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Novel wax esters and hydrocarbons in the cuticular surface lipids of the red harvester ant, *Pogonomyrmex barbatus*

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Abstract

The cuticular surface lipids of the red harvester ant, *Pogonomyrmex barbatus*, were found to contain minor amounts of novel wax esters, in addition to the major components, hydrocarbons. The wax esters ranged in carbon number from C19 to C31 and consisted of esters of both odd- and even-numbered alcohols and acids. Each wax ester with a given carbon number eluted at several different retention times indicating possible methyl branching in either the fatty acid or alcohol moiety, or in both moieties. Each eluting peak of wax esters consisted of a mixture of wax esters of the same carbon number in which the fatty acid moiety ranged from C8 to C18, and the alcohol moiety ranged from C8 to C17. Some wax esters were largely found on the head indicating they may be of a glandular origin. The hydrocarbons consisted of: *n*-alkanes, C23 to C33; odd-numbered *n*-alkenes, C27 to C35; and the major components, methyl-branched alkanes, C26 to over C49. Notable components of the methyl-branched alkanes were 2-methyltriacontane, and the novel trimethylalkanes with a single methylene between the first and second branch points, 13,15,19-trimethylhentriacontane and 13,15,21-trimethyltritriacontane. Published by Elsevier Science Inc.

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1. Introduction

Hydrocarbons are biologically stable common components of all life stages of arthropods (Nelson, 1978, 1993; Blomquist et al., 1987; Lockey, 1988; Nelson and Blomquist, 1995). They have been used as chemotaxonomic characters to distinguish closely related insect species, usually in the adult stage, but may also be applicable to distinguish larvae before visible species-specific characters have developed (Carlson and Yocom, 1986; Nelson and Buckner, 1995). Hydrocarbons of the postpharyngeal gland have been used in

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taxonomic studies of ants (Vander Meer, 1986; Dahbi et al., 1996a). The major hydrocarbons of the fire ant, *Solenopsis invicta*, were found to be the same whether obtained from the postpharyngeal gland (Thompson et al., 1981) or from the cuticle (Nelson et al., 1980). Cuticular hydrocarbons have been shown to be colony and caste-specific in many social insects (see for review: Howard, 1993; Clément and Bagnères, 1998; Singer, 1988; Wagner et al., 1998, 2000; Lahav et al., 1999; Haverty et al., 1999a).

Wax esters also may be a relatively stable and readily analyzed common component of the cuticular surface lipids of arthropods (Buckner, 1993). Long-chain wax esters are the major component of the cuticular surface lipids of whiteflies, hydrocarbons being present only in minor amounts (Buckner et al., 1994; Nelson et al., 1994, 1997, 1998, 1999, 2000). Long chain wax esters have been reported on the cuticle of *Pteronarcys californica* (Armold et al., 1969), on the pea aphid *Acyrthosiphon pisum* (Stránský et al., 1973), and grasshoppers, e.g. *Melanoplus packardii* (Blomquist et al., 1972). Both wax esters and hydrocarbons are components of pupal *Heliothis virescens* and *Helicoverpa zea* (Buckner et al., 1996). There are few reports of the presence of wax esters in social insects although acetate esters are frequently found. Wax esters were first reported on the cuticle of the imported red fire ants, *Solenopsis invicta* and *S. richteri,* with carbon numbers from 22 to 32 but the acid and alcohol moieties were not identified (Lok et al., 1975). True wax esters, esters of fatty acids with fatty alcohols, have also been reported to be constituents of the Dufour's gland of the ant, *Lasius niger* (Attygalle et al., 1987), *Cataglyphis niger* (Soroker et al., 1995), in the surface lipids of *P. barbatus* (Wagner et al., 2000), and in Dufour's gland of honey bees and bumble bees (Blum et al., 1983; Tengö et al., 1991; Katzav-Gozansky et al., 1997).

The observation of these wax esters in field-collected ants maintained in the laboratory raised the question whether these components were novel or contaminating substances from the rearing procedures. Initial mass spectra indicated that the components were short-chain wax esters, which were not detected in any of the rearing components. In this paper we report a more detailed study than previous reports (Wagner et al., 1998, 2000) on the composition and identification of the hydrocarbons and wax esters found on the surface of laboratory-kept red harvester ants, *Pogonomyrmex barbatus*. We demonstrate the presence of newly described wax esters on ants, and describe additional novel hydrocarbons (tri-methyl-branched) in this species.

2. Materials and methods

2.1. Insects

Ants, *Pogonomyrmex barbatus*, were collected near Portal, AZ and kept in the laboratory in Stanford, CA as described (Gordon and Mehdiabadi, 1999). They were kept in boxes connected to an open arena, in which the walls were coated with Fluon (Northern Products Inc., Woonsocket, RI) to prevent the ants from escaping. All boxes were filled with plaster and interconnected with Tygon tubing. The diet was based on a modified diet of Keller et al. (1989). A few birdseeds (whole) were also added to the diet.

2.2. Lipid extraction

After killing the ants from two different colonies by freezing the day before at $-80°C$, surface lipids were removed from four groups of 12 ants each. Three groups came from a colony that had been without a queen for a year, and one group was from a colony with queen. The lipids were obtained by immersion of the ants in 3 ml of pentane for 10 min in a clean glass vial. The pentane was removed under a stream of nitrogen and the sample analyzed on a Hewlett–Packard (HP) 5990 series II gas chromatograph at Stanford before shipping the dry sample overnight to the Biosciences Research Laboratory, USDA-ARS, Fargo, ND for mass spectral analysis (see below).

After separating heads from the rest of the body, pentane extractions of the two different parts were performed as for the intact animals. Four heads or four thoraxes plus abdomens were combined for the extraction in one vial. Twenty-four ants of the Sawyer colony in total were used in six replicates, and 12 ants from the Remedios colony in three replicates. After drying the extracts under nitrogen, the head extracts were re-

suspended in 50 μl chloroform, and the rest of the body in 100 μl chloroform.

All rearing materials were tested as a possible source of contamination of the insects' cuticle by extracting them with pentane as follows: an amount of food equivalent in volume to an ant, Tygon tubing, Fluon, plastic from the boxes, and whole and crushed seeds were extracted with 1-ml pentane for 10 min.

2.3. Chromatography and structural identification

All extractions of control materials from the holding boxes were analyzed using a gas chromatograph with flame ionization detectors (HP5990 series II). Aliquots of 2 μl were introduced by splitless injection onto a DB-1 capillary column (30 m, 0.25 mm ID, 0.25-μm film thickness; J. and W. Scientific, Folsom, CA). The injector was purged after 1 min. The injector and detectors were held at 300°C. The oven was initially at 170°C and then increased at 25°C/min to 220°C and then at 3°C to 300°C and held at the final temperature for 5 min (Wagner et al., 1998).

GC-MS (capillary gas chromatography-mass spectrometry) was performed on a HP 5890A gas chromatograph equipped with a pressure programmable cool on-column injection port. The column consisted of a 1-m retention gap connected to a 12.5-m × 0.2-mm capillary column of cross-linked dimethyl silicone Ultra 1 (HP) and was coupled to a HP 5970B quadrupole mass selective detector. The carrier gas was He and the initial column temperature was between 150 and 200°C and was programmed to 320°C at 3 or 4°/min and held at 320°C for 20–120 min. The system was operated and data collected with a HP 59970C computer. Ant surface lipids were suspended in 50 or 100 μl of chloroform and 1 μl of this suspension was injected. Total ion current values were transferred to MS Excel® for the preparation of GC-MS elution traces. The graphics files were then imported into Freelance® for final editing and labeling.

After GC-MS analysis of the total sample, the hydrocarbon and wax ester fractions were obtained by TLC (thin-layer chromatography) on 10 × 10 cm plates of HPTLC-GHL from Analtech. The plates were developed in diethyl ether, dried, baked at 160°C for 45 min, then stored in a desiccator. Just before use, the plates were developed in hexane/diethyl ether (95:5, v/v), air dried, the sample applied, and then developed in hexanediethyl ether (98:2, v/v). The plates were then air dried for 10 min and the lipid bands visualized by placing the plates in an iodine tank. When the lipid bands were visible, a picture was taken on a photocopier, the location of the bands marked by scoring the gel, the gel scraped into small champagne columns (Supelco #5-8098; 7.5 cm in length and 4-mm i.d.), and the lipids eluted with 7 ml of chloroform.

Mass spectra of hydrocarbons were interpreted as previously described (Nelson, 1978, 1993; Blomquist et al., 1987; Bernier et al., 1998; Carlson et al., 1998). The identification of minor components was facilitated by using the 'background subtract' feature of HP Enhanced ChemStation™ G1701BA version B.01.00 with Windows NT™ operating system. Contaminants that appeared in the samples during processing and analysis were tentatively identified using the Wiley275 mass spectral library. The mass spectral data for the wax esters were analyzed by single-ion extraction of the data (Nelson et al., 2000). The values for the mass spectral fragment ions were transferred to MS Excel® for the preparation of figures showing the mass spectra. The graphics files were then imported into Freelance® for final editing and labeling.

2.4. Quantification of hydrocarbons and wax esters

The total ion current data were analyzed using a computer spreadsheet in Lotus123™ in which the detector response was corrected for lack of linearity by using a standard curve described by three equations (Nelson et al., 1998). The data to develop the equations were obtained with a standard mixture of tricosanyl acetate, 3-methyltricosane, octacosane, tetracontane, and tricosanyl heptadecanoate. The equations used for the ranges from 0 to 3 ng and from 100 to 1000 ng were linear; the mid-range from 3 to 100 ng was best described by a first order polynomial. The equations describing the dose–response for each component of the standard mixture were calculated using Prism 2.0™ (GraphPad Software, San Diego). The formula in the spreadsheet selected the equation to be used, based on the total ion current of the GC-MS peak being calculated, to determine the nanograms that the GC-MS peak represented.

Table 1
Percent composition of hydrocarbons from laboratory maintained *P. barbatus*[a]

GC-MS peak[b]	Percent composition[c]	Components[d]
20:1	0.5 ± 0.5	
23	6.0 ± 2.2	Tricosane
24	1.4 ± 0.2	Tetracosane
?	T	?
25:1	T	Pentacosene
25	20.1 ± 1.2	Pentacosane
25A	2.6 ± 1.7	?13- and t9-Methylpentacosanes
25A	1.5 ± 1.7	7-Methylpentacosane
25A	T	5-Methylpentacosane
25A′,B	0.7 ± 0.1	3-Methyl- and 7,13-dimethylpentacosane
26	1.4 ± 0.1	Hexacosane
26A	T	13-Methylhexacosane
27:1	0.7 ± 0.1	Heptacosene
27	4.7 ± 1.2	Heptacosane
27A	4.3 ± 0.6	13-Methylheptacosane and t9-methylheptacosane
27A	3.5 ± 0.1	7-Methylheptacosane
27A	0.5 ± 0.1	5-Methylheptacosane
27B	0.8 ± 0.1	9,13-Dimethylheptacosane
27B	3.0 ± 0.3	7,13-Dimethylheptacosane + t3-methylheptacosane
27B	T	5,13-Dimethylheptacosane
28	0.5 ± 0.1	Octacosane
28A	T	14- and t13-Methyloctacosanes
28A	T	8-Methyloctacosane
28A′	0.5 ± 0.1	2-Methyloctacosane
29:1	0.6 ± 0.1	Nonacosene
29	2.2 ± 0.3	Nonacosane
29A	5.3 ± 0.9	15-, t13- and 9-Methylnonacosanes
29A	0.9 ± 0.6	9-Methylnonacosane
29A	1.7 ± 0.3	7-Methylnonacosane
29A	0.8 ± 0.7	5-Methylnonacosane
29B	0.3 ± 0.2	11,15- and 13,17-Dimethylnonacosanes
29B	1.0 ± 0.1	9,13-Dimethylnonacosane
29B	1.9 ± 0.2	7,13-Dimethylnonacosane and t3-methylnonacosane
29B	T	5,13- and 5,15-Dimethylnonacosanes
30	0.6 ± 0.1	Triacontane
30A	1.1 ± 0.1	15-, 14-, 13-, 11-, 8- and 7-Methyltriacontanes
30A	T	?
30A′	1.1 ± 0.2	2-Methyltriacontane
31:1	1.0 ± 0.2	Hentriacontene
31	1.6 ± 0.4	Hentriacontane
31A	5.9 ± 0.3	15-, 13-, 11- and 9-Methylhentriacontanes
31A	1.1 ± 0.1	7-Methylhentriacontane
31A	T	5-Methylhentriacontane
31B	3.8 ± 0.5	13,17-, 11,15- and 9,13-Dimethylhentriacontanes
31B	0.7 ± 0.1	7,13-Dimethylhentriacontane
31A′	T	3-Methylhentriacontane
31C	0.5 ± 0.1	11,15,19-Trimethylhentriacontane
31C	T	13,15,19-Trimethylhentriacontane
32	T	Dotriacontane
32A	0.6 ± 0.1	15-, 14- and 13-Methyldotriacontanes
32B	0.5 ± 0.1	14,18-, 13,??- and isomers-Dimethyldotriacontanes
33:1	T	Tritriacontene
33	T	Tritriacontane
33A	2.0 ± 0.2	17-, 15-, 13-, 11-, 9- and 7-Methyltritriacontanes
33B	2.8 ± 0.2	15,19-, 13,19-, 11,21- and 7,13-Dimethyltritriacontanes
33C	0.5 ± 0.2	13,17,21- and 11,15,21-Trimethyltritriacontanes
33C	T	13,15,21-Trimethyltritriacontane

Table 1 (*Continued*)

GC-MS peak[b]	Percent composition[c]	Components[d]
34	T	?
34A	T	15-Methyltetratriacontane
34B	T	14,20-Dimethyltetratriacontane
35:1	T	Pentatricontene
35	T	?
35A	T	15-, 13- and 11-Methylpentatriacontanes
35B	0.5 ± 0.1	15,19- and 13,21-Dimethylpentatriacontanes
35B	T	7,13-Dimethylpentatriacontane
35C	T	13,17,23-Trimethylpentatriacontane
??	T	?
36A	T	?
36B	T	?
37A	T	13-Methylheptatriacontane
37B	T	?
39A	T	?
41A	T	15- and 13-Methylhentetracontanes
41B	T	?
43A	T	17-, 15-* and 13-Methyltritriacontanes
43B	T	15,19-* and 13,17-Dimethyltritriacontanes
44A	T	?
44B	T	?
45A	T	15- and 13-Methylpentatetracontanes
45B	0.6 ± 0.1	15,19-Dimethylpentatetracontane
45C	T	?
46A	T	?
46B	T	14,20-Dimethylhexatetracontane
47A	T	?
47B	0.5 ± 0.1	15,21-Dimethylhepatetracontane
47C	T	15,??,??- and 13,??,??-Trimethylheptatetracontanes
49B	T	?
49C	T	?

[a]Total per ant = 9.0 μg.

[b]The GC-MS Peak number (preceded by an 'h' in Fig. 1a) corresponds to the number of carbon atoms in the backbone (carbon chain) of the hydrocarbon. The letters A, B and C indicate one, two or three methyl branches, respectively. Thus, 25A has a total of 26 carbons, 25 in the backbone and one methyl group. A prime symbol indicates one of the methyl branches is near the end of the carbon chain, i.e. on carbon 2, 3 or 4.

[c]Percent composition was calculated from the integrated TIC areas as described in Section 2. The amount of trace components, less than 0.1%, are indicated by a 'T'.

[d]The hydrocarbons were identified from their electron impact mass spectra. The isomers are listed in order of elution as determined by evaluating sequential scans through each CGC-MS peak. If the major isomer could be estimated, it is indicated by an '*'. A 't' indicates that a trace amount of that isomer was present based on the presence of diagnostic ion(s) in the mass spectra. A '?' indicates the component(s) could not be determined with certainty from their mass spectra other than that they were an alkane. Their tentative identification is indicated by their GC-MS Peak Number.

The amount of the fatty acid and fatty alcohol moieties of the wax esters were estimated by measuring the intensity of the single ions in the mass spectra corresponding to the protonated saturated fatty acids, i.e. at m/z 201 for the C12 acid moiety (Nelson et al., 1994, 1998). These data were then converted to nanomoles. For each wax ester, the intensities of the single ions were then expressed as a percentage of all the single ions for each individual wax ester within an eluting peak. This percentage was multiplied by the nanomoles of each wax ester determined from the total ion current to give the amount of fatty acid and fatty alcohol present in each wax ester.

2.5. Data analysis

Presence of the four main wax esters (see Table 3) on either the head or the thorax/abdomen was assessed by performing separate lipid extraction

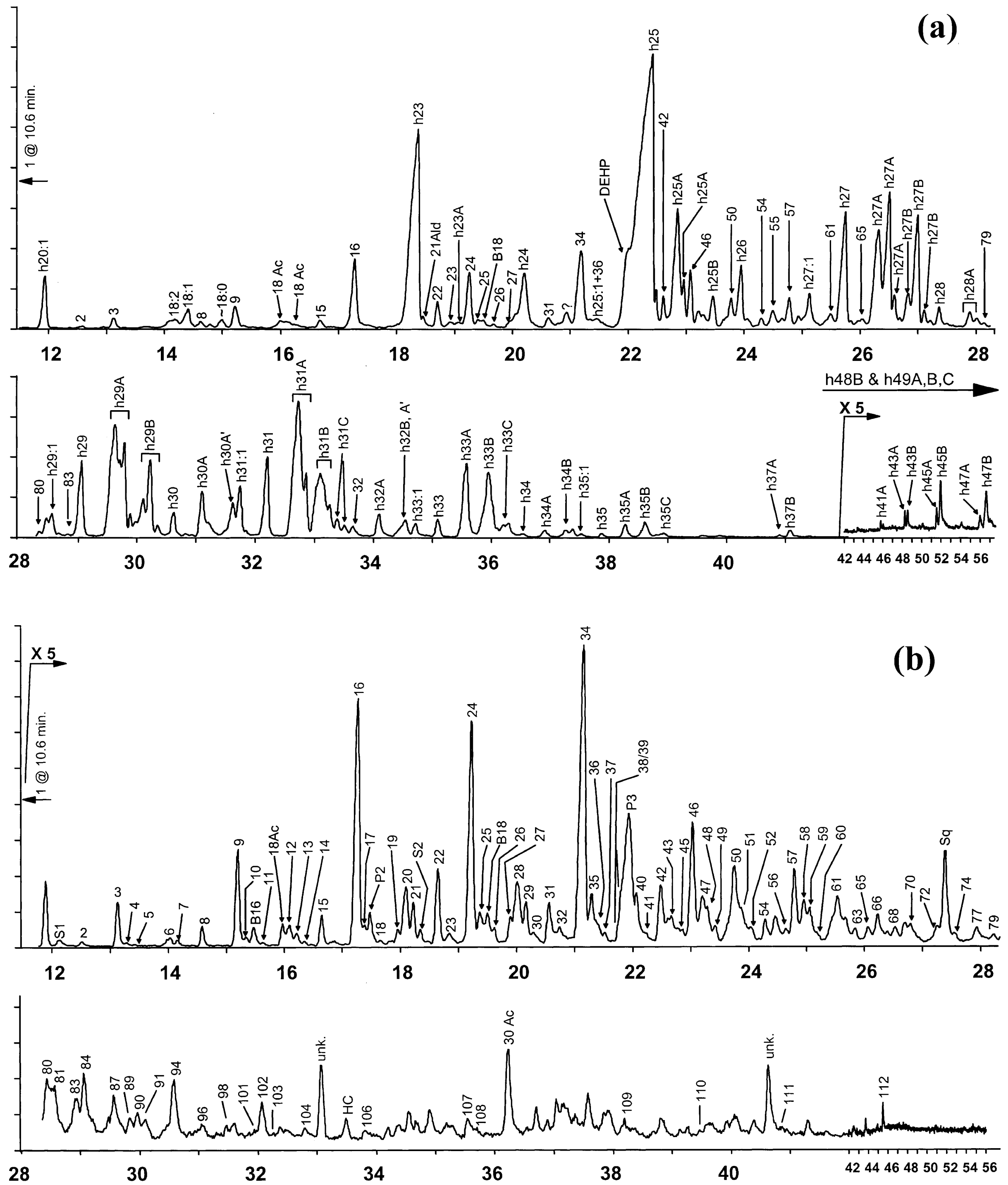

Fig. 1.

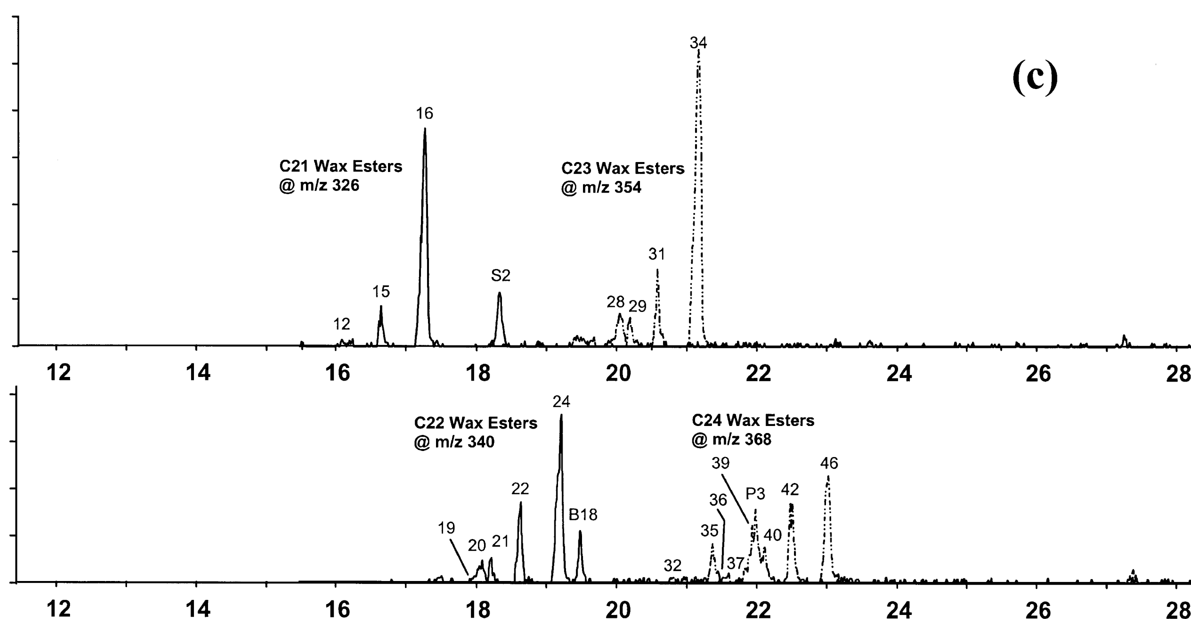

Fig. 1. (*Continued*).

of heads and decapitated ants (thorax plus abdomen). Extraction of four heads and four thoraxes plus abdomens, respectively, were performed in 0.5-ml pentane for 10 min and desiccated as described (see Section 2.2). This extraction was repeated six times for the queenless colony and three times for the queen-containing colony, for a total of nine samples for each different body part. After GC analysis (see Section 2.3), the area percentage of the wax esters was compared. As there was no significant difference between the two different colonies and no interaction between colony and body parts, (two-way ANOVA, colony and colony-body parts $P > 0.05$ for all wax esters), the data from the two colonies were combined. We then compared the area percentage between the head and the thorax plus abdomen for all four wax esters with a one-way ANOVA test.

3. Results

3.1. GC-MS analysis

GC-MS analysis of the total cuticular surface extract showed that the major components were alkanes (Fig. 1a and Table 1) ranging in size from C23 to approximately C52 (h49C). Consistent with previous work (Wagner et al., 1998), the major *n*-alkane was pentacosane (h25) and the major

Fig .1(a) GC-MS total ion current of the total ant cuticular surface lipids. Wax esters visible in the trace are marked sequentially by a number. Hydrocarbons are designated in bold type by an 'h' followed by a number, which is the number of carbon atoms in the backbone (carbon chain) of the molecule. Additional carbon atoms of the methyl branches are designated by the letters A, B and C, which indicate one, two and three methyl branches, respectively. Thus, h25A has a total of 26 carbons, 25 carbons in the backbone and one carbon in the methyl group. A letter with a prime symbol means that one of the methyl branches is near the end of the carbon chain, e.g. on carbon 2, 3 or 4. Identification of the hydrocarbons is summarized in Table 1. DEHP indicates bis(2-ethyhexyl)phthalate (common laboratory contaminant from plastics), 'Ac' indicates an acetate ester, 'Ald' indicates and aldehyde, 18:1 etc. are free fatty acids. (b) GC-MS total ion current of the TLC wax ester fraction. The wax esters are numbered sequentially starting with '1' and their characterization is summarized in Table 2. The letter 'S' indicates residue from daily analysis of a standard mixture: S1 is methyheptadecanoate, S2 is methylicosanoate. The letter 'B' indicates a butyl ester from the vial septum: B16 is butylhexadecanoate, B18 is butyloctadecanoate. The letter 'P' indicates a phthalate: P1 = butyl phthalate(eluted approx. 10 min); P2 = butyl phenylmethyl phthalate; P3 = DEHP [bis (2-ethylhexyl)phthalate], 'Sq' indicates a squalene-like contaminant from TLC. (c) Single ion plots of molecular weights showing the elution pattern of the isomers of the 4 major wax esters. Molecular weights for the C21 (m/z 326), C23 (m/z 354), C22 (m/z 340) and the C24 (m/z 368) wax esters. The trace for C21 is a solid line, and that for C23 is a dashed line. The trace for C22 is a solid line, and that for C24 is a dashed line.

Table 2
Percent composition of wax esters from laboratory maintained *P. barbatus*[a]

GC-MS Peak[b]	Carbon number[c]	Molecular weight[d]	Percent composition[e]	Acid moiety[f]
1	18	284	0.3 ± 0.2	8, 9
2	19	298	T	8, 9, 10
3	19	298	1.1 ± 0.2	8, 9, 10*
4	19	298	T	10
5	20	312	T	9, 10, 11
6	20	312	0.6	11
7	20	312	T	11
8	20	312	0.7	8, 9, 10*, 11
9	20	312	1.9 ± 0.4	9, 10*, 11, 12
10	20	312	0.3 ± 0.2	10, 11
11	20	312	T	11
12	21	326	0.7	11, 12
13	21	326	0.6	9, 10, 11, 12
14	21	326	T	10, 11, 12
15	21	326	0.9 ± 0.1	9, 10, 11, 12, 13
16**	21	326	6.0 ± 1.0	10*, 11, 12*
17	22	340	0.7 ± 0.1	10, 11, 12
18	22	340	T	10, 11, 12, 13
19	22	340	0.6	10*, 11
20	22	340	1.5 ± 0.1	11*, 12, 13
21	22	340	1.1 ± 0.1	10*, 11*, 12, 13
22	22	340	1.8 ± 0.2	10, 11*, 12*, 13
23	22	340	0.7	9, 10, 11, 12*, 13, 14
24**	22	340	5.4 ± 0.7	10, 11*, 12*, 13
25	22 + 23	340/354	1.1 ± 0.1	10, 11, 12*, 13, 14
26	23	354	0.7 ± 0.1	11*, 12, 13, 18
27	23	354	0.8	11, 12,
28	23	354	1.8 ± 0.2	10*, 11, 12, 13, 14
29	23	354	1.2 ± 0.1	11*, 12*, 13, 14
30	23	354	0.7 ± 0.1	10, 11, 12*, 13, 14
31	23	354	1.3 ± 0.1	10, 11*, 12*, 13*, 14
32	24	368	0.8 ± 0.1	11, 12, 13, 14, 15
33	24	368	0.3 ± 0.1	11, 13, 14
34**	23	354	8.3 ± 1.0	10, 12*, 14
35	24	368	1.3	10, 11, 12, 13*, 14, 15
36	24	368	0.9	10, 11, 12, 13, 14, 15
37	24	368	0.7 ± 0.1	11, 12*, 13, 14, 15
38	24	368	1.3 ± 0.2	11, 12*, 13
39	24	368	2.2 ± 0.4	10, 11, 12, 13*, 14, 15
40	24	368	1.5 ± 0.1	11, 12*, 13, 14, 15
41	25	382	0.7 ± 0.1	10, 11, 12, 13, 14
42	24	368	1.8 ± 0.2	11, 12*, 13*, 14, 15
43	25	382	1.2 ± 0.1	11, 12, 13, 14, 15
44	25	382	0.6 ± 0.1	11, 12, 13, 14
45	26	396	0.5 ± 0.3	11, 12, 13*, 14, 15
46**	24	368	3.3 ± 0.2	11, 12*, 13, 14
47	25	382	1.4 ± 0.1	10, 11, 12, 13, 14, 15, 16
48	25	382	1.1 ± 0.2	10, 11, 12, 13, 14, 15
49	25	382	0.8	11, 12, 13*, 14, 15
50	25	382	2.8 ± 0.2	11, 12*, 13, 14, 15, 16
51	25	382	1.1 ± 0.1	11, 12, 13*, 14, 15, 16
52	26	396	0.8 ± 0.2	11, 12, 13, 14, 15
53	26	396	0.3 ± 0.2	11, 12, 13, 14, 15
54	25	382	1.1 ± 0.1	11, 12*, 13*, 14*, 15
55	26	396	1.1 ± 0.1	11, 12, 13, 14, 15, 16
56	26	396	0.7	11, 12, 13, 14, 15

Table 2 (*Continued*)

GC-MS Peak[b]	Carbon number[c]	Molecular weight[d]	Percent composition[e]	Acid moiety[f]
57	25	382	2.4 ± 0.1	12*, 13, 14*, 15, 16
58	26	396	1.3 ± 0.1	11, 12*, 13, 14, 15, 16
59	26	396	1.1 ± 0.1	12, 13*, 14, 15, 16, 17
60	27	410	0.7 ± 0.1	12, 13, 14, 15*
61	26	396	2.1 ± 0.1	12*, 13, 14, 15, 16
62	26 + 27	396/410	0.9 ± 0.1	12, 13*, 14*, 15, 16
63	27	410	0.7 ± 0.1	12, 13*, 14, 15
64	27	410	0.3 ± 0.2	12, 13, 14
65	26* + 27	396/410	0.9 ± 0.1	12, 13, 14, 15, 16
66	27	410	1.2 ± 0.1	12, 13*, 14, 15, 16, 17
67	27	410	0.7 ± 0.1	12, 13, 14, 15, 16
68	26* + 27	396/410	0.9 ± 0.1	12, 13, 14*, 15, 16
69	27	410	0.9 ± 0.1	12, 13, 14, 15, 16, 17
70	27	410	1.0 ± 0.1	13*, 14, 15, 16, 17
71	28	424	0.5 ± 0.2	12, 13, 14, 15, 16, 17
72	27	410	0.9 ± 0.2	12, 13, 14, 15, 16
73	28	424	0.6 ± 0.3	13, 14, 15, 16
74	28	424	0.7 ± 0.1	13*, 14*, 15, 16
75	28	424	T	13, 14
76	27	410	0.3 ± 0.2	13, 14, 15, 16
77	28	424	0.9 ± 0.2	13, 14, 15, 16, 17
78	28	424	0.5 ± 0.1	14, 15, 16
79	27	410	0.7 ± 0.1	13, 14*, 15, 16
80	28	424	0.7 ± 0.1	12, 13*, 14*, 15, 16, 17, 18
81	29	438	0.7 ± 0.1	12, 13, 14*, 15*, 16, 17
82	29	438	T	14, 15, 16, 17
83	28	424	0.7 ± 0.1	13, 14*, 15, 16, 17
84	29	438	0.8 ± 0.1	14, 15*, 16,
85	29	438	T	14, 15, 16, 17
86	28	424	0.3 ± 0.3	14, 15, 16, 17, 18
87	29	438	0.7 ± 0.2	14, 15*, 16*, 17, 18
88	29	438	T	15, 16
89	28	424	0.3 ± 0.2	14, 15, 16, 17, 18
90	29	438	0.4 ± 0.2	10, 12, 14, 15, 16, 17, 18
91	29	438	0.5 ± 0.2	15*, 16, 17, 18
92	30	452	T	14, 15, 16, 17, 18
93	29	438	0.3 ± 0.2	14, 15, 16, 17, 18
94	30	452	0.7 ± 0.1	15*, 16*, 17, 18
95	29	438	T	11, 15, 16, 17, 18
96	30	452	0.4 ± 0.2	15, 16, 17, 18
97	29	438	T	11, 15, 16, 18
98	30	452	0.3 ± 0.3	14, 15, 16, 17, 18
99	31	466	0.4 ± 0.2	14, 16, 17, 18
100	30	452	T	16, 18
101	30	452	T	16, 18
102	31	466	0.5 ± 0.2	16*, 17, 18
103	30	452	T	14, 15, 16, 18
104	30	452	T	12, 14, 16, 18
105	31	466	T	14, 15, 16, 17, 18, 20
106	31	466	T	13, 15, 16, 18
107	32	480	T	14, 16, 18, 20
108	32	480	T	14, 16, 18
109	34	508	T	14, 16, 18, 20

Table 2 (*Continued*)

GC-MS Peak[b]	Carbon number[c]	Molecular weight[d]	Percent composition[e]	Acid moiety[f]
110	35	522	T	14, 16
111	36	536	T	14, 16, 18
112	40	592	0.5 ± 0.3	16, 17, 18, 20

[a] Total wax esters per ant = 1.3 μg.

[b] The GC-MS peak number (see Fig. 1) starts with the first detected wax ester eluting, and all subsequent wax esters are numbered sequentially. Those marked with a double asterisk correspond to wax esters reported by Wagner et al. (2000): Our peaks 16, 24, 34 and 46 correspond to their peaks 1, 3, 5 and 9, respectively.

[c] The GC-MS carbon number is the number of carbon atoms in the wax ester as determined from its molecular weight. An asterisk indicates the major component.

[d] Molecular weight values were obtained from the electron impact mass spectra.

[e] Percent composition was calculated based on the integrated TIC and protonated acid ion areas as described in Section 2. The amount of trace components, less than 0.1%, are indicated by a 'T'. Where no S.D. is given, it was less than 0.05%.

[f] The values of the carbon numbers of the acid moieties of the wax esters was determined from single ion scans of the TIC data as described in Section 2. An asterisk indicates the major isomer(s) where they could reasonably be estimated.

n-alkene was hentriacontene (h31:1). The alkenes were odd-numbered and ranged from C25 to C35. Methyl-branched alkanes were dominant and ranged in size from C26 (h25A) to C52 (h49C). They consisted of mono-, di- and trimethyl-branched compounds.

Also present in minor amounts were numerous wax esters of relatively short chain lengths which frequently blended into the baseline or were masked by the hydrocarbons. The wax esters were then isolated by TLC and analyzed by GC-MS free of the hydrocarbons. The wax esters ranged in carbon number from C18 (peak 1) to C40 (peak 127) (Fig. 1b and Table 2). All of the results were similar between the colony that possessed a queen and the colony that did not. Therefore, we only refer to the laboratory-maintained colonies, without distinctions between the two colony conditions. The major wax esters were C21 (peak 16) and C23 (peak 34). Other major peaks were 24 and 46, which consisted of mixtures of C22 + C23 and C23 + C24, respectively (Table 2).

The wax esters were present in such sufficient quantities that molecular ions were usually evident. All molecular ions observed were from saturated wax esters in both the total sample and the TLC wax ester fraction. Molecular weight plots from single ion analysis of the GC-MS data for the four major wax esters, C21, C22, C23 and C24, showed that wax esters of the same molecular weight eluted at three or more positions on the GC-MS trace (Fig. 1c). This finding indicated that branching was present in either the alcohol or acid moieties or in both. Positions of wax

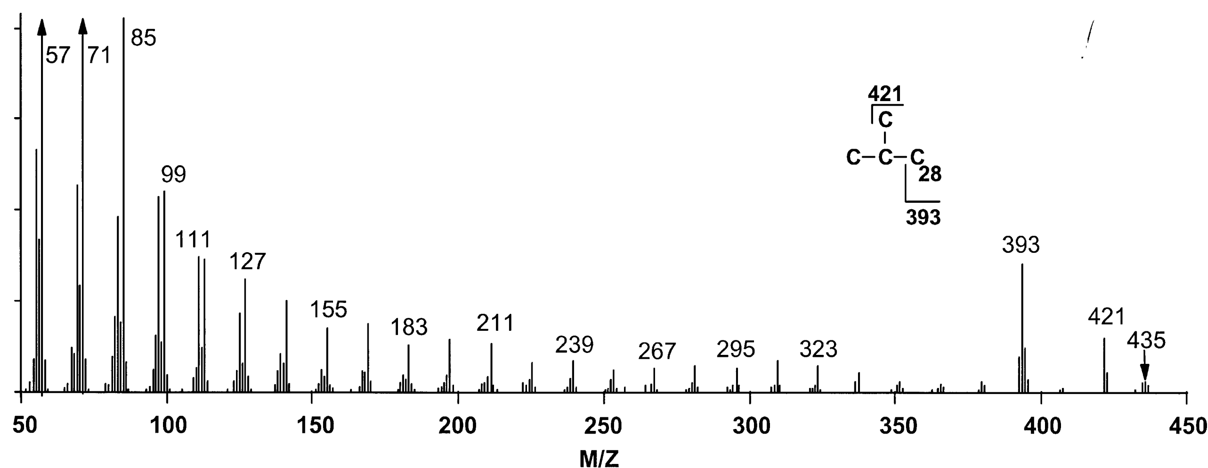

Fig. 2. Mass spectrum of GC-MS peak h30A′, 2-methyltriacontane.

esters could also be determined by single ion analysis using the values for their protonated acids. This was of less value because generally the wax esters had similar mixtures of acid moieties.

Control extracts of all materials and food to which the ants were exposed in the laboratory were analyzed by GC. No GC peaks were detected in any of these extracts that were equiva-

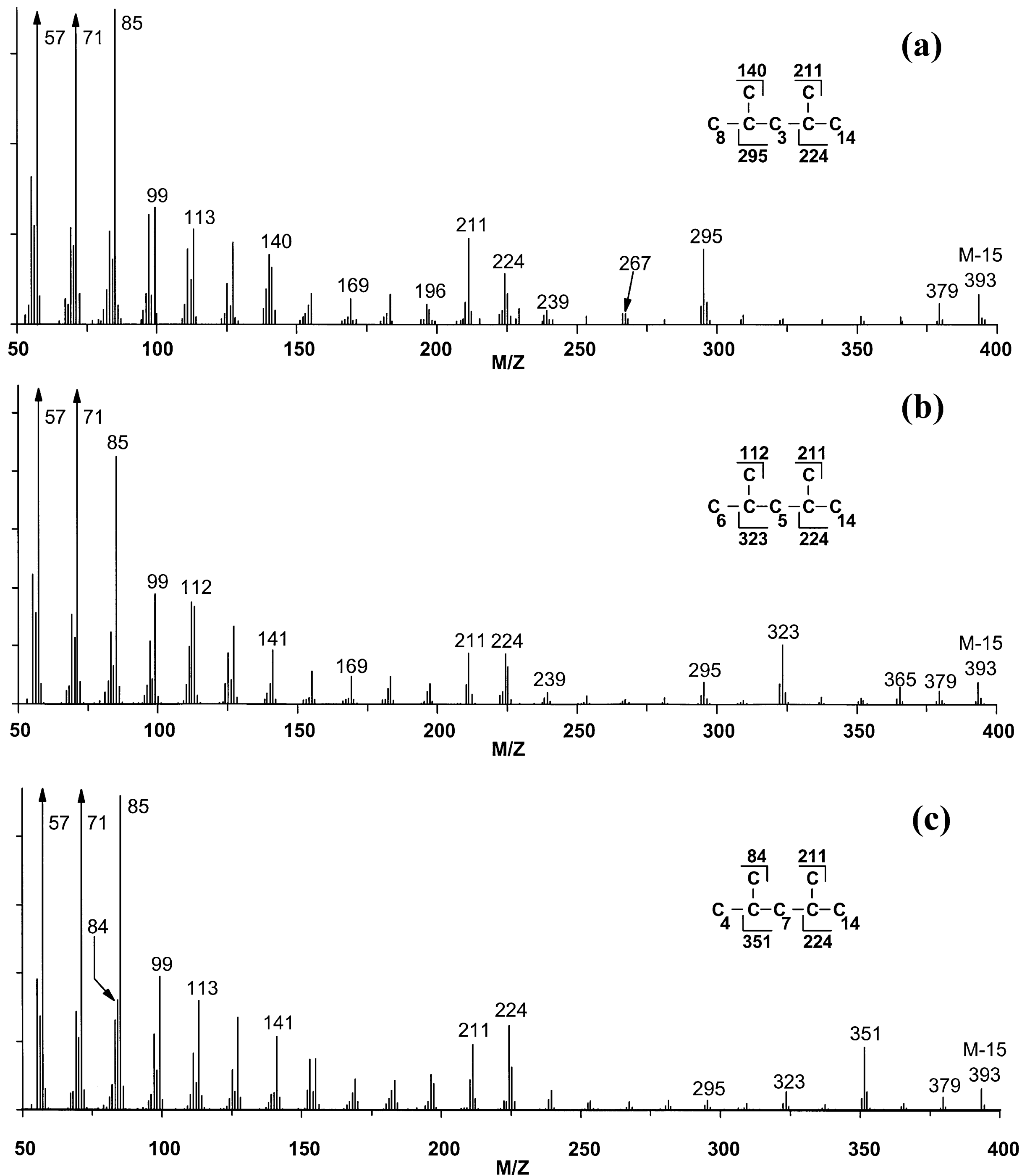

Fig. 3. Mass spectra of sequential GC-MS peaks of h27B: (A) 9,13-dimethylheptacosane; (B) 7,13-dimethylheptacosane (10 scans after A); and (C) 5,13-dimethylheptacosane (six scans after B).

lent to those observed in extracts of the cuticular lipids. Thus, the wax esters were not due to a contamination from the ants' boxes or diet.

3.2. Mass spectra of the hydrocarbon A series

The A series consisted of monomethylalkanes and ranged in size from 25A (the hydrocarbon and the peak number are the same) to at least 47A (Table 1). Most of the hydrocarbon mass spectra were readily interpreted unless the ion intensity was too low. The size of the monomethylalkanes and the positions of the methyl branches were the same as those reported for monomethylalkanes from many other insect sources.

Minor amounts of terminally branched monomethylalkanes, A′, were present: 3-methylpentacosane, 2-methyloctacosane, 2-methyltriacontane and 2-methyldotriacontane. Trace amounts of 3-methylalkanes were detected corresponding to 27A′ and 29A′. A 3-methylalkane elutes with an elution time between that of a 7,13- and a 5,13-dimethylalkane and contributes fragment ions to the mass spectrum of the dimethylalkanes which are unambiguous for the identification of the presence of the 3-methylalkane.

The assignment of a structure such as that of a 2-methyl- vs. a 4-methyl-alkane from the mass spectrum is sometimes difficult or impossible in practice. The elution of 2- and 4-methylalkanes often occurs on the leading edge of a dimethylalkane peak, which is frequently larger. The 2- and 4-methylalkanes essentially elute together, although on a high-resolution column the 4-methyl isomer does elute just ahead of the 2-methyl isomer. However, both isomers fragment to give the same major diagnostic fragment ion and the minor fragment ions necessary to distinguish them may not be apparent. In this case, the

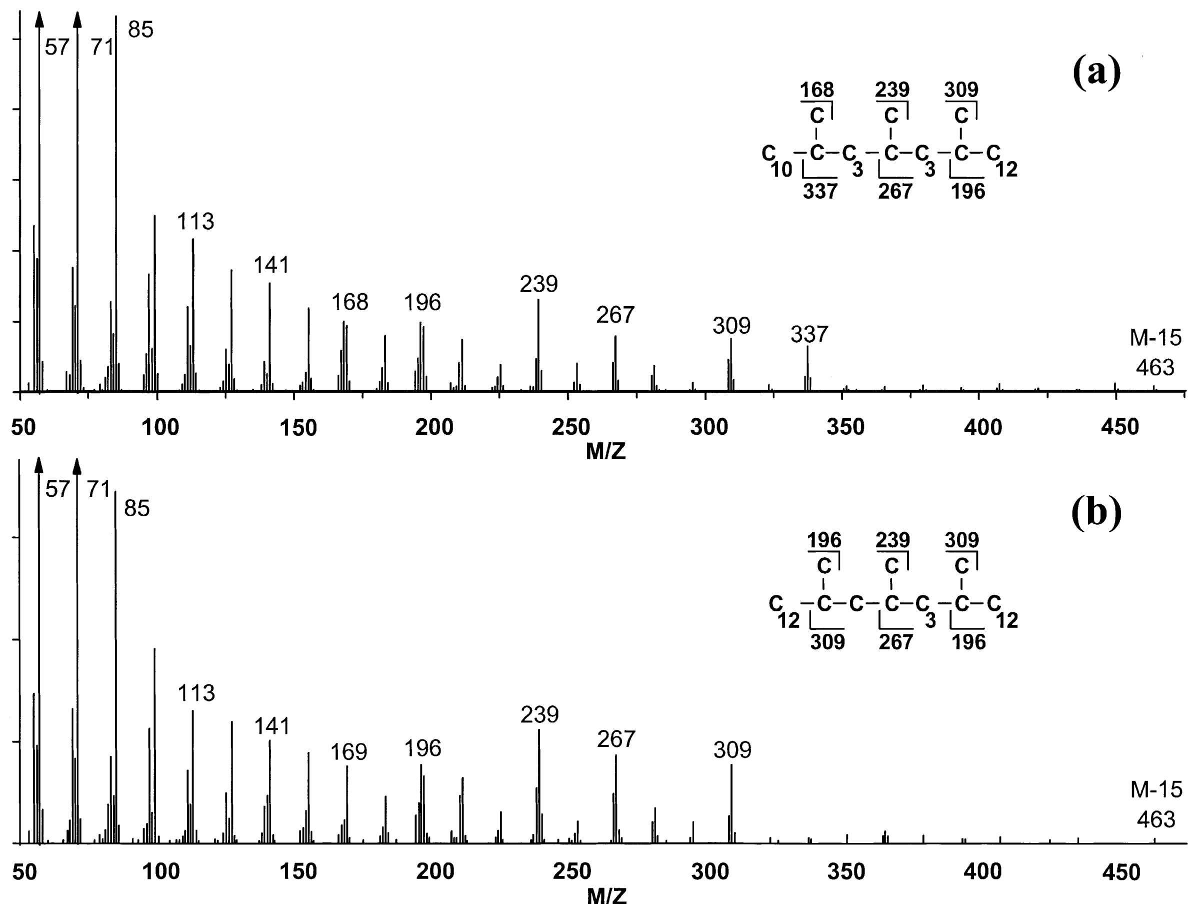

Fig. 4. Mass spectra of sequential GC-MS peaks h31C: (A) 11,15,19-trimethylhentriacontane; (B) 13,15,19-trimethylhentriacontane (six scans after A).

mass spectrum of peak 28A′ and of 30A′ (Fig. 2) showed that they were 2-methylalkanes. The fragmentations depicted in Fig. 2 do not show any fragment ions of diagnostic intensity at m/z 70 and 364/365 that would be expected from a 4-methyl isomer.

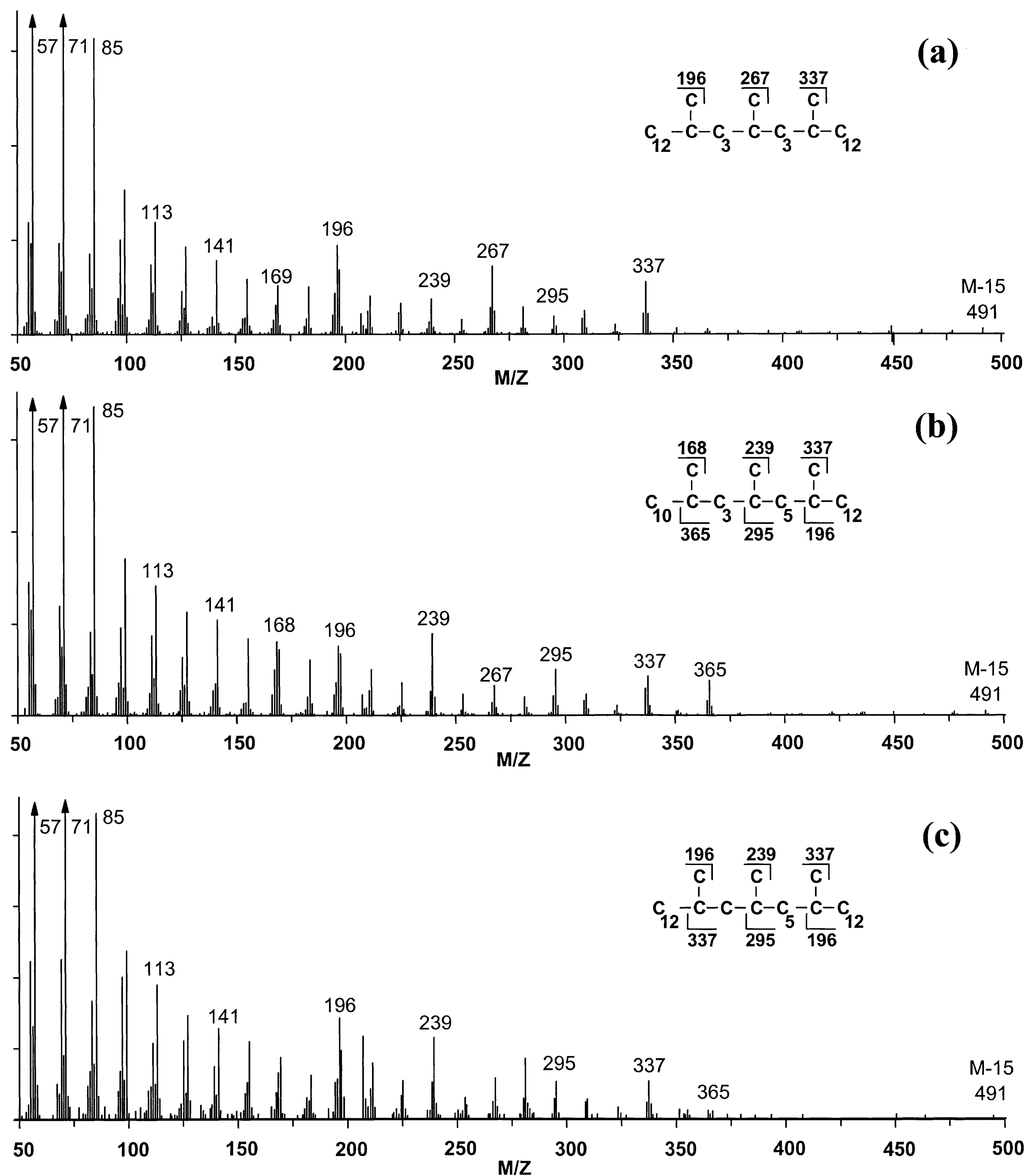

Fig. 5. Mass spectra of GC-MS peak h33C: (A) 13,17,21-trimethyltritriacontane; (B) 11,15,21-trimethyltritriacontane (six scans after A); and (c) 13,15,21-trimethyltritriacontane (six scans after B, and on the tailing edge of the peak; the asterisked ions at m/z 207 and 281 are from column bleed).

3.3. Mass spectra of the hydrocarbon B series

The dimethylalkanes from *P. barbatus* ranged in size from C27 (25B) to at least C51 (49B) (Fig. 1 and Table 1). The dimethylalkane peaks largely consisted of a single isomer. The dimethylalkane isomers exhibited a somewhat unique pattern with respect to the positions of the methyl groups; the group located internally on the carbon chain kept its position while the group located nearest to the end of the carbon chain changed position. Fig. 3 shows the mass spectra of the three isomers of 27B which eluted as three partially resolved peaks (Fig. 1). The first isomer to elute is 9,13-dimethylheptacosane in which the methyl branch points are separated by three methylenes (Fig. 3a). The second isomer to elute is 7,13-dimethylheptacosane in which the first methyl group is 2 carbons closer to the proximal end of the carbon chain, and the methyl branch points are now separated by 5 methylenes (Fig. 3b). The last isomer to elute, 5,13-dimethylheptacosane, has the first methyl group an additional two carbons closer to the proximal end of the chain and the methyl branch points are now separated by seven methylenes (Fig. 3c).

A similar sequence of change in the methyl branch positions was noted for the 29B isomers, where the second methyl position remained on carbon 13, except that some 5,15-dimethylnonacosane was detected. As the chain length of the dimethylalkanes increased to 31B, isomers 13,17- and 11,15- were both present, as is usually found in insects. Peak 33B was a mixture of isomers in which the positions of both methyl groups changed. It is notable that the 7,13-isomer was a major isomer irrespective of chain length in the B series, but only for odd-numbered carbon chain backbones from 25B to 31B.

3.4. Mass spectra of the hydrocarbon C series

Minor amounts of internally branched trimethylalkanes were identified (Table 1): two peaks of 31C (Fig. 4a,b), and broad peaks for 33C (Fig. 5a,b) and 35C. The mass spectrum of the first eluting peak of 31C was that of 11,15,19-trimethylhentriacontane (Fig. 4a). An ion for M-15 was present at m/z 463 and the proposed structure gave the expected fragment ions. This structure is commonly found in trimethylalkanes.

The second GC-MS peak of 31C to elute gave an unusual mass spectrum (Fig. 4b). The mass spectrum was completely compatible with that expected for the fragmentation of 13,15,19-trimethylhentriacontane. This is a structure, rarely found, in which the first two methyl branch points (as the structure is drawn) are separated by a single methylene.

The next group of trimethylalkanes occurred at GC-MS peak 33C in which the isomers were not resolved but eluted as a broad peak (Fig. 1). Examination of each scan through the peak showed that at least three isomers were present. The first to elute was a trimethylalkane with a symmetrical structure, 13,17,21-trimethyltritriacontane, in which the methyl branch points are separated by three methylenes, i.e. a 3,3 branching sequence (Fig. 5a). The second isomer to elute was tentatively assigned the structure of 11,15,21-trimethyltritriacontane (Fig. 5b). This compound has the first and second methyl branch points separated by three methylenes, and the second and third methyl branch points separated by 5 methylenes; a 3,5 branching sequence. The third isomer, which eluted on the tailing slope of the peak, was tentatively identified as 13,15,21-trimethyltritriacontane (Fig. 5c), a structure very rarely found, in which the first two methyl branch points are separated by one methylene and the second and third branch points by 5 methylenes; a 1,5 branching sequence.

The validity of this interpretation is supported by the identification of a similar structure in the trimethylalkane homologous series. GC-MS peak 31C, 13,15,19-trimethylhentriacontane (Fig. 4b), also had one methylene between the first and second branch points. For both homologues, the first two methyl branch points are on carbons 13 and 15, and the third methyl branch point is 13 carbons from the distal end of the carbon chain, i.e. on carbon 19 for 31C, forming a 1,3 branching sequence, and on carbon 21, a 1,5 branching sequence, for the 2-carbon longer homologue, 33C.

3.5. Mass spectra of wax esters

The GC-MS of the wax ester fraction consisted of almost a continuous elution of isomers beginning with C18 (Fig. 1b). The major wax ester was C23 (peak 34) with lesser amounts of C21, C22 and C24 (Fig. 1b,c; Table 2). In addition, for each of these major peaks, there were two or more

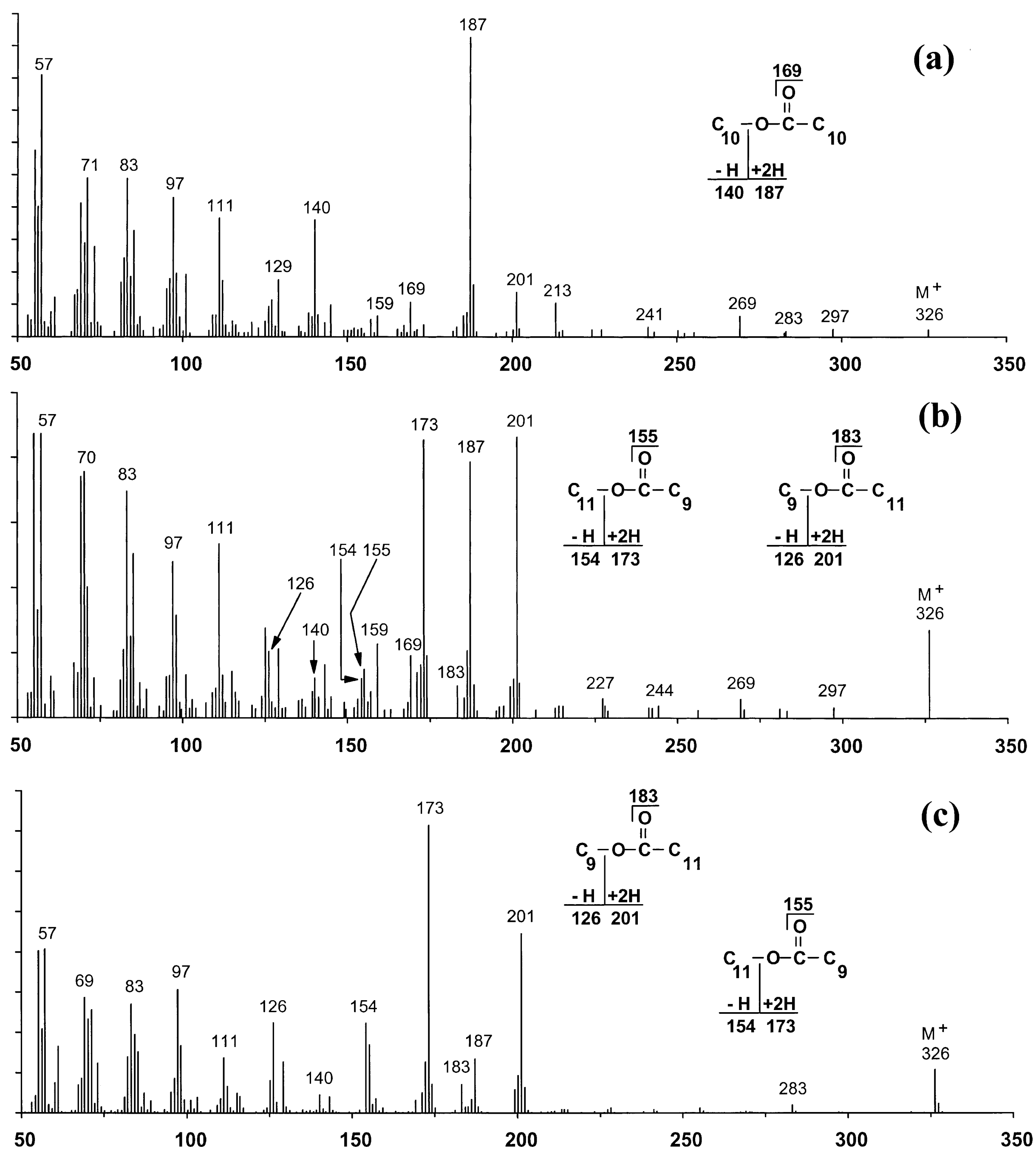

Fig. 6. Mass spectra of C21 wax esters (m/z 326; see Fig. 1c): (A) Peak 12; (B) Peak 15; and (C) Peak 16.

minor peaks of the same carbon number. Presumably, this was due to branching at different positions in the ester. A molecular ion was observed for the majority of the wax esters and these ions always corresponded to that for a saturated wax ester so differences in elution were not due to unsaturation.

Three examples of mass spectra for the major C21 (m/z 326) wax esters (see Fig. 1) are shown in Fig. 6. The spectra are similar but different. The mass spectrum of peak 12 (Fig. 6a) shows that it is largely a single wax ester isomer composed of an 11 carbon acid moiety (m/z 187) and a 10 carbon alcohol moiety (m/z 140). The ex-

Table 3
Percent composition of the major wax esters on the head vs. the thorax plus abdomen[a]

GC peak	Wax ester	Head	Thorax-abdomen
Peak 16	21	0	0.9 + 0.3
Peak 24	22	0	0.6 + 0.3
Peak 34	23	0	1.2 + 0.3
Peak 42	24	12.1 + 2.3	0.8 + 0.2
Peak 46	24	0.8 + 0.1	0.8 + 0.0

[a] The percentage composition is based on the total of the hydrocarbons plus wax esters in the cuticular surface extract of the head and of the thorax plus abdomen.

pected acylium ion is present at m/z 169. This wax ester is identified as decanoyl undecanoate. The ion at m/z 201 indicates that a minor amount of nonanoyl dodecanoate is also present.

The mass spectrum (Fig. 6b) for the next major C21 peak to elute (peak 15) clearly shows the molecular ion at m/z 326 and three major protonated acids at m/z 173, 187 and 201 corresponding to 10, 11 and 12 carbon acid moieties, respectively, showing that this peak is a mixture of at least three wax ester isomers. However, the ions for the alcohol moieties expected at m/z 154, 140 and 126, respectively, are not obvious as they are in Fig. 6a,c. Thus, although the number of carbon atoms in the alcohol and acid moieties is known, because the effect of the putative branching on fragmentation is not known, the structure of the wax esters could not be deduced.

The major C21 wax ester peak (peak 16 in Fig. 1) to elute clearly shows the major protonated acids at m/z 173 and 201 (Fig. 6c). However, in this spectrum, the ions for the alcohol moieties are again clearly evident, for these isomers at m/z 126 and 154, similar to the mass spectrum in Fig. 6a. Also, the acylium ions are evident at m/z 155 and 183. The presence of a minor amount of the 11-carbon acid and 10-carbon alcohol moieties is indicated by the ions at m/z 187 and 140. This peak had been previously identified as dodecanoicacid nonylester (Wagner et al., 2000).

3.6. Head wax esters

We examined whether the major wax esters might be of glandular origin and thus occur largely on either the head or the abdomen. To assess this, we performed separate cuticular lipid extractions from the heads only and from decapitated bodies (referred as thorax plus abdomen) of ants. A comparison of the major wax esters found on the two different body parts (head vs. thorax plus abdomen) is presented in Table 3. Most of the wax esters found on the thorax plus abdomen were absent from the head. However, one wax ester with a chain length of 24 carbons appeared almost solely on the head of the ants ($F_{1,16} = 24.1$, $P = 0.0002$, Table 3), indicating that at least some wax esters may be of glandular origin.

4. Discussion

Wax esters were minor components of the cuticular surface lipids of red harvester ants (Wagner et al., 2000). Whereas most of them could easily be considered part of the baseline 'garbage' and overlooked during the analysis of the hydrocarbons, a few of them were readily apparent. The locations of all the wax esters in the GC-MS chromatograms were readily determined by scanning the total ion current (Nelson et al., 2000) for the fragment ions corresponding to protonated acids (Ryhage and Stenhagen, 1963). Most of the wax ester peaks were present in sufficient amounts so that a molecular ion was observed in the mass spectrum. The molecular ions, all representing saturated wax esters, were also useful in determining their presence and location in the GC-MS chromatogram. The GC-MS chromatograms showed that wax esters of the same molecular weight eluted at up to at least three positions. This finding indicated that branching was present in either the alcohol or acid moieties or in both. The effect of branching on the elution times of wax esters has not been characterized. However, it is known from studies on methyl-branched hydrocarbons that the effects are significant, and dependent both on number and position of the methyl groups (Mold et al., 1966; Nelson and Sukkestad, 1970; Nelson, 1978; Blomquist et al., 1987; Carlson et al., 1998). The overall composition of the fatty acids found in the wax esters was 11, 14, 32, 17, 11, 6 and 5% for C10-C16, respectively, and the alcohol composition was 9, 13, 29, 20, 14, 10 and 5% for C9–C15, respectively. Thus, the major fatty acid moiety was dodecanoic acid and the major alcohol moiety was undecanol.

Wax esters in ants were first reported in the Dufour gland of *Lasius niger* (Attygalle et al.,

1987). The wax esters of *L. niger* and those reported herein, were unique from those known from other insects (Buckner, 1993; Buckner et al., 1996) in that they were of a relatively short chain length, and consisted of similar amounts of odd- and even-chain-length alcohols and acids, some of which were branched. Wax esters, if present, are sometimes not reported in the literature (Akino et al., 1996) or are removed from the extract prior to assaying it for biological activity or characterizing it chemically. An unusual series of wax esters of medium-chain length fatty acids with long-chain secondary alcohols were identified in the hymenopteran ecto parasitoid, *Diglyphus isaea*, which may have a role in mating behavior of the male (Finidori-Logli et al., 1996). Wagner et al. (2000) reported four wax esters eluting from the gas chromatograph as individual peaks in the surface lipids of *P. barbatus* all consisting of esters of short-chain alcohols (C9 to C12) with dodecanoic acid. We found those same wax esters and in addition numerous other wax esters present in lesser amounts.

We found striking differences in the distribution of various wax esters on different body parts of ants. As C21, C22 and C23 wax esters account only for a small proportion of the total lipid extraction, it is possible that they are also present on the surface of the head, but undetected. However, the proportion of C24 is clearly different on the head than on the rest of the body. This could indicate a very specific functional character of this wax ester compound. Whether this wax ester is synthesized by a pharyngeal gland remains to be assessed. No evident pheromonal role has been shown until now for this particular wax ester, due to a lack of purity of the compound after fractionation. They also have been reported in the Dufour's gland of the queen and the egg-laying workers of the honeybee (Katzav-Gozansky et al., 2000). Wax esters are thought to play a role as an egg marker, so that the worker bees can discriminate between queen-layed and worker-layed eggs. In *Lasius* ants, Bergström and Löfqvist (1970) identified acetate esters of fatty alcohols in Dufour's gland and tried to show a behavioral effect on *Lasius* ants, but the workers did not respond significantly to the compounds. No wax esters were found, but the acetate and propionate esters of tetradecanol were identified in the mandibular and the postpharyngeal glands of the jumping ant *Harpegnathos saltator* (Do Nascimiento et al., 1993).

The hydrocarbons consisted of *n*-alkanes, alkenes, and methyl-branched alkanes as previously reported (Wagner et al., 1998). The alkenes were odd-numbered and ranged from C25 to C35. The double bond position was not determined. In *Dinoponera quadriceps*, the alkene 9-hentriacontene was characteristic of the alpha ant (Monnin et al., 1998). There were similar amounts of alkanes and methylalkanes although the amount of pentacosane is difficult to estimate due to the almost ubiquitous presence in the laboratory of DEHP which elutes just before the alkane. The mono- and dimethylalkanes were of the same structures as those reported from many other species of insects (Nelson and Blomquist, 1995). Some minor components listed in Table 1 do not correspond to an earlier report in which the major hydrocarbon components were used for a linear discriminant analysis between nestmates (Wagner et al., 1998). The mass spectra of some components may have been misinterpreted due to the complexity of the isomers present. In this paper we have focused on the characterization of as many hydrocarbon components as possible.

Two minor peaks of 2-methylalkanes were found, 2-methyloctacosane and 2-methyltriacontane. The 2-methylalkanes are somewhat unique for two reasons: they are less frequently found in insect hydrocarbons than are 3-methylalkanes, and they are difficult to distinguish from 4-methylalkanes. The 2- and 4-methylalkanes have almost identical elution times although the 4-methyl isomer can be observed to elute just ahead of the 2-methyl isomer on a high resolution column (Pomonis et al., 1989). They can be distinguished in their mass spectra if present in sufficient quantities so that the ion diagnostic for the 4-methyl isomer at m/z 70 and the ion pair at M-70/M-71 are visible. In this study, the mass spectra were clearly that of the 2-methylalkanes. Other findings of 2-methylalkanes in insects are reviewed (Blomquist et al., 1987; Lockey 1988) and also include those identified in tsetse flies (Nelson and Carlson, 1986; Nelson et al., 1988; Sutton and Carlson, 1997), and in the termites *Drepanotermes perniger* (Brown et al., 1996), *Zootermopsis* (Haverty et al., 1988), *Coptotermes formosanus* (Haverty et al., 1990, 1996b), *Nasutitermes acajutlae* (Haverty et al., 1996c) and *Reti-*

culitermes (Haverty et al., 1996a, 1999b; Haverty and Nelson, 1997). The 4-methyl branched alkanes are infrequently identified. They have been reported in the ant *Messor barbarus* but no mass spectra were published (Provost et al., 1994).

Trimethylalkanes in insects were first reported in the tobacco hornworm, *Manduca sexta* (Nelson and Sukkestad, 1970), and in the ant, *Atta colombica* (Martin and MacConnell, 1970). The hydrocarbons of *P. barbatus* are distinguished by the presence of trimethylalkanes with unique structures at peaks 31C and 33C. Both peaks had an isomer in which the first two methyl branch points were separated by a single methylene, i.e. 13,15,19-trimethylhentriacontane and 13,15,21-trimethyltritriacontane. It seems unusual for two methyl-branched hydrocarbons differing by the addition of one acetate unit during biosynthesis, that the addition occurred between the second and third methyl branch points instead of by simply elongating the carbon chain beyond the third branch point. This fact, and the presence of few isomers in these ants, indicates that the biosynthetic pathway has a process of chain elongation and incorporation of methyl branches (from methylmalonylCoA) that is under tight control. Methylalkanes with spacing of the methyl branch points by a single methylene are usually not found, but 3,5-dimethyldodecane has been reported in the volatile constituents of Dufour's gland from *P. rugosus* and *P. barbatus* (Regnier et al., 1973). A trimethylalkane was reported to be in the post-pharyngeal gland of the desert ant, *Cataglyphis niger* (Soroker et al., 1995), however, no mass spectra were published.

Two dimethylalkanes with one methylene between their branch points, 13,15-dimethyl-nonacosane and 13,15-dimethylhentriacontane, were reported in *P. barbatus* (Wagner et al., 1998). We could find no indication of 13,15-dimethylhentriacontane in ants from either the field or laboratory and we were not able to verify the presence of 13,15-dimethylnonacosane because it would elute with the mixture of 13,17- and 11,15-dimethylnonacosanes. However, it is reasonable to suspect that monomethylene interrupted branch points could be present in dimethylalkanes because of the presence of such structures in the trimethylalkanes. Dimethylalkanes with one methylene between the branch points were reported in hemolymph of larvae of the Japanese beetle, *Popillia japonica* (Bennet et al., 1972) and in the polydomous ant, *Cataglyphis iberica*, (Dahbi et al., 1996b), however, no mass spectra were published. In a later study of *P. japonica*, no monomethylene interrupted branch points were found (Nelson et al., 1977). Similarly, one methylene interrupted branch points were reported in *Solenopsis* spp. (Lok et al., 1975) and in *Bombyx mori* (Murata et al. 1974). It was later shown that the published elution position and mass spectrum of the *Solenopsis* methylalkane was better interpreted as 3,11-dimethyltricosane, but that 13,15-dimethylpentacosane and 13,15-dimethylheptacosane were present (Nelson et al., 1980). The mass spectrum from *B. mori* was likely that of a mixture of monomethylalkanes. Similar dimethylalkanes were listed in a table of hydrocarbons from *Reticulitermes*, but they appear to be monomethylalkanes, and the entries were likely typographical errors (Takematsu and Yamaoka, 1999). Haverty's group has identified monomethylene interrupted branch points in termites: i.e. 13,15-dimethylnonacosane (published mass spectrum) and 13,15,17-trimethylnonacosane in *C. formosanus* (Haverty et al., 1990, 1996a), and 11,13- and 13,15-dimethylalkanes were reported in *Reticulitermes* (Haverty et al., 1996b). A short-chain trimethylalkene, 3,5,7-trimethylundecatetraene, was identified as the male-produced aggregation pheromone of *Carpophilus obsoletus* (Petroski et al., 1994).

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