Volatile Components of Alfalfa Leaf-Cutter Bee Cells

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The vacuum steam volatile oil of alfalfa leaf-cutter bee (*Megachile rotundata* Auct.) cells has been analyzed by capillary GLC–MS. A total of 60 components have been identified. The major component was caryophyllene epoxide. Other components in relatively large amounts included 2-phenylethanol, geranylacetone, (*Z*)-3-hexenol, 1-octen-3-ol, (*E,Z*)-2,4-heptadienal, 2-methyl-2-hepten-6-one, caryophyllene, and 1-pentadecene. Unusual components included butyl phenylacetate, dihydrocoumarin, 1-pentadecene, and an unidentified pentadecadiene.

The alfalfa leaf-cutter bee (*Megachile rotundata* Auct.) is the most effective pollinator of alfalfa (Bohart and Koerber, 1972). The management and control of this leaf-cutter bee is an important industry in the Western United States. Recently a fungus infection (*Ascophaera* sp.) has caused considerable losses of the larval stages of the leaf-cutter bee in western seed growing regions of the United States. The spores of this fungus are transferred from old nesting sites to new nesting sites by the newly emerged bees. One way to reduce the level of infection is to sterilize the leaf cells and then use sterile nesting materials. The emerging bees, however, tend to return to old nesting sites instead of the new ones. There is some indication that the bees are attracted by the characteristic odor of their old nesting material. If the new nesting material could be given a similar odor to the material from which the bees emerged, it seems reasonable that the new nesting material might be more attractive to the bees.

The present study was carried out to determine the nature of the volatile (odor) compounds associated with the leaf cells from which the bees emerged and to initiate field tests on selected compounds.

EXPERIMENTAL SECTION

Materials. The leaf-cutter bee cells were obtained from a number of different natural nesting sites in the Logan, UT, area. Two main lots of combined cells were examined.

Authentic chemical compounds were generally obtained from commercial sources (e.g., Aldrich Chemical Co.) or synthesized by established methods. Authentic samples of sesquiterpenes were separated from the hydrocarbon fraction of hop oil. All samples were purified by gas–liquid chromatography (GLC) separation before determination of their spectra.

Isolation of Volatile Oil. The intact bee cells (1.3 kg) were placed in a 12-L round-bottom flask together with 6 L of odor-free water (preboiled to remove volatiles and then cooled). The isolation of volatiles was carried out under reduced pressure (100 mmHg) with the bee cells at ~51 °C, using a Likens–Nickerson steam distillation continuous extraction head. The method used is essentially the same as that described previously by the authors for other products [e.g., Buttery and Kamm (1980)]. The hexane extract obtained was dried by freezing out the water and concentrated under reduced pressure (100 mmHg) to give the bee cell volatile oil which was stored at −20 °C with a trace of ethyl antioxidant 330.

Separation into Hydrocarbon and Oxygenated Fractions. The bee cell volatile oil (50 μL) was placed on a column (12 × 100 mm) of silica gel (Mallinkrodt SilicAR CC-7). The hydrocarbon fraction was eluted with pentane (200 mL). The oxygenated fraction was then eluted with freshly distilled diethyl ether (200 mL). Solvent was removed by distillation (warm water bath) using low hold up distillation columns.

Capillary GLC–Mass Spectral Analysis. Two types of capillary GLC columns were used. These were a 150 m × 0.64 mm i.d. Pyrex glass capillary coated with Carbowax 20-M and a 150 m × 0.64 mm i.d. Pyrex glass capillary coated with Silicone OV-3. The GLC programming conditions used were to hold the column at 50 °C for 30 min after the injection and then to temperature program the column at 1 °C/min from 50 to 170 °C. A single-stage Lewellyn-Littlejohn silicone rubber membrane molecular separator was used to couple the end of the capillary column to the mass spectrometer (a modified CEC 620 cycloidal type). Electron ionization voltage was 70 eV.

Packed Column GLC–Infrared Spectral Analysis. Samples were separated from the whole bee cell volatile oil and from the hydrocarbon fraction by using a 3 m × 0.64 cm o.d. stainless steel column packed with 80–100-mesh Chromosorb G-DMCS coated with 20% Carbowax 20-M. The column was linearly temperature programmed at 2 °C/min from 50 to 170 °C. Samples were collected in 3 mm o.d. × 14 cm Pyrex tubes. The infrared spectra were measured as thin film between ultramicro salt plates or as solutions in CS_2 in ultramicrocavity cells by using a reflecting beam condensor with a Perkin-Elmer Model 237 instrument.

RESULTS AND DISCUSSION

The volatile oil obtained by vacuum steam distillation continuous extraction of the leaf-cutter bee cells amounted to 50 parts per million (ppm) of the cells. Figure 1 shows a capillary GLC analysis of this oil. Capillary GLC–MS analysis was carried out on several different samples of the whole oil and on hydrocarbon fractions and oxygenated fractions (separated on silica gel). The capillary GLC–MS analyses were also carried out by using both polar (Carbowax 20-M) and nonpolar (Silicone OV-3) coated Pyrex capillary GLC columns. The results from these analyses are summarized in Table I. The peak numbers in the first column of Table I correspond to those peaks indicated in Figure 1. Components without a peak number were not found as separate peaks in the Carbowax 20-M GLC analysis shown in Figure 1, apparently occurring under other larger components. These were identified in the hydrocarbon or oxygenated fractions or when using the silicone capillary column.

Some studies using separation by packed column gas chromatography were also carried out, and identifications

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Table I. Compounds Identified in the Vacuum Steam Volatile Oil of Leaf-Cutter Bee Cells

peak no. (Figure 1)	compound[a]	characteristic mass spectral ions[b]	Kovat's GLC index[c]	rel %
		Alkanals		
1a	hexanal	44, 56, 72, 82, 67, *100*	1108	1.9
3	heptanal	44, 70, 81, 86, 96, *114*	1190	1.6
8	octanal	43, 44, 84, 100, 110, *128*	1290	0.3
14	nonanal	57, 44, 98, 82, 114, *142*	1390	0.4
		Alkenals		
5	(*E*)-2-hexenal[d]	41, 42, 55, 69, 83, *98*	1230	0.5
10	(*E*)-2-heptenal	41, 55, 83, 70, 68, *112*	1330	0.7
16	(*E*)-2-octenal[d]	41, 55, 70, 83, 97, *126*	1430	1.0
23	(*E*)-2-nonenal	41, 70, 83, 96, 111, *140*	1530	0.6
31	(*E*)-2-decenal	43, 70, 83, 97, 110, *154*	1630	0.2
		Alkadienals		
19	(*E,Z*)-2,4-heptadienal	81, 39, 53, 67, 95, *110*	1480	3.5
20	(*E,E*)-2,4-heptadienal[d]	81, 39, 53, 67, 95, *110*	1660	1.4
	(*E,E*)-2,4-decadienal	81, 41, 67, 95, *152*, 123	1790	0.1
		Aliphatic Ketones		
	heptan-2-one	43, 58, 71, 59, *114*, 99	1190	0.1
22	(*E,Z*)-3,5-octadien-2-one	43, 95, 81, 53, *124*, 109	1500	2.3
23	(*E,E*)-3,5-octadien-2-one[d]	43, 95, 81, 53, *124*, 109	1550	2.3
		Aliphatic Alcohols		
	3-methylbutanol	55, 42, 43, 70, 31, 57	1180	1.0
	pentanol	42, 31, 55, 70, 57	1230	0.6
	heptan-2-ol	45, 43, 55, 31, 83, 101	1290	0.2
12	hexanol	56, 43, 42, 31, 69, 84	1330	1.6
13	(*Z*)-3-hexenol	41, 67, 55, 82, 69, *100*	1370	4.0
18	1-octen-3-ol	57, 72, 85, 81, 99, 110	1420	4.0
		Alkanoic Acids		
	hexanoic acid	60, 73, 57, 45, 55, 87	1880	0.7
42	heptanoic acid[d]	60, 73, 55, 45, 87, 101	1990	0.5
47	octanoic acid	60, 73, 45, 85, 101, 115	2100	1.0
49	nonanoic acid	60, 73, 45, 115, 129, *158*	2200	0.5
52	dodecanoic acid	60, 73, 43, 129, 157, *200*	2500	1.0
		Terpenoids		
2a	myrcene	41, 69, 93, 79, 121, *136*	1160	0.2
4	limonene	68, 93, *136*, 79, 107, 121	1180	2.6
9	2,2,6-trimethylcyclohexanone	82, 56, 69, 55, *140*, 97	1320	0.2
11	2-methyl-2-hepten-6-one	43, 69, 55, 108, 58, *126*	1340	3.1
	α-cubebene[e]	161, 119, 105, 120, 91, *204*		0.1
20a	α-copaene	161, 119, 105, 93, 81, *204*	1460	1.4
	linalool oxide A (2-methyl-2-vinyl-5-(2-hydroxy-2-propyl)-tetrahydrofuran)	59, 43, 94, 111, 155, 137	1440	0.5
	linalool oxide B (isomer of A)	59, 43, 94, 121, 155, 137	1470	0.1
27	caryophyllene	69, 93, 79, 133, 161, *204*	1570	3.0
	2-methyl-2,4-heptadien-6-one	109, 43, 81, 53, *124*, 79	1590	1.0
	humulene	93, 121, 80, 147, 107, *204*	1642	0.1
	β-farnesene	69, 93, 79, 133, 120, *204*	1650	0.1
29	β-cyclocitral	137, 109, 81, 67, *152*, 123	1600	0.7
	γ-muurolene	161, 105, 41, 93, 119, *204*	1655	3.1
	γ-cadinene	161, 105, 91, 119, 133, *204*	1680	0.2
	α-terpineol	59, 93, 81, 121, 136, 139	1710	0.1
35	δ-cadinene	161, 134, 105, 119, *204*, 91	1730	0.4
39	geranylacetone[d]	43, 69, 93, 136, 151, *194*	1850	4.0
	β-ionone	177, 43, 122, 91, 135, *192*	1920	0.6
43a	caryophyllene epoxide[d]	79, 69, 93, 95, 109, *220*	1950	19.0
	epoxy-β-ionone[e]	123, 43, 135, 109, 95, *208*		0.3
		Benzene and Furan Compounds		
6	2-pentylfuran	81, 53, *138*, 39, 95, 68	1240	0.2
	furfural	39, *96*, 95, 67, 42, 50	1450	0.1
21	benzaldehyde	77, 105, *106*, 51, 50, 39	1520	1.1
30	phenylacetaldehyde	91, 92, *120*, 65, 39, 51	1650	1.0
40	2-phenylethanol[d]	91, 92, *122*, 65, 39, 51	1890	5.4
	butyl phenylacetate	91, 57, 92, 136, 65, *192*	1970	0.3
	dihydrocoumarin[d]	*148*, 120, 91, 78, 51, 65	2290	0.4
	coumarin	*146*, 118, 90, 63, 51, 45	2460	1.0
	2-hydroxyacetylbenzene	121, *136*, 43, 65, 93, 53	1810	2.0
	2-methoxyacetylbenzene	135, 77, 43, *150*, 92, 51	2020	0.1
	phenylacetonitrile	*117*, 90, 116, 89, 39, 51	1920	0.2
	p-vinylguaiacol	*150*, 135, 39, 77, 107, 51	2160	0.1

Table I (*Continued*)

peak no. (Figure 1)	compound[a]	characteristic mass spectral ions[b]	Kovat's GLC index[c]	rel %
		Aliphatic Hydrocarbons		
25	1-pentadecene[d]	43, 55, 69, 83, 97, 111	1540	2.3

[a] Mass spectrum (complete spectrum) and Kovat's GLC retention index of all compounds listed are consistent with that of authentic samples except for footnote *e*. [b] Not necessarily the most intense ions but six of those considered the most characteristic for that compound. Ions are listed in descending order of intensity with the most intense ion first and molecular ion in italic type. [c] Kovat's index for the Carbowax 20-M coated Pyrex capillary described under Experimental Section. [d] Infrared absorption spectrum also consistent with that of authentic samples. [e] No authentic sample available. Mass spectrum consistent with published data.

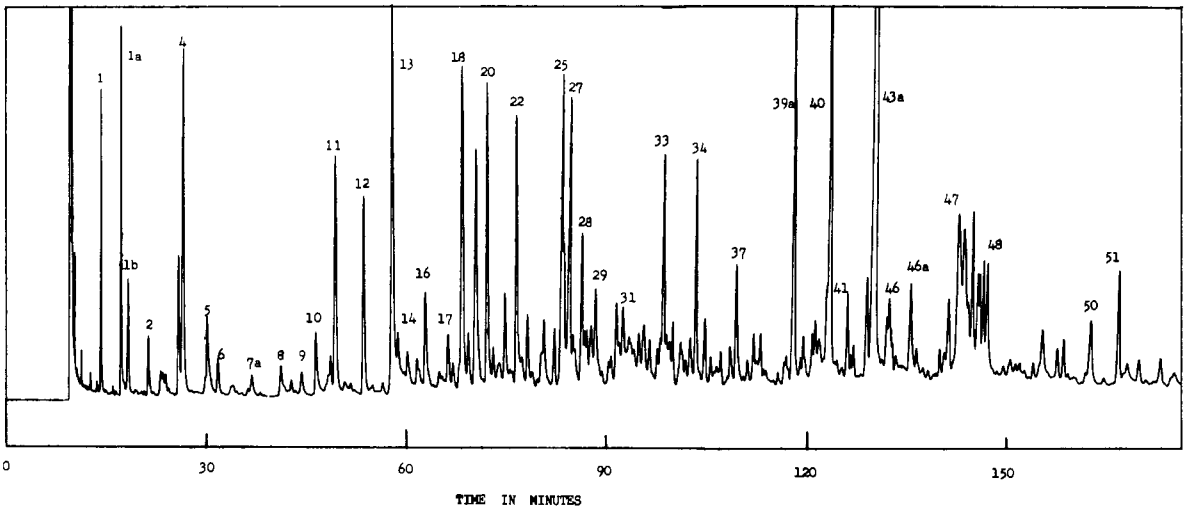

Figure 1. Capillary GLC analysis of the vacuum steam volatile oil from leaf-cutter bee cells using a Carbowax 20-M coated Pyrex capillary column. For GLC conditions, see the text.

of a few components were corroborated by measurement of their infrared absorption spectra. These are indicated by footnote *d* in Table I. Some idea of the relative percentages of the components found in the volatile oil (based on GLC peak areas) is also listed in the last column of Table I.

The major component (19%) of the volatile oil was identified as caryophyllene epoxide. Other major components included 2-phenylethanol (5.4%), geranylacetone (4%), (*Z*)-3-hexenol (4%), 1-octen-3-ol (4%), (*E*,*Z*)-2,4-heptadienal (3.5%), 2-methyl-2-hepten-6-one (3.1%), caryophyllene (3%), and 1-pentadecene (2.3%). Some of these compounds are fairly commonly occurring in plant materials. Caryophyllene is a common component of some plant "essential oils" (Masada, 1976). Caryophyllene epoxide readily results from air oxidation of caryophyllene. Caryophyllene has been implicated (Flint et al., 1979) as an attractant for the green lace wing insect (*Chrysopa carnea*). One of the authors has identified caryophyllene in small amounts in alfalfa flowers (Buttery, 1980).

Unusual components identified in the bee cell volatile oil include butyl phenylacetate, dihydrocoumarin, 1-pentadecene, and a pentadecadiene. The positions of the double bonds in the pentadecadiene were not established. Infrared absorption spectra confirmed that the double bond was at the end of the chain with the 1-pentadecene and indicated that the pentadecadiene probably also had one double bond at the end of the chain and in addition eliminated the possibility of an acetylene.

The butyl phenylacetate is of some interest because its homologue ethyl phenylacetate has long been used as a synthetic honey flavor (Guenther, 1949).

The C_{15} hydrocarbons are of interest in relation to some of the types of compounds found in beeswax (Downing et al., 1961) which include 16% C_{25}–C_{33} generally straight chain hydrocarbons as well as esters of long straight chain alcohols (ca. C_{30}) with long straight chain acids (principally C_{16}). One major beeswax acid is 14-hydroxypalmitic acid (Downing et al., 1961) which could, in theory, be dehydrated and decarboxylated to 2-pentadecene, a very similar compound to the 1-pentadecene found in the present work. Some C_{31} and C_{33} unsaturated hydrocarbons with the double bond in the 10 position have been identified in beeswax (Streibl et al., 1966).

Tests with Bees. Two test mixtures, namely, (1) caryophyllene epoxide and (2) caryophyllene epoxide, caryophyllene, and 2-phenylethanol were field tested to determine their influence on acceptance of new nesting material by the bees. More bee cells were made in the new and impregnated nesting material than in either the unimpregnated or old nesting materials. However, the differences among the replicates of the impregnated and unimpregnated materials were not statistically significant. These preliminary tests may have been influenced by other biological parameters (height and placement of nesting material within the domicile) effecting nest orientation behavior. Also, the effect of these compounds on the bees may have been masked due to their volatile nature since replicates of the test were adjacent on one wall of a field domicile. We are planning additional tests of these and other volatile compounds under isolated field conditions.

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Received for review February 2, 1981. Revised manuscript received May 18, 1981. Accepted May 18, 1981. Reference to a company and/or product named by the U.S. Department of Agriculture is only for purposes of information and does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

Pyrrolizidine Alkaloids in Honey from *Echium plantagineum* L.

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Honey produced from stands of *Echium plantagineum* L. (Paterson's Curse or Salvation Jane) contained pyrrolizidine alkaloids to the extent of 0.27–0.95 ppm. The main alkaloid present was echimidine, with smaller amounts of 7-acetyllcopsamine, 7-acetylintermedine, echiumine, uplandicine, lycopsamine, intermedine, and a new alkaloid which is probably acetylechimidine.

Many species of the family Boraginaceae are frequented by bees as a source of nectar and pollen. The purple-flowered herb, *Echium plantagineum* L., known as Paterson's Curse in Victoria and New South Wales and as Salvation Jane in South Australia, is an annual weed forming dense stands over thousands of square miles of southeastern Australia. It is worked by apiarists for brood building in the spring and as a major source of honey (Purdie, 1968). Species of Boraginaceae are also sources of pyrrolizidine alkaloids (Bull et. al., 1968). *E. plantagineum* contains a number of alkaloids of which echiumine, echimidine (Culvenor, 1956), lycopsamine, intermedine, and 7-angelylretronecine (Frahn, 1979) have been identified. A recent, unpublished study of the alkaloids of this species by the authors has also disclosed three alkaloids recently found for the first time in *Symphytum* × *uplandicum* (Russian comfrey), 7-acetyllycopsamine, 7-acetylintermedine and uplandicine (Culvenor et al., 1980), and a new alkaloid which is probably acetylechimidine. The possibility that these alkaloids may be transmitted to honey derived from *E. plantagineum* was investigated by us in 1957 with negative results. It has now been reinvestigated, using more sensitive detection methods, following the demonstration by Deinzer et al. (1977) that pyrrolizidine alkaloids are present in honey produced from *Senecio jacobaea* L. (Ragwort) at the level of 0.3–3.9 ppm.

MATERIALS AND METHODS

Honey. Samples of ~2 kg of fresh honey produced from stands of *E. plantagineum* were obtained from four suppliers who named as the source localities Tocumwal, Young, Stockinbingal, and Southern Riverina, all in New South Wales. The samples from Young and Stockinbingal were still in the comb. A fifth sample of unknown source locality, but labeled as *Echium* honey, was purchased in an Adelaide department store.

Extraction of Alkaloids. The honey (1 kg) was diluted with water (1 L) and divided into two equal portions. One part was acidified with H_2SO_4 (23 mL of concentrated acid) and stirred with zinc dust for 4 h. Both parts were then basified with concentrated aqueous NH_3 to pH 9, saturated with NaCl, and separately extracted with chloroform. Emulsions formed and were broken by centrifuging at 2000 rpm. The extracts, after removal of solvent, were taken up in 0.5 N H_2SO_4, and the solutions washed 3 times with chloroform before basifying to pH 9 with NH_3, saturating with NaCl, and extracting with chloroform. The unreduced extract was assumed to represent the tertiary base alkaloids and the reduced extract the combined tertiary bases and *N*-oxides.

In two instances, the honey (2 kg) was diluted, adjusted to pH 9, and extracted with chloroform to give the tertiary base alkaloids. The aqueous residue was then reduced with $Zn-H_2SO_4$, filtered, adjusted to pH 9, and extracted with chloroform to give the parent alkaloids of the *N*-oxides.

Identification and Quantitation by Gas Chromatography–Mass Spectrometry (GC–MS). *GC–MS Conditions.* The instrument used was a Varian MAT-111 gas chromatograph–mass spectrometer containing a 1 m × 2 mm i.d., glass-lined stainless steel column packed with 1% SE30 (U/P) on Chromosorb W (HP), mesh size 80–100. The injector temperature was 210 °C, and the oven temperature was programmed from 150 to 220 °C at 6 °C/min. The GC–MS interface temperature was 220 °C, and the carrier gas (helium) flow rate was 15 mL/min.

Identification of Alkaloids. The alkaloid extracts were examined underivatized as well as after conversion to butylboronates and trimethylsilyl derivatives. Retention times and mass spectra were compared with those of authentic samples.

Derivatization. For butylboronation, the alkaloid extracts were taken up in pyridine containing butylboronic acid (10 μg/μL) and used immediately. For trimethylsilylation, the alkaloids were taken up in a mixture of pyridine and *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) (1:1) and kept for 30 min–8 h at room temperature before injection.

Quantitation. The butylboronate derivatives were used for quantitation. With the exception of lycopsamine and intermedine, they give sharp, reproducible GLC peaks for the main alkaloids. The almost identical retention time (RT) of 7-acetylintermedine butylboronate (6.1 min) and uplandicine butylboronate (6.1 min) and the similar RT of 7-acetyllycopsamine butylboronate (5.9 min) (Figure 1) made it necessary for these three alkaloids to be quantitated together as group A. The GLC peaks of echiumine butylboronate (RT 8.8 min) and echimidine butylboronate (RT 8.5 min) also overlap (Figure 1), and these two alkaloids were therefore also grouped for quantitation as group B.

Quantitation was based on a comparison of peak areas with those produced by approximately equal amounts of authentic samples of either 7-acetyllycopsamine butylboronate (group A) or echimidine butylboronate (group

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0021-8561/81/1429-0958$01.25/0