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Physical properties of insect cuticular hydrocarbons: The effects of chain length, methyl-branching and unsaturation

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The waterproofing abilities of insect cuticular lipids, consisting mainly of hydrocarbons, are thought to depend upon their biophysical properties. However, little is known regarding the effects of specific structural changes upon cuticular lipid properties. We examined the phase behavior of pure hydrocarbons differing in chain length, methyl-branching pattern, and unsaturation, using Fourier transform infrared spectroscopy. Melting temperatures (T_m) of 21–40 carbon *n*-alkanes increased by 1–3°C for an increase in backbone chain length of one carbon atom. The effects of methyl-branching on hydrocarbon properties depended upon the location of the methyl group along the molecule. Melting temperatures of 25-carbon long methylpentacosanes decreased by over 30°C as the location of the methyl moiety was shifted from the terminal portion of the molecule to more internal positions. Addition of a second methyl branch had additional effects on T_m. Unsaturation decreased T_m by 50°C or more.

Key words: Chain length; Cuticular lipid; Hydrocarbon; Methyl-branching; Phase transition; Unsaturation.

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Introduction

Insects contain a wide variety of hydrophobic lipids on the surface of their cuticles. These lipids provide the primary passive barrier to evaporative water loss and are a major factor allowing insects and other land arthropods to thrive in terrestrial environments. The chemical composition of cuticular lipids has received considerable attention. Variation in composition occurs at all levels of organization, from among species to within a single individual. Long-chain hydrocarbons (>20 carbon atoms) predominate in many species (Blomquist *et al.*, 1987; Lockey, 1988; de Renobales *et al.*, 1991). These can be straight-chain *n*-alkanes, methyl-branched alkanes, or unsaturated alkenes. Additional constituents include wax esters, fatty acids, long-chain alcohols, sterols, and so forth.

A great deal of effort has been directed toward understanding the functional significance of variation in cuticular lipids. In some insects, certain compounds serve in chemical communication, both intra- and interspecifically (Howard, 1993). However, no such role is known for most cuticular lipids, nor have these lipids been implicated in communication in numerous insect species. Physiologists have focused upon the role of surface lipids in water balance (Edney, 1977; Hadley, 1994). A common observation has been that the typically low water loss rates of insects

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increase rapidly above a "critical" or "transition" temperature (Wigglesworth, 1945; Loveridge, 1968; Davis, 1974). This phenomenon has been hypothesized to reflect melting of the surface lipids, with a consequent increase in cuticular permeability (Wigglesworth, 1945). Several biophysical studies, using a variety of techniques, have provided evidence consistent with this interpretation (Beament, 1945; Chefurka and Pepper, 1955; Holdgate and Seal, 1956; Toolson *et al.*, 1979).

Besides biophysical studies, comparisons of the surface lipid composition of insects from different habitats appear to be consistent with the proposed importance of lipid phase behavior in water balance. Individuals and species living in warmer, drier environments tend to have longer chain-length hydrocarbons (Hadley, 1977, 1978; Toolson and Hadley, 1977, 1979; Hadley and Schultz, 1987). These would be predicted to melt at higher temperatures. However, methyl-branching and unsaturation patterns may also change (Toolson, 1982; Gibbs and Mousseau, 1994). In phospholipids, these structural changes have significant effects on lipid physical properties, tending to lower T_m (Stubbs and Smith, 1984). However, there is no *a priori* reason to expect similar behavior in hydrocarbons. The physical properties of phospholipids are strongly dependent upon their interactions with water to form oriented monolayers, bilayers, and multilamellar structures (Crowe *et al.*, 1992), and results based on these compounds may not generalize to other lipid classes.

Given the apparent relationship between cuticular permeability and the physical properties of surface lipids, an understanding of the functional physiological consequences of lipid variation requires knowledge of the relationship between lipid structure and physical properties. Hydrocarbons are the most abundant and most well-described cuticular lipids. Of those hydrocarbons occurring naturally on insects, only the properties of straight-chain *n*-alkanes have been studied in any detail (Maroncelli *et al.*, 1982; *CRC Handbook*, 1992). The purpose of this study was to examine how specific changes in hydrocarbon structure affect phase behavior. We used Fourier transform infrared (FTIR) spectroscopy to investigate the effects of chain length, methyl-branching, and unsaturation on the physical properties of pure hydrocarbons. Melting temperatures increased with chain length, for *n*-alkanes, methylalkanes, and alkenes. Our analysis of the complete series of 25-carbon long methylpentacosanes indicated that the presence of a single methyl branch can lower T_m by over 30°C. These effects were strongly dependent upon the position and number of methyl groups. The effects of unsaturation were even greater; the insertion of a single double bond reduced T_m by ~50°C.

Materials and Methods

Chemicals

Alkenes and most straight-chain alkanes (those with 21–30 and 33 carbons) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Other *n*-alkanes were purchased from PolyScience Corp. (Niles, IL). Methylpentacosanes were synthesized as described by Pomonis *et al.* (1989). Other hydrocarbons (primarily dimethylalkanes) were a generous gift from Dr. Robert F. Doolittle. Unless stated otherwise, all compounds were at least 98% pure as indicated by gas chromatography.

Infrared spectroscopy

Biophysical properties of hydrocarbons were examined using FTIR spectroscopy, as described by Gibbs and Crowe (1991). Five to fifty micrograms of lipid in hexane were spotted as a thin film onto an infrared-transparent CaF_2 window. After the solvent evaporated, the window was placed in a temperature-controlled cell holder in a Perkin-Elmer Systems 2000 FTIR spectrometer. The sample temperature was ramped at 1–2°C intervals from at least 20°C below the solid-liquid phase transition temperature to ~10°C above the transition temperature. Five scans were averaged at each temperature, and the scanning interval was 1.0 cm^{-1}. We note that the scanning interval simply indicates the density of data used to calculate the Fourier transform, and that this interval still allows peak locations to be determined to within <0.1 cm^{-1} (Braiman and Rothschild, 1988). We used the frequency of the $-CH_2-$ symmetric stretching absorbance peak as our index of the progress of lipid melting. This peak shifts to higher frequencies as lipids melt, from ~2849 cm^{-1} to ~2853 cm^{-1} (Blazyk and Rana, 1987; Crowe *et al.*, 1989; Gibbs and Crowe, 1991). Hydrocarbon melting temperatures were determined by probit analysis.

Results

Straight-chain alkanes

Representative lipid melting curves for 21- to 40-carbon *n*-alkanes are depicted in Fig. 1. Most compounds exhibited at least two shifts in $-CH_2-$ symmetric stretch vibration frequency. The first transition occurred several degrees below the capillary melting point, when the absorbance peak shifted from ~2849

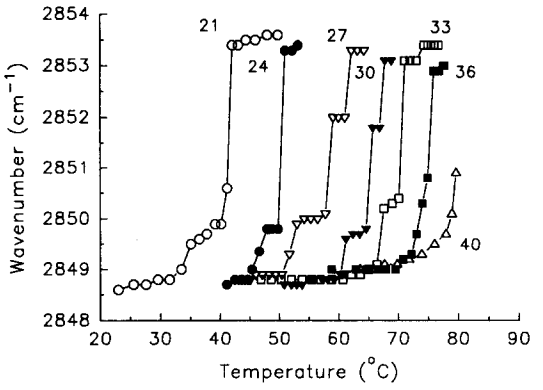

Fig. 1. Representative melting curves for *n*-alkanes. Numbers indicate chain lengths.

cm^{-1} to ~2850 cm^{-1}. The most dramatic frequency change occurred near the capillary melting temperature, when the frequency increased by ~3 cm^{-1} over a span of <2°C. In intermediate-length compounds (25–33 carbon atoms), an additional high-temperature transition was also apparent. For each compound, T_m was estimated as the midpoint of the main transition. Lipid melting temperatures measured by FTIR increased with chain length, by 1–3°C per additional methylene unit, and were within 1–2°C of capillary melting point and calorimetric estimates of T_m (Fig. 2).

Methyl-branched alkanes

All methyl-branched pentacosanes melted at lower temperatures than 25-carbon *n*-pentacosane, but the effects of methyl-branching upon T_m were highly position-dependent. Two-methylpentacosane melted ~10°C lower than *n*-pentacosane, and T_m values decreased by an additional 25°C as the methyl branch was shifted from the 2-position to more internal locations (Fig. 3). Six- to twelve-methylpentacosanes melted at 17–21°C, but 13-methylpentacosane melted at nearly 30°C. Rather than seeing two or three distinct transitions as for the *n*-alkanes, methyl-branched pentacosanes tended to exhibit a single major transition, occurring over a range of 5–10°C (Fig. 4).

To investigate further the effects of chain length on hydrocarbon physical properties, we analyzed additional methylalkanes with greater chain lengths, on one or the other side of the methyl group. Lipid melting temperatures increased with chain length, no matter which end of the carbon backbone was extended (Fig. 5).

Dimethylalkanes studied are listed in Table 1. These differed in both chain length and methyl branch position. Differences in chain length could be generated in three ways: by changing the number of carbon atoms on either side of the backbone, or by insertion of methylene units between the methyl branches.

We analyzed five 11,15-dimethylalkanes differing in the length of the longer end of the backbone. Melting points varied between 15°C and 24°C as the chain length increased from 31 to 37 carbons (Fig. 6, upper panel). We also examined four dimethylalkanes with a 20-carbon chain on the longer end of the methyl branches, differing in the length of the shorter end. If one numbers the carbon backbone in the reverse of convention (i.e., starting from the longer end of the molecule), these could

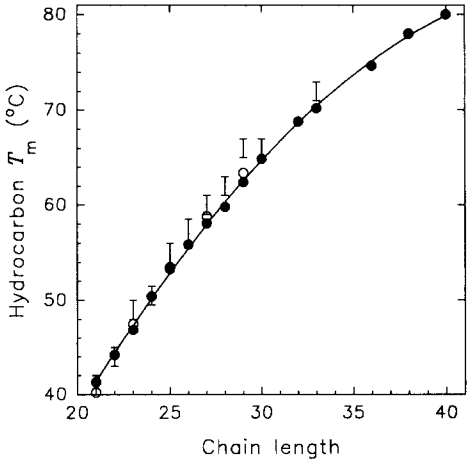

Fig. 2. Effects of chain length upon T_m of *n*-alkanes. Filled symbols indicate FTIR determinations. Open symbols are T_m values determined using differential scanning calorimetry (from Maroncelli *et al.* (1982)). Vertical bars indicate T_m ranges from the *CRC Handbook* (1992) or provided by Aldrich Chemical Co. (capillary melting point determinations). Melting temperatures for 38- and 40-carbon *n*-alkanes were estimated; we could not determine the end of the phase transition because our temperature controller could not operate above 80°C.

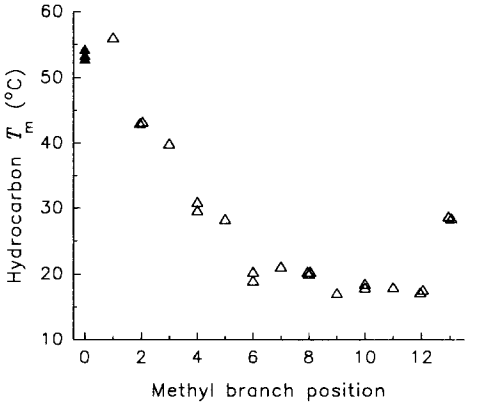

Fig. 3. Effects of methyl-branch position on T_m of 25-carbon chain length methylpentacosanes. Each point represents a separate determination of T_m. Filled symbols: *n*-pentacosane; open symbols: methylpentacosanes. The "1-" isomer was *n*-hexacosane.

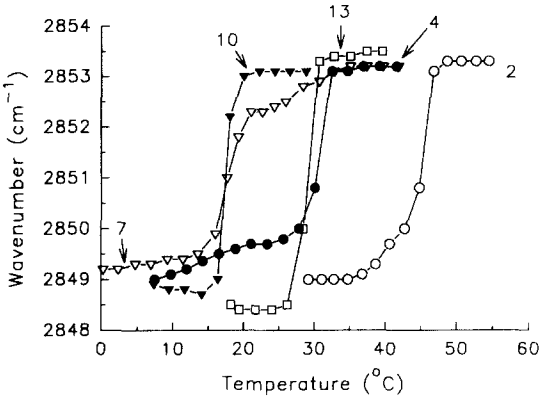

Fig. 4. Representative melting curves for methylpentacosanes. Numbers indicate methyl-branch positions.

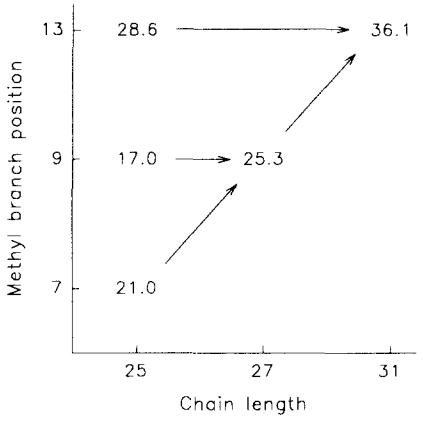

Fig. 5. Effects of chain elongation upon T_ms of methyl-branched alkanes. Values are T_m estimates for each combination of chain length and branch position. Horizontal arrows indicate elongation of the longer end of the carbon backbone, and diagonal arrows indicate elongation of the shorter end.

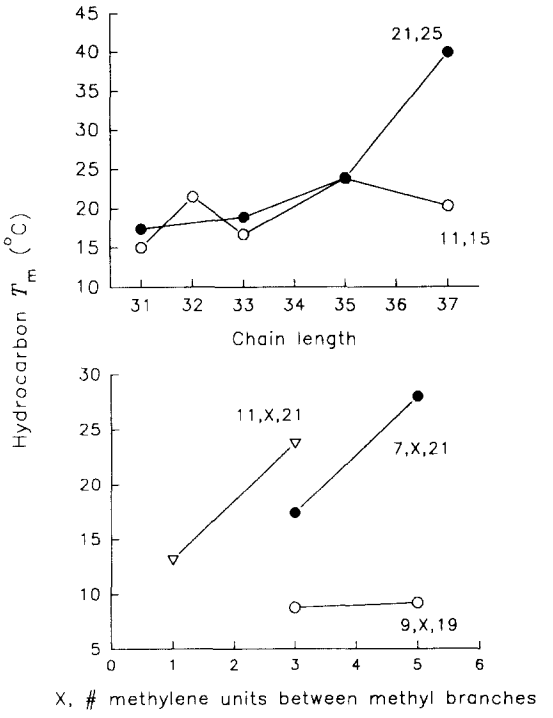

Fig. 6. Effects of chain elongation on T_ms of dimethylalkanes. Upper panel: Compounds differing in chain length on either end of the carbon backbone. Open symbols: 11,15-dimethylalkanes; filled symbols: dimethylalkanes with 20 carbon atoms after the second methyl branch. Counting backwards from the longer end of the molecule, these can be considered 21,25-dimethylalkanes. Lower panel: Compounds differing in the number of methylene units between methyl branches. Numbers indicate number of carbon atoms on either side of the interbranch segment. Summation of the three numbers provides the length of the carbon backbone. For example, 11,1,21 represents 11,13-dimethyltritriacontane (chain length equals 33 carbons), and 11,3,21 represents 11,15-dimethylpentatriacontane (35 carbons long).

Table 1. Dimethylalkanes analyzed in this study.

Carbon backbone length	Methyl branch positions	Purity
Hentriacontanes:		
31 carbons	7,11	87.6
	9,13	97.8
	11,15	96.3
Dotriacontane:		
32 carbons	11,15	93.4
Tritriacontanes:		
33 carbons	7,13	96.4
	7,15	91.7
	9,13	96.2
	9,15	79.0
	11,13	100.0
	11,15	97.8
	15,18	90.3
Pentatriacontane:		
35 carbons	11,15	94.0
Heptatriacontanes:		
37 carbons	11,15	88.5
	13,17	98.4

Purities were determined using gas chromatography.

be considered as 21,25-dimethylalkanes, again 31 to 37 carbons in length. For this series, T_m increased uniformly with chain length (Fig. 6, upper panel). Three pairs of dimethylalkanes were available in which chain length was increased by insertion of two methylene units between the methyl branches. For two of these pairs, the T_m of the longer hydrocarbon was over 10°C higher (Fig. 6, lower panel).

As indicated by our analysis of the methylpentacosanes, lipid physical properties can be affected significantly by changes in methyl-branch position, in the absence of chain length variation. Figure 7 depicts melting temperatures of seven dimethyltritriacontanes (33 carbons long), arranged as two series for which the position of one methyl branch is held constant and the position of the other is varied. No consistent pattern was observed; T_m increased or decreased as the second methyl branch changed position.

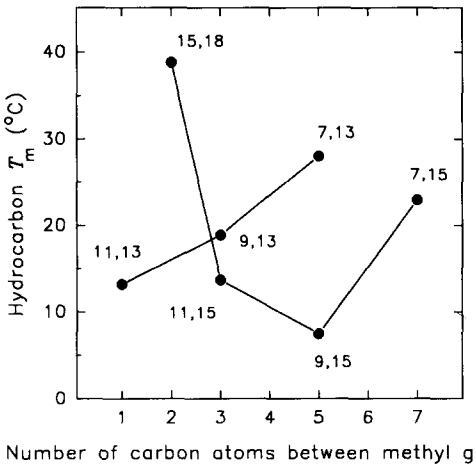

Fig. 7. Effects of methyl branch position on T_ms of dimethyltritriacontanes (chain length equals 33 carbons). Numbers indicate methyl branch positions for each compound. *n*-Tritriacontane melted at ~70°C (Fig. 1).

Effects of unsaturation

We examined two unsaturated *n*-alkenes, (*Z*)-9-heneicosene (21 carbon atoms) and (*Z*)-9-tricosene (23 carbon atoms). Their melting curves are depicted in Fig. 8, along with those of *n*-alkanes having the same chain lengths. Unsaturated hydrocarbons melted at much lower temperatures than their corresponding *n*-alkanes. Calculated T_m values were −0.6°C for tricosene and −10.0°C for heneicosene.

Discussion

The absorbance frequency of $-CH_2-$ symmetric stretching vibrations indicates the extent of *trans-gauche* isomerization about carbon–carbon bonds along the hydrocarbon chain (Maroncelli *et al.*, 1982). As lipids undergo solid–liquid phase transitions, the relative proportion of *gauche* conformers increases. Infrared spectra of *n*-alkanes exhibited substantial changes (~3 cm^{-1}) in the frequency of $-CH_2-$ symmetric stretching vibrations, at temperatures similar to their melting points determined by other techniques (Fig. 2). These observations confirm the validity of FTIR as a tool for biophysical analyses of hydrocarbons.

Besides lipid melting, additional biophysical changes could be detected using FTIR. Most *n*-alkanes exhibited a change in $-CH_2-$ symmetric stretching frequency below the main transition, and those with 25–33 carbons also underwent a third transition above the capillary melting point (Fig. 1). One possible explanation is that this is an artefact of the scanning interval (1.0 cm^{-1}) used in this study. This interval should still allow peak locations to be determined with resolution better than 0.1 cm^{-1} (Braiman and Rothschild, 1988). However, visual inspection of hydrocarbon melting curves led us to suspect that some optical artefact might cause peaks to tend to appear at integral or half-integral frequency values. To test this possibility, we examined *n*-pentacosane using a scanning interval of 0.2 cm^{-1}, the best resolution possible with our instrument. The melting curve at the higher resolution differed little from those obtained at 1.0 cm^{-1} resolution (Fig. 9). We therefore conclude that the step changes in frequency reflected actual physical events.

The low temperature shift in wavenumber probably indicated a solid–solid transition event, possibly the introduction of "end-gauche" or "kink" packing defects (Maroncelli *et al.*, 1982; Blazyk and Rana, 1987). Previous FTIR studies of *n*-alkanes have characterized these transitions using data from the conformationally sensitive region (1400–1700 cm^{-1}) of the infrared spectrum (Maroncelli

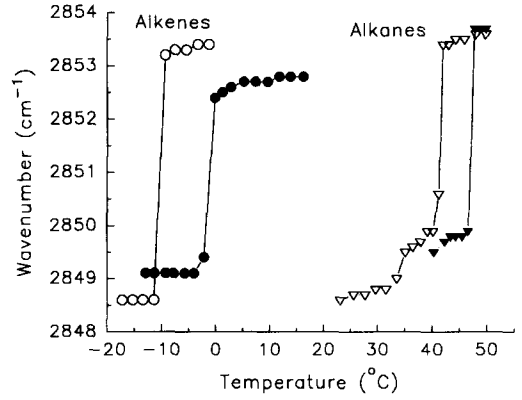

Fig. 8. Effects of unsaturation on melting curves of (*Z*)-9-unsaturated hydrocarbons. Open symbols: chain lengths were 21 carbons; filled symbols: chain lengths were 23 carbons.

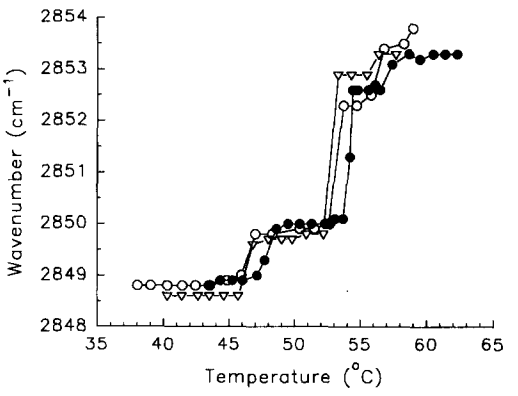

Fig. 9. Melting curves for *n*-pentacosane obtained using a scanning interval of 1.0 cm^{-1} (open symbols) or 0.2 cm^{-1} (filled symbols).

et al., 1982), or have employed shorter chain length hydrocarbons than those reported in this study (Blazyk and Rana, 1987). Our results extend their findings to additional hydrocarbons and demonstrate that solid–solid phase transitions can also be detected from the $-CH_2-$ symmetric stretching vibrational frequency. It is unclear what physical event the third transition represented, or why it was absent in the longest hydrocarbons studied. Perhaps in longer compounds the main and upper transitions converged and could not be distinguished.

Terrestrial arthropods contain many other cuticular hydrocarbons besides *n*-alkanes. Because of their limited availability, long-chain methylalkanes have not been investigated by biophysicists. We found that methyl-branching had significant effects on hydrocarbon physical properties. For 25-carbon long methylpentacosanes, T_ms decreased greatly as the methyl branch was moved to more internal positions along the carbon backbone (Fig. 3). We did not observe the discrete frequency shifts seen for *n*-alkanes. Instead, most methylalkanes exhibited broader transitions occurring over a 5–10°C range, often preceded by a continuous increase in wavenumber at lower temperatures (Fig. 4). A high temperature transition was not seen. The observation that terminal methyl-branching had relatively little effect on T_m indicates that the long straight-chain portion dominates the physical properties of these molecules. Internal branching is more disruptive of hydrocarbon packing. Our data confirm the hypothesis that disruption of lipid packing by methyl branches broadens the phase transition and reduces T_m (Lockey, 1988).

The ~10°C increase in T_m from 12- to 13-methylpentacosane (Fig. 3) was surprising. Possible explanations for this could concern the symmetric structure of 13-methylpentacosane. Except for the 2- and 13-methylpentacosanes, our branched alkane series contained enantiomeric mixtures, which could have affected T_m. Thus, it is possible that T_m values for methylpentacosanes were underestimated due to interactions between stereoisomers. Even so, it is clear that the physical properties of methylalkanes are strongly dependent upon methyl-branch position. We note that it is unknown whether or not insects synthesize one or both enantiomers of methylalkanes (Howard, 1993).

The situation for the dimethylalkanes is even more complex, because these consisted of mixtures of four different stereoisomers. Also, they were somewhat variable in purity (Table 1). As was the case for *n*-alkanes (Fig. 2) and methylalkanes (Fig. 5), an increase in chain length often resulted in an increase in T_m (Fig. 6). However, chain length had little effect on T_m in the 11,15-dimethylalkane series. The variability among dimethylalkane series may result from the fact that increasing chain length in one part of the molecule will change the positions of the methyl branches relative to the carbon backbone. Clearly, T_m is highly dependent upon methyl branch position (Fig. 3). We can only draw the general conclusion that T_m is usually correlated with chain length. The data shown in Fig. 7, for seven dimethylalkanes, all having a 33-carbon backbone, demonstrate why more specific predictions are untenable. Melting temperatures differed by up to 30°C among these compounds, depending on the locations of the methyl branches.

We were able to obtain only two alkenes in pure form, (*Z*)-9-tricosene and (*Z*)-9-heneicosene. We note that (*Z*)-9-tricosene is the main component of the female sex pheromone of houseflies (Carlson *et al.*, 1971; Adams and Holt, 1987). Each of these compounds melted more than 50°C below the corresponding *n*-alkane (Fig. 8). Unfortunately, we could not examine the effects of double bond position directly. A somewhat relevant published value of T_m is available for a terminally unsaturated alkene, 1-eicosene (chain length equals 20 carbons), which melts at 28.5°C, whereas saturated *n*-eicosane melts at 36.8°C (*CRC Handbook*, 1992). Based on this limited data set, it appears that the effects of double bond position mirror those of methyl-branch position; internal unsaturation reduces T_m more than does a terminal double bond.

We have only considered here the effects of structural changes on the physical properties of pure hydrocarbons. However, surface lipids of insects and other arthropods do not consist of a single pure compound, nor even only of hydrocarbons. Melting of natural lipid mixtures occurs over a broad temperature range (15–25°C; Gibbs and Crowe, 1991; Gibbs *et al.*, 1991), whereas the pure compounds analyzed in this study exhibited relatively sharp phase transitions. An important question to be addressed is whether or not the physical properties of natural surface lipid mixtures can be predicted from the properties of the individual components.

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