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Isolation and determination of absolute configurations of insect-produced methyl-branched hydrocarbons

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Although the effects of stereochemistry have been studied extensively for volatile insect pheromones, little is known about the effects of chirality in the nonvolatile methyl-branched hydrocarbons (MBCHs) used by many insects as contact pheromones. MBCHs generally contain one or more chiral centers and so two or more stereoisomeric forms are possible for each structure. However, it is not known whether insects biosynthesize these molecules in high stereoisomeric purity, nor is it known whether insects can distinguish the different stereoisomeric forms of MBCHs. This knowledge gap is due in part to the lack of methods for isolating individual MBCHs from the complex cuticular hydrocarbon (CHC) blends of insects, as well as the difficulty in determining the absolute configurations of the isolated MBCHs. To address these deficiencies, we report a straightforward method for the isolation of individual cuticular hydrocarbons from the complex CHC blend. The method was used to isolate 36 pure MBCHs from 20 species in nine insect orders. The absolute stereochemistries of the purified MBCHs then were determined by digital polarimetry. The absolute configurations of all of the isolated MBCHs were determined to be (*R*) by comparison with a library of synthesized, enantiomerically pure standards, suggesting that the biosynthetic pathways used to construct MBCHs are highly conserved within the Insecta. The development of a straightforward method for isolation of specific CHCs will enable determination of their functional roles by providing pure compounds for bioassays.

cuticular hydrocarbons | methyl-branched hydrocarbons | absolute configuration | isolation | contact pheromone

The use of chemical signals is highly developed within insects, with semiochemicals mediating a wide variety of inter- and intraspecific behaviors. Volatile pheromones, such as sex and aggregation pheromones, are the most well-known types, but insects also use nonvolatile cuticular lipids as contact pheromones (1–4). The cuticular lipids consist of a complex blend of *n*- and methyl-branched alkanes, alkenes, and lesser amounts of more polar compounds such as esters and alcohols. The lipid layer acts primarily as a waterproofing barrier (5), but individual lipid components have evolved secondary roles as signals that mediate a variety of behaviors and physiological changes (1, 2, 6). For example, solitary insects use cuticular hydrocarbons (CHCs) to identify the species and sex of mates (7, 8) whereas, in social insects, CHCs mediate identification of nestmates (9, 10), recognition of castes, and task allocation within the colony (11). Social insect queens also use CHCs to signal fecundity and dominance status within the colony, inhibiting development of workers into reproductives (12–14).

Determining the roles of specific CHCs as signals has been hindered by three interlinked problems. First, CHCs typically consist of a large number of compounds, which can be difficult to isolate in pure form to test their bioactivities. Specifically, 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 insoluble in the aqueous-organic solvent systems typically used with reverse phase chromatography. Whereas most CHCs can be separated to some degree by analytical gas chromatography (GC), isolation by preparative GC is challenging 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.

In contrast, reverse phase high performance liquid chromatography (RP-HPLC) using nonaqueous solvent systems has excellent potential for isolating CHCs from complex blends because the separation depends primarily on hydrophobic interactions between the solutes and the stationary phase. Thus, homologous CHCs should be readily separable on the basis of chain length, presence or absence of double bonds, and chain branching. However, there is a second problem, one of detection, because most HPLC detectors are poorly sensitive or insensitive to CHCs. HPLC coupled to mass spectrometry also is problematic because alkanes and alkenes are only poorly ionized or not ionized at all by electrospray or other ionization methods typically used with HPLC-MS, and so are invisible to the mass spectrometer.

The third problem concerns the inherent chirality of methyl-branched CHCs, most of which can exist in two or more stereoisomeric forms, depending on the number of branches and their positions. The correct stereochemistry is integral to the activity of most bioactive molecules. Insects are no exception, with numerous examples known of the natural stereoisomer of a volatile pheromone eliciting the expected bioactivity whereas other stereoisomers can vary from eliciting hyperactivity to being strongly inhibitory (3, 4).

Methyl-branched cuticular hydrocarbons (MBCHs) have been identified or implicated as contact pheromones in a number of insect species (15–27), but most studies involving MBCHs have ignored the issue of stereochemistry and used only racemic MBCHs in bioassays to assess function. The linked questions as to whether insects biosynthesize MBCHs enantioselectively and whether insects can discriminate between the stereoisomeric

Significance

Methyl-branched hydrocarbons (MBCHs) are widely used for inter- and intraspecific communication by insects. MBCHs can exist as two or more stereoisomers, but it remains unknown which stereoisomer(s) insects produce, nor is it clear whether insects can distinguish MBCH stereoisomers. To address these questions, we developed a generic method for isolation of MBCHs (and other lipids) from a range of insect species. Polarimetric analyses of 36 isolated MBCHs showed that their stereochemistry is conserved across nine orders of Insecta, with all compounds having the (*R*)-configuration, regardless of methyl branch position or chain length. These results will enable further explorations of the role of stereochemistry in contact chemical communication, and the molecular mechanisms involved in recognition of hydrocarbons.

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forms of MBCHs are still largely unknown (28). This deficiency is due to the difficulty in determining the absolute configurations of insect-produced MBCHs, particularly when produced in only nanogram to microgram amounts per individual. In particular, MBCH enantiomers do not separate on chiral stationary phase chromatography columns due to their lack of functional groups or other structural features that interact strongly enough with the chiral stationary phase to permit resolution (29–31).

As an alternative, the specific rotations of compounds can be measured with a polarimeter and the absolute stereochemistry determined by comparing the measured rotations to those of enantiopure standards. However, this method is useful only if compounds can be isolated in pure form. Furthermore, the specific rotations of MBCHs are small (a few degrees-cm^2/g for 3-methylalkanes) and decrease further as the methyl branch is moved toward the middle of the chain, requiring isolation of milligram quantities of each MBCH to obtain a measurable rotation. Thus, a method of isolating individual, pure MBCHs in milligram amounts is a prerequisite to the determination of their stereochemistry.

We describe here 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 known fractionation methods (32–34). Reverse phase HPLC with nonaqueous solvent systems and an evaporative light-scattering detector (ELSD), a "universal" detector that detects any nonvolatile molecule, then allowed isolation of individual components from these fractions. This methodology was used to isolate MBCHs from 20 insect species from nine orders, including adults of both sexes from both holometabolous and hemimetabolous species, and of several different life stages, and the absolute configuration of each compound was determined by polarimetry. The separation protocol was extended to the isolation of individual alkenes from CHC extracts to demonstrate its general utility in isolating pure components from the CHC blend.

Results

Isolation of Pure Compounds from Cuticular Hydrocarbon Extracts. As proof of concept, the separation protocol was first tested with extracts of male and female American cockroaches, *Periplaneta americana*. The sexes have similar cuticular lipid profiles containing ∼25 compounds (35). Because the sexes could conceivably produce different stereoisomers of individual MBCHs, the CHCs of each sex were analyzed separately. Thus, crude extracts from 14 males and 16 females, respectively, were first fractionated on a silver nitrate-impregnated silica gel column. Sequential elution with hexane, 5% (vol/vol) cyclohexene in hexane, and diethyl ether yielded fractions containing alkanes, alkenes, and more polar compounds, in sequence. The alkane fraction in isooctane was then treated with 5-Å molecular sieves, resulting in adsorption of the straight-chain alkanes into the sieve matrix (34). Filtration or centrifugation of the resulting slurry gave a filtrate containing only branched-chain alkanes. This fraction was further fractionated by RP-HPLC, eluting with ethyl acetate/methanol (1:1). The most abundant MBCH, 3-methylpentacosane, was collected in multimilligram amounts, in 99% purity, from adults of both sexes (Table 1).

We then tested the method with extracts from insects with more complex CHC profiles. Thus, a crude extract of females of the cockroach *Blaberus giganticus* was fractionated using the steps described above, followed by RP-HPLC of the MBCH fraction. Isocratic elution with a more polar solvent (2:3 ethyl acetate/methanol) was required in light of the increased complexity of this fraction in comparison with those from the American cockroaches. The separation sequence is illustrated in Fig. 1 *A–D*, with multimilligram amounts of 3-methylhentriacontane being isolated from adults of both sexes.

Crude extracts of the cerambycid beetle *Monochamus titillator* were even more complex than those of *B. giganticus*. Here, RP-HPLC analysis of the MBCH fraction with a gradient of increasing amounts of ethyl acetate in methanol was required for adequate resolution, allowing isolation of 9-methylpentacosane, 9-methylheptacosane, and 3-methylhentriacontane in one pass (Fig. 1 *E–H*).

The method was 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. In total, 36 chiral MBCHs were isolated from 20 species, in amounts from 0.7 mg to 13.7 mg (Table 1). For all species tested, one or more MBCHs were isolated in high purity, in approximately milligram or higher amounts. Representative chromatograms are shown in Figs. S1–S4.

As a test of its generality, the method was applied to the isolation of individual alkenes from a CHC extract from female *Thasus neocalifornicus*. The alkene fraction was first separated into subfractions containing monoenes, dienes, and trienes with a silver ion-loaded ion-exchange column. Individual monoalkenes then were isolated by isocratic RP-HPLC (*SI Text* and Fig. S5).

Determination of the Absolute Configurations of Methyl-Branched Hydrocarbons. The absolute configurations of the 36 isolated MBCHs were determined with a digital polarimeter with a small-volume optical cell. Scattered reports in the literature indicated that monomethyl-branched alkanes with the (*R*)-configuration all exhibited negative optical rotations (36–44). These data were confirmed by measuring the specific rotations of ∼50 synthesized standards of both (*R*)- and (*S*)-configurations, with chain lengths from 25 to 35 carbons and methyl branches on carbons 3–13 (Table S1). Measurement of the specific rotations of the MBCHs isolated from the various insect species determined that all 36 compounds had negative specific rotations, and thus the (*R*)-configuration (Table 1). The amount of each MBCH isolated and polarimetric details are listed in Table S2.

Discussion

Cuticular hydrocarbons are known to mediate behavioral and social interactions in a number of insect species, and they have been implicated as contact pheromones and kairomones in many more. CHC profiles also have been used in studies examining speciation (45–48). However, to date, the lack of methods for the isolation of pure compounds from CHC 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, without being able to confirm that any one or any group of these compounds do indeed have a signaling role. Other studies have bioassayed synthesized CHCs to probe the roles of specific compounds, with some success (15–22). However, this approach is limited by the large number of compounds in CHC extracts so that a single study might require syntheses of numerous standards to assess the functional roles of all possible components. The problem is compounded when contact pheromones consist of blends of components (22–24), each of which may have no activity when presented individually (25–27).

As an alternative, we have shown that isolation of pure compounds from complex CHC extracts is feasible, using simple fractionations combined with RP-HPLC. For cases in which the pheromone consists of multiple components, subtractive recombination of individual CHCs will permit elucidation of the subset of CHCs that are necessary and sufficient to elicit biological activity (49). Whereas we used extracts from large numbers of insects in some cases, these numbers were dictated by the sensitivity of the polarimeter, rather than by the amounts required for bioassays, which would typically be one insect-equivalent or less. For smaller quantities, the separation method is limited only by the sensitivity of the ELSD detector. Conversely, the method is scalable to quantities larger than a few milligrams by batchwise operation or by switching to larger diameter columns. Although our focus was on isolation of MBCHs, the method is also amenable to isolation of other types of CHCs, as demonstrated by isolation of individual

Table 1. Methyl-branched hydrocarbons isolated from various insect species and life stages, with measured specific rotations (±SD, $n = 10$ measurements) and absolute configurations. In all cases, the absolute configuration was (*R*)

Genus	Species	Order	Sex/life stage	Isolated compound	Specific rotation $[\alpha]_D^{25}$
Periplaneta	*americana*	Blattodea	♀	3MeC25	−3.19 ± 0.01
Periplaneta	*americana*	Blattodea	♂	3MeC25	−3.17 ± 0.01
Periplaneta	*brunnea*	Blattodea	♂	3MeC23	−3.25 ± 0.03
Periplaneta	*australasiae*	Blattodea	♂	3MeC23	−3.21 ± 0.04
Periplaneta	*fuliginosa*	Blattodea	♂	3MeC23	−3.23 ± 0.03
Blaberus	*giganticus*	Blattodea	♀	3MeC31	−2.91 ± 0.01
Blaberus	*giganticus*	Blattodea	♂	3MeC31	−2.95 ± 0.03
Blaberus	*discoidalis*	Blattodea	Nymph	3MeC29	−2.99 ± 0.03
				3MeC31	−2.89 ± 0.07
Blaberus	*discoidalis*	Blattodea	♀	3MeC29	−2.96 ± 0.03
				3MeC31	−2.85 ± 0.01
Blaberus	*discoidalis*	Blattodea	♂	3MeC29	−2.98 ± 0.07
				3MeC31	−2.85 ± 0.03
Incisitermes	*minor*	Isoptera	Worker	3MeC23	−3.23 ± 0.01
Extatosoma	*tiaratum*	Phasmatodea	♀	11MeC23	−0.11 ± 0.05
Thasus	*neocalifornicus*	Heteroptera	♀	3MeC29	−2.97 ± 0.05
Thasus	*neocalifornicus*	Heteroptera	♂	3MeC29	−2.99 ± 0.06
Brothylus	*gemmulatus*	Coleoptera	♀	3MeC25	−3.15 ± 0.08
Tenebrio	*molitor*	Coleoptera	♀	11MeC23	−0.10 ± 0.01
Tenebrio	*molitor*	Coleoptera	♂	11MeC23	−0.08 ± 0.03
Xylotrechus	*colonus*	Coleoptera	♀	3MeC25	−3.19 ± 0.10
Monochamus	*clamator*	Coleoptera	♀	3MeC23	−3.11 ± 0.12
Monochamus	*titillator*	Coleoptera	♀	9MeC25	−0.19 ± 0.02
				9MeC27	−0.27 ± 0.01
				3MeC31	−3.01 ± 0.07
Monochamus	*titillator*	Coleoptera	♂	9MeC25	−0.20 ± 0.03
				9MeC27	−0.29 ± 0.02
				3MeC31	−3.11 ± 0.07
Calisoma	*protector*	Coleoptera	♀	3MeC25	−3.15 ± 0.09
Zophobas	*morio*	Coleoptera	Larvae	3MeC25	−3.19 ± 0.03
				3MeC27	−3.01 ± 0.07
Linepathema	*humile*	Hymenoptera	Worker	3MeC25	−3.15 ± 0.09
				3MeC27	−2.99 ± 0.10
Hemileuca	*eglanterina*	Lepidoptera	♂	5MeC27	−0.67 ± 0.03
Musca	*domestica*	Diptera	♂	3MeC25	−2.89 ± 0.08*
Myrmeleon	*sp.*	Neuroptera	♂	3MeC25	−3.13 ± 0.10

*Purity of isolated compound, 96%.

alkenes from *Thasus neocalifornicus* (*SI Text* and Fig. S5). In addition, other lipid classes such as triglycerides are amenable to RP-HPLC separation with nonaqueous solvent systems (50). Triglycerides and related compounds have recently been identified as contact pheromones for *Drosophila melanogaster*, and it is likely that similar lipids will be discovered as contact pheromones in other insect species (51, 52).

The determination of the absolute configurations of 36 insect-produced MBCHs provides insight into the biosynthesis of MBCHs. The species that were used in our study were chosen essentially randomly, on the basis of availability and to some extent size, rather than on the basis of taxonomy. Each compound, regardless of chain length or branch point, was shown to have the (*R*)-configuration, in eusocial and solitary insects of different species, sexes, and life stages, and in both hemimetabolous and holometabolous insects. The fact that all 36 MBCHs had the same configuration provides strong evidence that the enantioselectivity of the biosynthetic pathway for these molecules is highly conserved throughout the Insecta.

It must be pointed out that the accuracy of the determination of the enantiomeric purities of the isolated compounds depended on the magnitudes of their specific rotations and the sensitivity of the polarimeter. Whereas the measured rotations clearly showed that the major proportion of each isolated compound must be the (*R*)-enantiomer, polarimetry does not allow us to state that each compound was 100% enantiomerically pure because trace amounts of the corresponding (*S*)-enantiomers might go undetected.

Here, we focused on monomethyl-branched alkanes, but our separation methods should be equally applicable to the isolation of dimethyl- or multimethyl-branched CHCs. However, determination of the absolute configurations of such compounds would be further complicated because of the increased numbers of possible isomers. For example, a trimethyl-branched compound could have eight stereoisomers, four with positive and four with negative specific rotations. In such cases, determination of absolute configurations by measurement of specific rotations would be uncertain at best, even with standards of known configuration for comparison, because of the chances that two or more stereoisomers could have similar specific rotations.

In most insects, MBCHs are biosynthesized in specialized oenocyte cells in a process analogous to vertebrate fatty acid biosynthesis (53). The hydrocarbon chains are formed by microsomal 3-ketoacyl-acyl carrier protein (ACP) synthase-catalyzed Claisen condensations of malonyl-CoA substrates, with the methyl branches being introduced by insertion of a methyl malonyl-CoA into the growing hydrocarbon chain (54). The stereochemistry of the methyl branch is likely controlled by a stereoselective NADPH-catalyzed reduction of the resulting α,β-unsaturated thioester by the enoyl-ACP reductase domain of the fatty acid synthase (FAS) (Fig. 2) (55). Subsequent elongation steps followed by

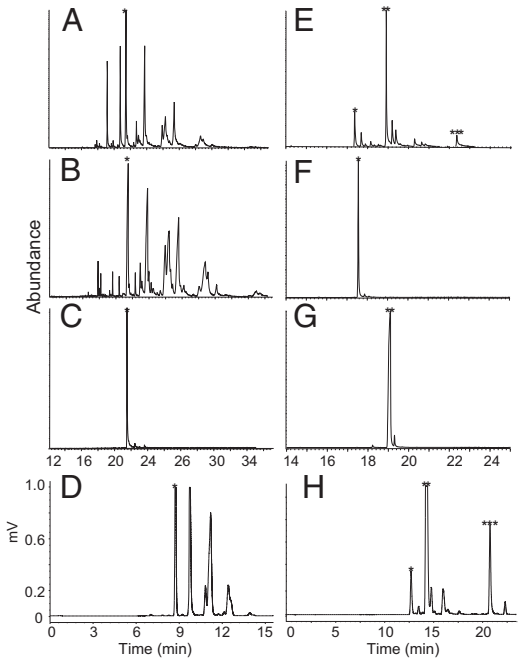

Fig. 1. Isolation of MBCHs from ♀ *B. giganticus* (*A–D*) and ♂ *M. titillator* CHC extracts (*E–H*). GC chromatograms of (*A*) the saturated alkanes fraction, (*B*) methyl-branched alkanes after removal of *n*-alkanes, (*C*) 3MeC31* isolated by RP-HPLC separation of the MBCH fraction, and (*D*) HPLC-ELSD chromatogram of the MBCH fraction. Male and female *B. giganticus* had identical alkane fractions. (*E–H*) GC chromatograms of (*E*) the methyl-branched alkanes fraction, (*F*) isolated 9-methylpentacosane* (9MeC25), (*G*) isolated 9-methylheptacosane** (9MeC27), and (*H*) HPLC-ELSD chromatogram of the MBCH fraction of ♀*M. titillator*. The compounds isolated are indicated with *, **, or *** symbols.

cytochrome-P450–mediated decarbonylation result in internally branched chiral MBCHs (2, 56). Although the enoyl-ACP reductase domain has yet to be isolated from insects and there have been only a few reports of microsomal FAS isolation (54, 57), FAS isolated from fungal species show highly conserved NADPH binding sites and genetic homology within the enoyl reductase domain (58), suggesting that this domain may also be conserved within the Insecta. Such conservation would explain the lack of stereochemical diversity in our isolated MBCHs and the enantiospecificity in their biosynthesis. It must be noted 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 biosynthesis 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*)]. Nevertheless, the available evidence suggests that, at least for monomethyl-branched compounds, the methyl branches are inserted relatively early during chain construction (59–62). Further insight into the stereospecificity of insect MBCH biosynthesis will require the isolation and expression of microsomal FAS and its enoyl-ACP reductase domain from insects, and interrogation of these enzymes with labeled substrates to confirm the stereochemical course of the enoyl-reduction.

It was pointed out more than two decades ago and reiterated recently that the possible importance of stereochemistry in MBCHs used as pheromones was essentially unknown (2). To date, only a handful of studies have probed the effects of MBCH chirality in the context of the behavioral responses elicited. For example, the parasitic wasp *Ooencytrus kuvanae* uses a blend of 5-methylheptacosane and 5,17-dimethylheptacosane as a contact pheromone for mate recognition (63). Superficially, both sexes seemed to produce the same two compounds that apparently constitute the pheromone. However, bioassays with enantiopure synthetic MBCHs suggested that the pheromone consisted of (*S*)-5-methylheptacosane and (5*R*,17*S*)-dimethylheptacosane whereas the combination of (*R*)-5-methylheptacosane and (5*R*,17*S*)-dimethylheptacosane inhibited attraction. The other stereoisomers of the dimethyl-branched component were inactive. Because the (*S*)-configuration of the monomethyl-branched compound 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 apparently produced by most other insects. It has not yet been possible to corroborate these findings by determination of the absolute configurations of the compounds produced by male and female wasps, respectively.

In similar fashion, Schlamp et al. indirectly identified the absolute stereochemistry of female-produced contact pheromone components of the peach twig borer moth, *Anarsia lineatella* (38). In bioassays with female decoys treated with a single enantiomer or a racemic mixture of 11-methyltricosane and octadecyl acetate, males responded only to decoys treated with (±)-11-methyltricosane and octadecyl acetate. Decoys treated with (*S*)-11-methyltricosane did not elicit responses whereas decoys treated with the (*R*)-enantiomer inhibited responses by males.

More recently, the results of bioassays with synthetic standards have suggested that females of the cerambycid beetles *Tetropium fuscum* and *Tetropium cinnamopterum* use mixtures of (*S*)-11-methylheptacosane and (*Z*)-9-heptacosene as their contact sex pheromones (64). However, the specific rotation reported for the synthetic (*S*)-11-methylheptacosane used in that study, $[\alpha]_D = -0.06$ degree-cm^2/g (c = 3.33, hexanes), actually matches the specific rotation of the (*R*)-enantiomer, suggesting that the stereochemical identification of these compounds may be incorrect. The insect-produced 11-methylheptacosane has not yet been isolated to determine its absolute configuration by analytical methods.

It must be pointed out that only one of the methyl-branched hydrocarbons isolated in this study is a known contact pheromone (3-methylpentacosane, for the cerambycid beetle *Xylotrechus colonus*) (24); the other 35 compounds have no known signaling roles. Thus, it may be that MBCHs used only for maintaining water balance or other protective purposes are biosynthesized in one enantiomeric form (*R*) whereas compounds used for sexual

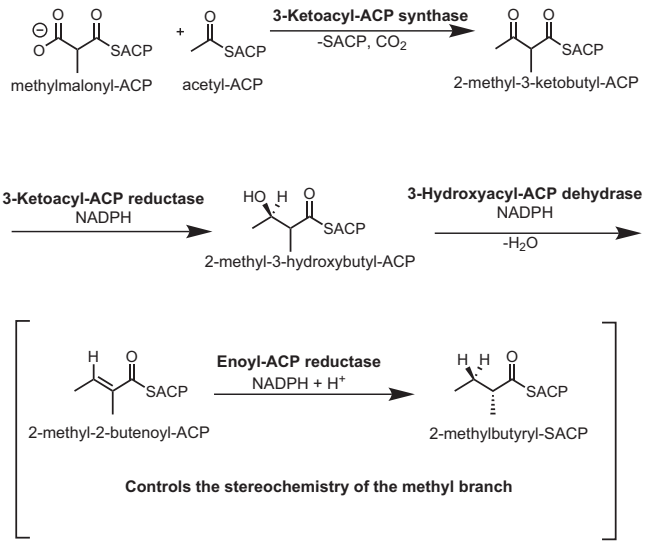

Fig. 2. Suggested biosynthetic pathway for methyl-branched hydrocarbon biosynthesis by microsomal fatty acid synthase (FAS) in insects. Absolute stereochemistry of methyl-branched hydrocarbons is hypothesized to be controlled by the NADPH-catalyzed enoyl-reductase reduction of the α,β-unsaturated thioester.

discrimination and other signaling functions may be biosynthesized in various stereoisomeric forms to differentiate them from the general MBCHs that have no functions as signals. This variation in stereochemistry could be achieved by the insertion of a methyl branch toward the end of hydrocarbon biosynthesis, which results in the production of compounds with opposite stereochemistry than those whose methyl branch point is inserted early in the hydrocarbon chain, assuming that the methyl branch is inserted on the same face of the growing chain. Overall, the examples above suggest that the chirality of MBCHs is crucial to their activity when used as signal molecules and that determination of their chirality will be essential to fully decipher their functional roles.

Although our generic method was successful in isolating a number of pure MBCHs from a variety of insect species, there are limitations. Isolation of pure compounds from simple to moderately complex CHC 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 system in combination with using high-resolution columns with <2-μm stationary phase particle sizes (so called Ultra-HPLC). The methodology could also be improved by combining RP-HPLC with an advanced laser polarimeter detector (PDR Chemical) (65), which we were not able to access for the study reported here. Such a detector should allow the determination of optical rotations in real time as part of the HPLC analysis, allowing assessment of the absolute configurations of a number of methyl-branched hydrocarbons in an extract in one pass.

Methods

Sources of Insects. *Periplaneta americana*, *Periplaneta australasiae, Periplaneta brunnea, Periplaneta fuliginosa, Blaberus giganticus, Blaberus discoidalis* adults, *Blaberus discoidalis* nymphs, *Extatosoma tiaratum, Tenebrio molitor, Incistermes minor* workers, *Zophobas morio* larvae, and *Musca domestica* were obtained from populations maintained in the Department of Entomology, University of California, Riverside (UCR). *Linepathema humile* workers were collected from a citrus grove on the UCR campus. *Brothylus gemmulatus* and *Monochamus clamator* were collected during late spring 2013 from traps in the San Bernardino National Forest in California. *Xylotrechus colonus* were collected during summer 2013 from traps near Urbana, IL. *Hemileuca eglanterina* were collected in the spring of 1999–2003 from pheromone-baited traps in the San Bernardino National Forest and were frozen at −20 °C until extracted. *Thasus neocalifornicus*, *Calosoma protector,* and an unidentified *Myrmeleon* sp. were collected in late summer of 2013 from Sedona, AZ. *Monochamus titillator* was collected in summer 2010 in traps in Kisatchie National Forest, Catahoula Ranger District, Louisiana. For those species for which both sexes were collected, insects were separated by sex before extraction. The numbers of specimens extracted for each species are listed in Table 1.

Preparation, Fractionation, and Analysis of Cuticular Extracts. Composite samples of specimens were extracted in *n*-hexane (50 mL) for 5 min, followed by a second hexane rinse (50 mL) for 3 min. The combined extracts were concentrated, reconstituted in ∼500–1,000 μL of hexanes, and loaded onto liquid chromatography columns containing 300–500 mg of silica gel impregnated with 10% (wt/wt) $AgNO_3$ (+230 mesh; Aldrich Chemical Co.). Columns were eluted with 4 × 1 mL hexanes to elute alkanes, then 4 × 1 mL 1:19 cyclohexene/hexanes to elute alkenes, and then 2 × 1 mL diethyl ether to recover more polar compounds. Fractions were analyzed by coupled GC-MS, using an HP6890 gas chromatograph (Hewlett-Packard, now Agilent) equipped with a DB-17MS capillary column (25 m × 0.20 mm, 0.33 film thickness; J&W Scientific, Inc.) coupled to an HP5973 mass selective detector run in full-scan mode, with electron impact ionization (70 eV), and using helium carrier gas. Samples were analyzed in splitless injection mode with an injector temperature of 280 °C, and a temperature program of 100 °C/1 min, then 5 °C or 10 °C/min to 280 °C, hold for 20 min. Compounds were identified by their retention indices relative to straight-chain alkane standards and interpretation of their mass spectra (66, 67).

Separation of Methyl-Branched from Straight-Chain Alkanes. The alkane fractions were pooled in a tared 20-mL vial, concentrated, and weighed. Samples were reconstituted in 5 mL of isooctane and 100 mg of activated 5Å molecular sieves (Aldrich Chemical Co.) per mg of sample were added. The vials were flushed with argon, sealed, and magnetically stirred overnight. The resulting slurry was briefly centrifuged to pellet the molecular sieves and the supernatant containing the MBCHs was removed. The pellet was resuspended in 10 mL of isooctane, then centrifuged. The combined supernatants were filtered through a glass wool plug into a tared vial, concentrated, and weighed. Compounds were identified by GC-MS as described above.

Reverse-Phase HPLC Isolation of Pure MBCHs. The MBCH fraction was concentrated and reconstituted in EtOAc (500 μL) and then fractionated on an Infinity 1220 HPLC coupled to a 380-Evaporative Light Scattering Detector (both from Agilent Technologies). The HPLC was equipped with an Eclipse XDB-C18 reverse-phase column (5-μm particles, 4.6 mm i.d. × 250 mm; Agilent Technologies) and a 100-μL sample loop (25-μL injection volumes were used). Column oven temperature was 60 °C, and the ELSD was set to nebulize at 40 °C and evaporate at 70 °C, with a stripping gas flow rate of 1.2 standard liters/min (SLM). The eluent was split 80:20 between the fraction collection and the detector. Collected fractions were analyzed by GC-MS to determine purity. Fractions from replicated analyses were checked to confirm identity and purity and then pooled and concentrated for polarimetric analysis. The typical isocratic solvent system used for the separation of individual compounds was 2:3 EtOAc/methanol at a flow rate of 1 mL/min. The specific conditions used for each analysis are summarized in Table S3.

Synthesis of Chiral Standards for Polarimetric Comparisons. The syntheses of both enantiomers of a series of monomethyl-branched alkane standards are described in *SI Text* and Fig. S6. The full list of synthesized standards, along with their specific rotations, is shown in Table S1.

Polarimetric Analysis of Isolated Methyl-Branched Hydrocarbons. Specific rotations of isolated MBCHs were obtained with an Autopol IV Digital Polarimeter (Rudolph Research Analytical) operated in high accuracy specific rotation mode at 25 °C, with the light source set at 589 nm. Isolated MBCHs were dissolved in 250 μL of chloroform and transferred to a T32 micro sample cell (2.5 mm i.d. × 50 mm length; 250 μL volume; Rudolph Research Analytical). Specific rotation values were obtained 10 times and averaged, and SDs were calculated. The sample was removed from the sample cell by syringe, and the cell was rinsed five times with chloroform and dried with compressed air before being used for another sample.

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1. Blomquist GJ, Bagnères A-G (2010) Introduction: History and overview of insect hydrocarbons. *Insect Hydrocarbons: Biology, Biochemistry, and Chemical Ecology*, eds Blomquist GJ, Bagnères A-G (Cambridge Univ Press, Cambridge, UK), pp 3–18.
2. Howard RW, Blomquist GJ (2005) Ecological, behavioral, and biochemical aspects of insect hydrocarbons. *Annu Rev Entomol* 50:371–393.
3. Mori K (2007) Significance of chirality in pheromone science. *Bioorg Med Chem* 15(24):7505–7523.
4. Mori K (1998) Chirality and insect pheromones. *Chirality* 586:578–586.
5. Gibbs AG (1998) Waterproofing properties of cuticular lipids. *Am Zool* 38:471–482.
6. Blomquist GJ (2010) Structure and analysis of insect hydrocarbons. *Insect Hydrocarbons: Biology, Biochemistry, and Chemical Ecology*, eds Blomquist GJ, Bagnères A-G (Cambridge Univ Press, Cambridge, UK), pp 19–34.
7. Ginzel MD (2010) Hydrocarbons as contact pheromones of longhorned beetles (Coleoptera: Cerambycidae). *Insect Hydrocarbons: Biology, Biochemistry, Chemical Ecology*, eds Blomquist GJ, Bagnères A-G (Cambridge Univ Press, Cambridge, UK), pp 375–389.
8. Singer TL (1998) Roles of hydrocarbons in the recognition systems of insects. *Am Zool* 38(2):394–405.
9. van Zweden JS, d'Ettorre P (2010) Nestmate recognition in social insects and the role of hydrocarbons. *Insect Hydrocarbons: Biology, Biochemistry, and Chemical Ecology*, eds Blomquist GJ, Bagnères A-G (Cambridge Univ Press, Cambridge, UK), pp 222–243.
10. Vander Meer RK, Morel L (1998) Nestmate recognition in ants. *Pheromone Communication in Social Insects*, eds Vander Meer RK, Breed MD, Winston ML (Westview Press, Boulder, CO), pp 79–103.

11. Greene MJ, Gordon DM (2003) Social insects: Cuticular hydrocarbons inform task decisions. *Nature* 423(6935):32.
12. Liebig J (2010) Hydrocarbon profiles indicate fertility and dominance status in ant, bee, and wasp colonies. *Insect Hydrocarbons: Biology, Biochemistry, and Chemical Ecology*, eds Blomquist GJ, Bagnères A-G (Cambridge Univ Press, Cambridge, UK), pp 254–281.
13. Liebig J, Peeters C, Oldham NJ, Markstädter C, Hölldobler B (2000) Are variations in cuticular hydrocarbons of queens and workers a reliable signal of fertility in the ant *Harpegnathos saltator? Proc Natl Acad Sci USA* 97(8):4124–4131.
14. Van Oystaeyen A, et al. (2014) Conserved class of queen pheromones stops social insect workers from reproducing. *Science* 343(6168):287–290.
15. Silk PJ, Ryall K, Barry Lyons D, Sweeney J, Wu J (2009) A contact sex pheromone component of the emerald ash borer Agrilus planipennis Fairmaire (Coleoptera: Buprestidae). *Naturwissenschaften* 96(5):601–608.
16. Lelito JP, et al. (2009) Behavioral evidence for a contact sex pheromone component of the emerald ash borer, *Agrilus planipennis* Fairmaire. *J Chem Ecol* 35(1):104–110.
17. Spikes AE, et al. (2010) First contact pheromone identified for a longhorned beetle (Coleoptera: Cerambycidae) in the subfamily Prioninae. *J Chem Ecol* 36(9):943–954.
18. Ruther J, Döring M, Steiner S (2011) Cuticular hydrocarbons as contact sex pheromone in *Dibrachys cavus*. *Entomol Exp Appl* 140:59–68.
19. Holman L, Jørgensen CG, Nielsen J, d'Ettorre P (2010) Identification of an ant queen pheromone regulating worker sterility. *Proc R Soc B* 277(1701):3793–3800.
20. Zhang A, et al. (2003) Evidence for contact sex recognition pheromone of the Asian longhorned beetle, *Anoplophora glabripennis* (Coleoptera: Cerambycidae). *Naturwissenschaften* 90(9):410–413.
21. Olaniran OA, et al. (2013) A male-predominant cuticular hydrocarbon, 7-methyltricosane, is used as a contact pheromone in the western flower thrips Frankliniella occidentalis. *J Chem Ecol* 39(4):559–568.
22. Lacey ES, Ginzel MD, Millar JG, Hanks LM (2008) 7-Methylheptacosane is a major component of the contact sex pheromone of the cerambycid beetle, *Neoclytus acuminatus acuminatus*. *Physiol Entomol* 33(3):209–216.
23. Böröczky K, et al. (2009) Monoalkenes as contact sex pheromone components of the woodwasp *Sirex noctilio*. *J Chem Ecol* 35(10):1202–1211.
24. Ginzel MD, Blomquist GJ, Millar JG, Hanks LM (2003) Role of contact pheromones in mate recognition in *Xylotrechus colonus*. *J Chem Ecol* 29(3):533–545.
25. Fukaya M, Yasuda T, Wakamura S, Honda H (1996) Reproductive biology of the yellow-spotted longicorn beetle,Psacothea hilaris (pascoe) (Coleoptera: Cerambycidae). III. Identification of contact sex pheromone on female body surface. *J Chem Ecol* 22(2):259–270.
26. Doi M, Nemoto T, Nakanishi H, Kuwahara Y, Oguma Y (1997) Behavioral responses of males to major sex pheromone component (*Z,Z*)-5,25-hentriacontadiene, of *Drosophila ananassae* females. *J Chem Ecol* 23(8):2067–2078.
27. Rutledge CE, Millar JG, Romero CM, Hanks LM (2009) Identification of an important component of the contact sex pheromone of Callidiellum rufipenne (Coleoptera: Cerambycidae). *Environ Entomol* 38(4):1267–1275.
28. Hefetz A, Wicker-Thomas C, Bagnères A-G (2010) Future directions in hydrocarbon research. *Insect Hydrocarbons: Biology, Biochemistry, and Chemical Ecology*, eds Blomquist GJ, Bagnères A-G (Cambridge Univ Press, Cambridge, UK), pp 477–485.
29. Leal WS, Shi X, Liang D, Schal C, Meinwald J (1995) Application of chiral gas chromatography with electroantennographic detection to the determination of the stereochemistry of a cockroach sex pheromone. *Proc Natl Acad Sci USA* 92(4):1033–1037.
30. Meierhenrich UJ, Nguyen M-J, Barbier B, Brack A, Thiemann WH (2003) Gas chromatographic separation of saturated aliphatic hydrocarbon enantiomers on permethylated β-cyclodextrin. *Chirality* 15(Suppl):S13–S16.
31. Mori K (1998) Separation of enantiomers and determination of absolute configuration. *Chemical Methods*, Methods in Chemical Ecology, eds Millar JG, Haynes KF (Kluwer, Dordrecht, The Netherlands), Vol 1, pp 295–338.
32. Nokolova-Damyanova B (1992) Silver ion chromatography and lipids. *Advances in Lipid Methodology-One*, ed Christie WW (Oily Press, Ayr, Scotland), pp 181–237.
33. Sonnet PE, Ubel EC, Lusby WR, Schwarz M, Miller RW (1979) Sex pheromone of the stable fly: Identification, synthesis, and evaluation of alkenes from female stable flies. *J Chem Ecol* 5(3):353–361.
34. O'Connor JG, Burow FH, Norris MS (1962) Determination of normal paraffins in C20-C32 paraffin waxes by molecular sieve adsorption: Molecular weight distribution by gas-liquid chromatography. *J Anal Chem* 34(1):82–85.
35. Saïd I, Costagliola G, Leoncini I, Rivault C (2005) Cuticular hydrocarbon profiles and aggregation in four *Periplaneta* species (Insecta: Dictyoptera). *J Insect Physiol* 51(9): 995–1003.
36. Bello JE, Millar JG (2013) Efficient asymmetric synthesis of long chain methyl-branched hydrocarbons, components of the contact sex pheromone of females of the cerambycid beetle, *Neoclytus acuminatus acuminatus*. *Tetrahedron Asymmetry* 24(13-14):822–826.
37. Kühbander S, Bello JE, Mori K, Millar JG, Ruther J (2013) Elucidating structure-bioactivity relationships of methyl-branched alkanes in the contact sex pheromone of the parasitic wasp *Lariophagus distinguendus*. *Insects* 4(4):743–760.
38. Schlamp KK, et al. (2005) Pheromone components from body scales of female *Anarsia lineatella* induce contacts by conspecific males. *J Chem Ecol* 31(12):2897–2911.
39. Mori K (2011) Pheromone synthesis. Part 247: New synthesis of the enantiomers of 13-methylheptacosane, the female sex pheromone of pear psylla, *Cacopsylla pyricola*. *Tet Asymm* 22:1006–1010.
40. Marukawa K, Takikawa H, Mori K (2001) Synthesis of the enantiomers of some methyl-branched cuticular hydrocarbons of the ant, *Diacamma sp*. *Biosci Biotechnol Biochem* 65(2):305–314.
41. Naoshima Y, Mukaidani H (1987) Synthesis of racemate and enantiomers of 15-methyltritriacontane, sex-stimulant pheromone of stable flyStomoxys calcitrans L. *J Chem Ecol* 13(2):325–333.
42. Schlenk W (1973) Konfigurativ nicht-stetige gitterzuordnung der gastmoleküle. *Liebigs Ann Chem* 1973:1179–1194.
43. Shirai Y, Seki M, Mori K (1999) Synthesis of all the stereoisomers of 7-methylheptadecane and 7,11-dimethylheptadecane, the female sex pheromone components of the spring hemlock looper and pitch pine looper. *Eur J Org Chem* 1999: 3139–3145.
44. Mori K (2009) Pheromone synthesis. Part 240: cross metathesis with Grubbs I (but not Grubbs II) catalyst for the synthesis of (*R*)-trogodermal (14-methyl-8-hexadecanal) to study the optical rotatory powers of compounds with terminal *sec*-butyl group. *Tetrahedron* 65:3900–3909.
45. Bagnères A-G, Wicker-Thomas C (2010) Chemical taxonomy with hydrocarbons. *Insect Hydrocarbons: Biology, Biochemistry, and Chemical Ecology*, eds Blomquist GJ, Bagnères A-G (Cambridge Univ Press, Cambridge, UK), pp 121–162.
46. Chapman RF, Espelie KE, Sword GA (1995) Use of cuticular lipids in grasshopper taxonomy: A study of variation in *Schistocerca shoshone* (Thomas). *Biochem Syst Ecol* 23:383–398.
47. Espelie KE, Berisford CW, Dahlsren DL (1990) Cuticular hydrocarbons of geographically isolated populations of *Rhopalicus pulchripennis* (Hymenoptera: Pteromalidae): Evidence for two species. *Comp Biochem Physiol* 96B:305–308.
48. Calderón Fernández G, et al. (2005) Cuticular hydrocarbon variability among Triatoma dimidiata (Hemiptera: Reduviidae) populations from Mexico and Guatemala. *J Med Entomol* 42(5):780–788.
49. Byers JA (1992) Optimal fractionation and bioassay plans for isolation of synergistic chemicals: The subtractive-combination method. *J Chem Ecol* 18(9):1603–1621.
50. Kühbandner S, Sperling S, Mori K, Ruther J (2012) Deciphering the signature of cuticular lipids with contact sex pheromone function in a parasitic wasp. *J Exp Biol* 215(Pt 14):2471–2478.
51. Chin JSR, et al. (2014) Sex-specific triacylglycerides are widely conserved in *Drosophila* and mediate mating behavior. *eLife* 3:e01751.
52. Yew JY, Soltwisch J, Pirkl A, Dreisewerd K (2011) Direct laser desorption ionization of endogenous and exogenous compounds from insect cuticles: Practical and methodologic aspects. *J Am Soc Mass Spectrom* 22(7):1273–1284.
53. Bagnères A-G, Blomquist GJ (2010) Site of synthesis, mechanism of transport, and selective deposition of hydrocarbons. *Insect Hydrocarbons Biology, Biochemistry, and Chemical Ecology*, eds Blomquist GJ, Bagnères A-G (Cambridge Univ Press, Cambridge, UK), pp 75–99.
54. Juárez P, Chase J, Blomquist GJ (1992) A microsomal fatty acid synthetase from the integument of Blattella germanica synthesizes methyl-branched fatty acids, precursors to hydrocarbon and contact sex pheromone. *Arch Biochem Biophys* 293(2): 333–341.
55. Smith S (1994) The animal fatty acid synthase: One gene, one polypeptide, seven enzymes. *FASEB J* 8(15):1248–1259.
56. Qiu Y, et al. (2012) An insect-specific P450 oxidative decarbonylase for cuticular hydrocarbon biosynthesis. *Proc Natl Acad Sci USA* 109(37):14858–14863.
57. Gu P, Welch WH, Guo L, Schegg KM, Blomquist GJ (1997) Characterization of a novel microsomal fatty acid synthetase (FAS) compared to a cytosolic FAS in the housefly, *Musca domestica*. *Comp Biochem Physiol B Biochem Mol Biol* 118(2):447–456.
58. Kajiwara S, Oura T, Shishido K (2001) Cloning of a fatty acid synthase component FAS1 gene from *Saccharomyces kluyveri* and its functional complementation of *S. cerevisiae* fas1 mutant. *Yeast* 18(14):1339–1345.
59. Dwyer LA, Blomquist GJ, Nelson JH, Pomonis JG (1981) A ^{13}C-NMR study of the biosynthesis of 3-methylpentacosane in the American cockroach. *Biochim Biophys Acta* 663(2):536–544.
60. Dillwith JW, Nelson JH, Pomonis JG, Nelson DR, Blomquist GJ (1982) A ^{13}C NMR study of methyl-branched hydrocarbon biosynthesis in the housefly. *J Biol Chem* 257(19): 11305–11314.
61. Chase J, Jurenka RA, Schal C, Halarnkar PP, Blomquist GJ (1990) Biosynthesis of methyl branched hydrocarbons in the German cockroach *Blattella germanica* (L.) (Orthoptera: Blatellidae). *Insect Biochem* 20:149–156.
62. Guo L, Blomquist GJ (1991) Identification, accumulation, and biosynthesis of the cuticular hydrocarbons of the southern armyworm *Spodoptera eridania* (Lepidoptera: Noctuidae). *Arch Insect Biochem Physiol* 16:19–30.
63. Ablard K, Gries R, Khaskin G, Schaefer PW, Gries G (2012) Does the stereochemistry of methylated cuticular hydrocarbons contribute to mate recognition in the egg parasitoid wasp Ooencyrtus kuvanae? *J Chem Ecol* 38(10):1306–1317.
64. Silk PJ, et al. (2011) Contact sex pheromones identified for two species of longhorned beetles (Coleoptera: Cerambycidae) *Tetropium fuscum and T. cinnamopterum* in the subfamily Spondylidinae. *Environ Entomol* 40(3):714–726.
65. Linder SW, Yanik GW, Bobbitt DR (2004) Evaluation of laser-based polarimetry for the determination of enantiomeric excess at extremes of the ee scale. *Microchem J* 76: 105–112.
66. Vandendool H, Kratz PD (1963) A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography. *J Chromatogr A* 11: 463–471.
67. Carlson DA, Bernier UR, Sutton BD (1998) Elution patterns from capillary GC for methyl-branched alkanes. *J Chem Ecol* 24(4):845–1865.