-methylheptacosane, and the mature male wasps may have odorant receptor neurons that perceive both enantiomeric forms. The presence of different odorant receptor neurons for both enantiomers of a pheromone has been seen previously with Japanese beetles, *Popillia japonica,* where males are known to have odorant receptors for both the female produced sex pheromone (*R*)-japonilure and its antipode (*S*)-japonilure.[27] The activation of the odorant receptor for the unnatural enantiomer (*S*)-japonilure, antagonizes the biological activity of the active enantiomer, resulting in loss of behavioral attraction. In the case of *L. distinguendus* wasps, the presence of odorant receptor neurons for both enantiomers of 3-methylheptacosane may allow the mature males to distinguish between newly emerged males and females, but other physiological factors may trump the importance of chirality in mate identification, inducing the mature male wasps to court both sexes of *L. distinguendus.* This may explain the preference of mature male *L. distinguendus* to (*S*)-3-methylheptacosane in two-dimensional filter paper assays, and the subsequent loss of this stereochemical preference when models or cadavers are utilized in behavioral bioassays.[26]

A possible second reason for the ability of both enantiomers of 3-methylheptacosane to elicit the wing –fanning courtship responses of male *L. distinuguendus* may be that the incorrect enantiomer is still perceived by the odorant receptor neuron of the contact pheromone. In fact, although (*R*)-3-methylheptacosane may be the naturally occurring enantiomer of the contact pheromone, its antipode, (*S*)-3-methylheptacosane may preferentially bind to the odorant receptor, acting as a superagonist, which results in the slight increase in behavioral activity of mature males to (*S*) vs. (*R*)-3-methylheptacosane in the homosexual courtship response recovery assays seen chapter 4 (see figure 4.1). A similar result is seen in the contact sex pheromone of the German cockroach *Blatella germanica*, where the naturally occurring (3*S*,11*S*)-dimethylheptacosan-2-one was shown to be the least biologically active stereoisomer of the contact sex pheromone..[28] Future studies are required to elucidate the reasons for the lack of stereochemical preference by male *L. distinguendus*. In addition, identifying the cause of the lack of enantiomeric discrimination for the contact sex pheromone of this parasitic wasp may help increase the understanding of stereochemistry-bioactivity relationships for other insect species that utilize methyl-branched hydrocarbons as their contact pheromones.

It is likely that methyl-branched hydrocarbons are used as contact pheromones by other arthropods. Thus, the results from this thesis should provide solid baseline data and standards for further investigations of the chemistry, biochemistry, molecular biology, and chemical ecology of these and related cuticular compounds. For example, isolation and cloning of the genes which encode the enoyl-ACP reductase domain of microsomal

FAS, followed by construction of recombinant enoyl-ACP reductases and labeled precursors, and analysis of labeled products formed by both the isolated enzymes and recombinant mutants would provide definitive evidence for the conserved stereochemistry within the microsomal enoyl-ACP reduction. My library of enantiopure MBCHs will form a resource for further in-house and collaborative studies of contact pheromones in numerous insect species, for example in probing the specificity of the pheromone-binding proteins and receptors for these types of compounds. With the methods and materials that have been developed during the course of my thesis work, it now should be possible to make progress in a major area of insect chemical ecology that has long been neglected.

5.1. References

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