UNIVERSITY OF SOUTHAMPTON

STRUCTURAL STUDIES ON BEHAVIOURALLY ACTIVE COMPOUNDS IN INSECTS

A thesis submitted for the degree of
Doctor of Philosophy

by

ALAN JONATHAN BACON

September 1985

With Love
To Mum and Dad

there is always some
little thing that is too
big for us

- archy

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UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF SCIENCE

CHEMISTRY

Doctor of Philosophy

STRUCTURAL STUDIES ON BEHAVIOURALLY ACTIVE COMPOUNDS IN INSECTS

by Alan Jonathan Bacon

An examination of aeration extracts from adult male and female melon flies, Dacus cucurbitae, and Oriental fruit flies, Dacus dorsalis, has revealed the presence of several female-specific 2-alkyl-8-methyl-1,7-dioxaspiro(5.5)undecanes, together with the non-specific amide, N-(3-methylbutyl)acetamide, and 2-methoxy-N-(3-methylbutyl)acetamide (specific to male D. cucurbitae). Tetramethylpyrazine and 2,3,5-trimethylpyrazine have been identified as male-specific components. In addition, extracts from male and female D. dorsalis contained the ethyl esters of C_{12}, C_{14} and C_{16} carboxylic acids as major components.

(E,E)-alpha-Farnesene has been synthesised from (E)-nerolidol utilising lithium aluminium hydride-promoted extrusion of sulphur dioxide from 2-(3,7-dimethyl-2,6-octadienyl)-3-methyl-2,5-dihydrothiophene-1,1-dioxide as the key step.

The structure of a proposed aggregation pheromone of the Oriental cockroach, Blatta orientalis, has been investigated and assigned as a primary sesquiterpene alcohol. The sesquiterpenoid was found to be inactive singly in the field. The aggregation pheromone system of B. orientalis has been reinvestigated by chemical analysis and behavioural bioassay. The pheromone is unstable under normal conditions, but is probably a complex mixture of compounds, in which certain carboxylic acids appear to play an important role.

Female Bruchid beetles, Callosobruchus chinensis, employ a sex pheromone to attract males. The structure of the sex pheromone has been tentatively assigned as a saturated ester of relative molecular mass 228 on the evidence of chemical analysis and behavioural bioassay.

ABBREVIATIONS

Solvents and Reagents

CSA	(+)-10-Camphorsulphonic acid
mCPBA	*meta*-Chloroperoxybenzoic acid
DHP	Dihydropyran
py	Pyridine
THF	Tetrahydrofuran
TPP	Triphenylphosphine

Derivatives

Ac	Acetyl
THP	Tetrahydropyranyl
TMS	Trimethylsilyl

General

Bu	Butyl
CI	Chemical ionisation (mass spectrometry)
EAG	Electroantennography
EI	Electron impact (mass spectrometry)
Et	Ethyl
GC	Gas chromatography
GC/MS	Gas chromatography - mass spectrometry
HPLC	High performance liquid chromatography
IR	Infra-red
M	Molecular ion (mass spectrometry)
Me	Methyl
m/z	Charge-to-mass ratio (mass spectrometry)
NMR	Nuclear magnetic resonance
O.D.	Outside diameter
Ph	Phenyl
Pr	Propyl
r.a.	Relative amplitude (mass spectrometry)
s.d.	Standard deviation
TLC	Thin layer chromatography

CHAPTER ONE

Chemical Analysis of Aeration Extracts from the Fruit Flies *Dacus cucurbitae* (Coquillet) (Melon Fruit Fly) and *Dacus dorsalis* (Hendel) (Oriental Fruit Fly), Diptera: Tephritidae

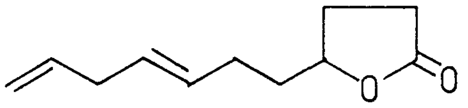
1.1 Introduction

1.1.1 Fruit fly pheromones

Little is known about the chemical composition of Dipteran pheromones, but biological and behavioural studies have demonstrated that many fruit fly species use pheromones to control a wide range of responses.[1] Hence pheromone-based control methods may prove useful in controlling these wide-ranging agricultural pests. Both the melon fly, *Dacus cucurbitae* Coquillet, and the oriental fruit fly, *Dacus dorsalis* Hendel, are distributed throughout East Africa, India, South East Asia and Hawaii,[2] and are serious pests of melon and other cucurbitacea.

The importance of pheromones for intraspecific communication during mating differs widely from species to species, because "other factors" (normally visual and auditory signals) can play an important part in controlling fruit fly behaviour. For example, the males may aggregate at a visual marker and then wait for the females to approach in response to the visual cue; the males may also assemble at an emergence, feeding or oviposition site where the two sexes can integrate in response to non-sexual stimuli.

Although no fruit fly species studied to date releases a pheromone that can elicit behavioural responses over the relatively large distances reported for some Lepidoptera, pheromones are used as long-range attractants in some species. Most species that produce a long-range sex pheromone synthesise and store it in specialised glands and release the pheromone at a specific time of day, generally controlled by the light intensity. These long-range attractants are normally emitted by the males (e.g. *Ceratitis capitata*,[3] *Dacus tryoni*[4] and *Anastrepha suspensa*[5]), but may be produced by the female (e.g. *Dacus oleae*[6]). The Queensland fruit fly (*Dacus tryoni*) is active at dusk, in common with most other species, and secretes a pheromone from glands associated with the posterior ventral regions of the rectum.[7] The Caribbean fruit fly (*Anastrepha suspensa*), olive fruit fly (*Dacus oleae*), oriental fruit fly (*Dacus dorsalis*) and melon fruit fly (*Dacus cucurbitae*) all have similar glands.[8,9] Jacobson has reported that male *Dacus dorsalis* and *Dacus cucurbitae* produce a visible "smoke" from their rectal glands at dusk, whilst fanning their wings and "calling".[10] The melon fly smoke was shown to contain unbranched C_{25}, C_{27} and C_{29} saturated hydrocarbons, together with a lactone, (E)-5-(3,6-heptadienyl)dihydro-2-(3H)-furanone (1):



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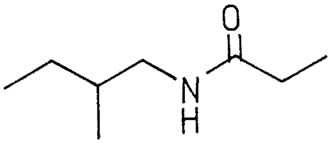
Jacobson also reported that oriental fruit fly smoke contained largely *n*-heptacosane and N-(2-methylbutyl)propanamide (2).



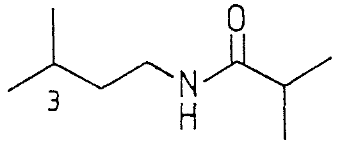
(2)

The secretion from the rectal glands of male Queensland fruit flies (*Dacus tryoni* and *Dacus neohumeralis*) is largely a mixture of six aliphatic amides.[11] The proportions of the various amides in the two species are similar (Table 1). In both cases the 3-methylbutylamide is present in far larger amounts than the corresponding 2-methylbutylamide (approximately 15:1, respectively).

Table 1. Proportions of 2-methylpropanamides (B), acetamides (A) and propanamides (P) in individual male flies of *D. tryoni* and *D. neohumeralis*.

	3B + 2B[a]	3A + 2A	3P + 2P
D. tryoni	5.2	20.8	74.0
D. neohumeralis	5.4	19.4	77.1

[a] 3 indicates a 3-methylbutyl and 2 denotes a 2-methylbutyl substituent.



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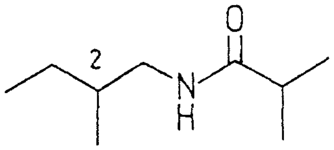

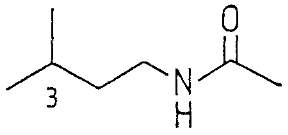

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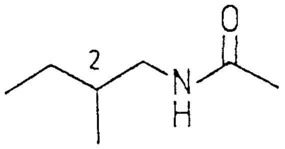

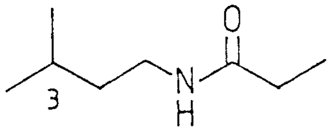

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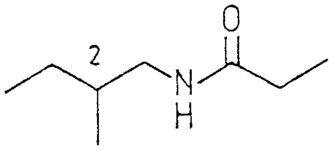
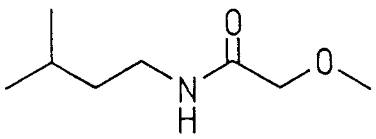


Behavioural studies have shown that these amides probably act as short-range attractants, increasing the sexual excitement of the females, rather than attracting females over a distance.

The volatile constituents of the rectal gland secretion of male *D. cucurbitae* have been shown to contain 2-methoxy-N-(3-methylbutyl)-acetamide (3) together with N-(3-methylbutyl)-acetamide (4), N-(2-methylbutyl)-acetamide (5), tetramethylpyrazine (6), methylpyrazine (7), 2,3,5-trimethylpyrazine (8) and 2-ethoxybenzoic acid (9).[12]



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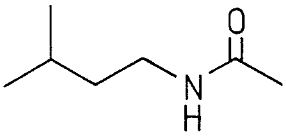

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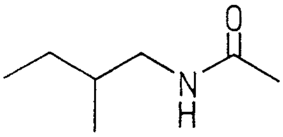

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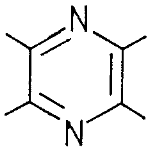

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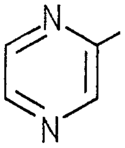

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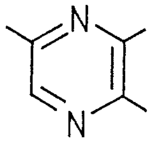

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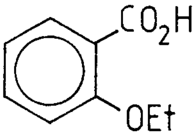

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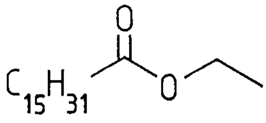

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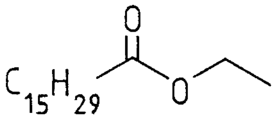

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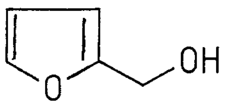
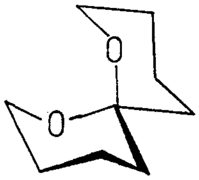


(12)

Solid sample GC/MS analysis of rectal glands from male D. dorsalis resulted in the identification of N-(3-methylbutyl)-acetamide (4) (major component), ethyl hexadecanoate (10), ethyl hexadecenoate (11), 2,3,5-trimethylpyrazine (8) and furfuryl alcohol (12).[13]

1,7-Dioxaspiro(5.5)undecane (13) has been shown to be the major component of the sex pheromone of the olive fly, Dacus oleae,[14] and the synthetic racemate has proved to be a potent attractant in the field. Nothing is known concerning the absolute configuration of the natural pheromone itself. The enantiomers of 1,7-dioxaspiro-(5.5)undecane (13) have recently been synthesised by Mori *et al*,[15] with deoxygenation of 4,10-dihydroxy-1,7-dioxaspiro(5.5)undecane (14) as the key step, and by Redlich and Francke[16] using D-(+)-glucose (dextrose) as the starting material. 8-(Hydroxymethyl)-1,6-dioxaspiro(4.5)decane (15) and 4-hydroxy-1,7-dioxaspiro(5.5)undecane (16) have also been identified by solid sample gas chromatography-mass spectrometry of the rectal glands of female D. oleae.[17] Compound (15) was later found to be a thermally-induced rearrangement product of 3-hydroxy-1,7-dioxaspiro(5.5)undecane (17). Mori has recently synthesised the enantiomers of 3-hydroxy-1,7-dioxaspiro(5.5)undecane (17)[18] and 4-hydroxy-1,7-dioxaspiro(5.5)undecane (16)[19] from (S)-malic acid.



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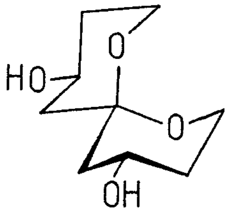

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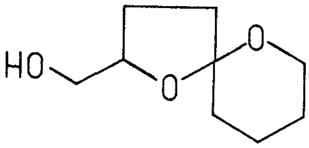

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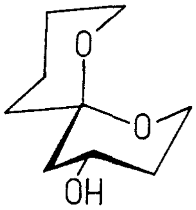

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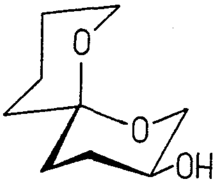
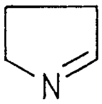


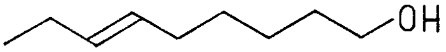
(17)

Recently, 3,4-dihydro-2H-pyrrole (Δ^1-pyrroline) (18) has been reported as the key component of the sex pheromone of the male Mediterranean fruit fly, *Ceratitis capitata*[20] (see Section 2.1.1).



(18)

Previously, aeration/cold-trapping enabled (E)-6-nonen-1-ol (19) and methyl (E)-6-nonenoate (20) to be identified from the volatiles produced by male *C. capitata*, and the latter was shown to be a male attractant in the field.[21]



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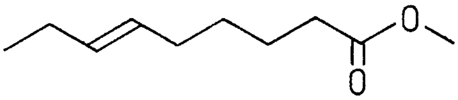
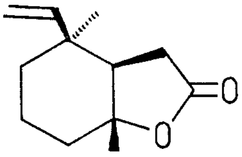


(20)

Two isomeric γ-lactones, anastrephin (21) and *epi*-anastrephin (22), have been reported as major components of the sex and aggregation pheromones of the Mexican and Caribbean fruit flies, *Anastrepha ludens* and *Anastrepha suspensa*.[22,23]



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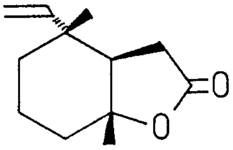
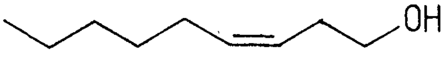


(22)

Nation has reported anastrephin (21) and *epi*-anastrephin (22), together with (Z)-3-nonen-1-ol (23) and (Z,Z)-3,6-nonadien-1-ol (24) as sex and aggregation pheromone components for *Anastrepha suspensa*.[24]



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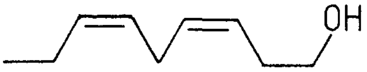
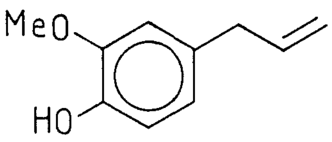


(24)

Fruit fly pheromones frequently appear to play a part in sexual behaviour at close range. Short range orientation is generally mediated by different chemical compounds than those involved in long-range attraction (for example Ceratitis capitata),[21] and these compounds are frequently incorporated into the cuticular waxes resulting in a continuous release of the pheromone (e.g. Musca domestica,[25] the active component being Z-9-tricosene).

1.1.2 Synthetic Attractants

Protein lures consisting of aqueous solutions of casein, gelatin, yeast, blood or egg white have been found to be attractive to the Central American fruit fly (Anastrepha striata), Ceratitis capitata, Dacus cucurbitae and Anastrepha suspensa. The amino acids glycine, racemic alanine and (S)-(-)-cysteine are also attractants.[26] Eugenol (25), methyl eugenol (26), methyl isoeugenol (27) and 3,4-dimethoxybenzoic acid are all reported as attractants for D. dorsalis.[27]



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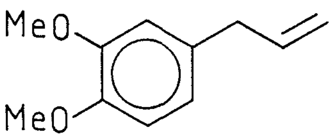

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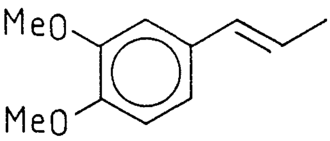

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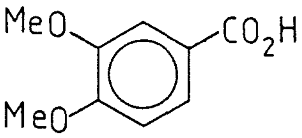

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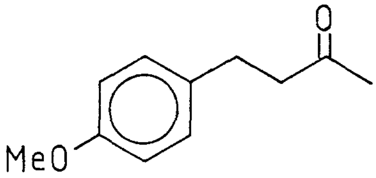

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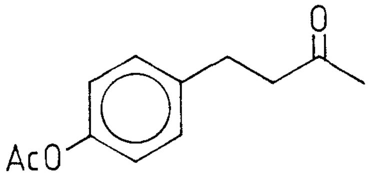


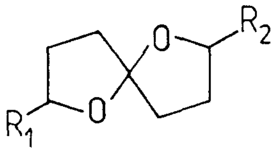
(30)

4-(*p*-Methoxyphenyl)-2-butanone (anisylacetone) (29) is a structurally related synthetic attractant for male *Dacus cucurbitae*.[28] An analogue, 4-(*p*-acetoxyphenyl)-2-butanone (30), is twenty times as effective as anisylacetone, and is used as the standard lure (cuelure) for male *D. cucurbitae* in population monitoring.[29]

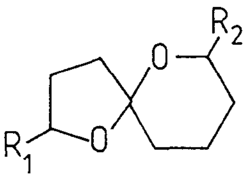
The aim of this project was to identify the chemical components present in the aeration extracts from both sexes of D. dorsalis and D. cucurbitae, with a view to aiding the long-term development of effective pheromone-based control systems for these important agricultural pests.

1.1.3 Spiroacetals in Insects

Since the identification of 2-ethyl-1,6-dioxaspiro[4.4]nonane as the principal aggregation pheromone of the bark beetle *Pityogenes chalcographus*,[30] five different types of spiroacetal structures have been found in insects: 1,6-dioxaspiro[4.4]nonanes (system 1), 1,6-dioxaspiro(4.5)decanes (system 2), 1,6-dioxaspiro(4.6)undecanes (system 3), 1,7-dioxaspiro(5.5)undecanes (system 4) and 1,7-dioxaspiro(5.6)dodecanes (system 5).[31] Most of these compounds occur as mixtures of E/Z diastereoisomers in nature.[32] All the known spiroacetal pheromones show unbranched carbon skeletons and, with one exception, contain 9, 11 or 13 carbon atoms, thus showing close similarities to a large number of other pheromones which are presumably derived from carboxylic acids.[33]



system 1



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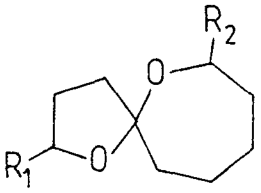
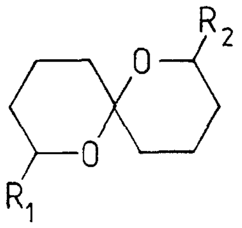
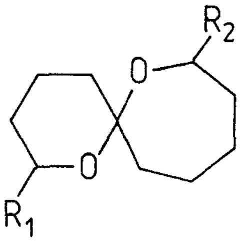


system 3



system 4



system 5

Following the identification of system 2 in several species of common wasps,[34] the existence of compounds of system 3 was postulated from general considerations on a possible biogenesis of spiroacetals. These suggestions were confirmed by the identification of both 2,8-dimethyl-1,7-dioxaspiro(5.5)undecanes, as one of the main components in the cephalic secretions of the solitary bee *Andrena wilkella*[35] and as the sex pheromone of the olive fly, *Dacus oleae*.[14] Table 2 shows the spiroacetals identified to date and their function as pheromones.[36]

The identification of microamounts of spiroacetals by GC/MS has been facilitated by the development of general fragmentation schemes.[34,37,38] Pure enantiomers would be expected to racemise readily at the spirocentre, but under normal conditions spiroacetals are quite stable with the exception of system 1.

The enantiomers of some selected

Table 2. Spiroacetals in insects.

System 1		System 2		System 3		System 4		System 5		Insect	Function
R^1	R^2	R^1	R^2	R^1	R^2	R^1	R^2	R^1	R^2	Species	(Production Site)
H	Et									*Pityogenes*	Aggregation
		H	Me							*chalcographus*	pheromone[30] (hindgut?)
		Me	H							*Paravespula*	
										vulgaris	
		Me	Et							*Paravespula*	Alarm pheromones?[34]
		Et	Me							*germanica*	(abdomen)
										Dolichovespula	
		H	Me							*saxonica*	
										Leperisinus	Anti-aggregation
										varius	pheromone[34]
						H	H			*Dacus oleae*	Sex pheromone[14]
											(anal gland)
Et	Et	Me	Et			Me	Me			*Andrena*	Scent mark[35]
										wilkella	(mandibular gland)
Me	Pr	Me	Et	Me	Me	H	Pr	Me	H	*Andrena*	Scent mark[40]
Pr	Pr	Et	Me	Me	Bu	Me	Pr			*haemorrhoa*	(mandibular gland)

spiroacetals have been resolved by complexation GC on chiral transition metal β-ketoenolates, recently reviewed by Weber and Schurig.[39] The syntheses of optically active spiroacetals have also been reviewed recently, this time by Mori.[40] The enantiomers of (E,E)- and (Z,E)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane have recently been separated by HPLC on a triacetylcellulose column.[41]

It has been concluded from NMR data that alkyl substituents attached α- to oxygen in any tetrahydropyranyl ring of corresponding acetals are likely to be in an equatorial position. The diaxial orientation of oxygen atoms at the spirocentre is favoured by the anomeric effect.[42-44]

The mode of action of spiroacetals at the receptor site is unknown. It is possible that either the spiroacetals may be opened on the cell membrane, loosing one chiral centre, thereafter functioning as acyclic pheromones, or else the round cage-like molecules may act as ionophores.

The formation of spiroacetals from the appropriate ketodiol precursors is common in plants and animals. (Z)-2,6-Nonadien-4-olide, which has been suggested as a possible male sex pheromone of a pyralide moth,[45] shows all the functionalities needed for biosynthetic transformation into a spiroacetal.

1.2 Results and Discussion

1.2.1 Collection of Extracts

The fruit fly extracts were obtained by aeration of virgin males, virgin females, or mixed mated males and females (see Section 1.3.4). Charcoal, Porapak Q and Tenax were all used as adsorbents, but charcoal was found to be far superior to the other two.

1.2.2 Analysis of Extracts

The concentrated extracts were initially examined by GC and subsequently by GC/MS using polar (FFAP) and non-polar (OV101) columns.

1.2 .3 Male *Dacus cucurbitae*

GC analysis of the concentrated aeration extract indicated the presence of a mixture of four main components (Figure 1); these components were identified with the aid of GC/MS as tetramethylpyrazine (6), 2,3,5-trimethylpyrazine (8), N-(3-methylbutyl)acetamide (4) and 2-methoxy-N-(3-methylbutyl)acetamide (3) (Table 3).

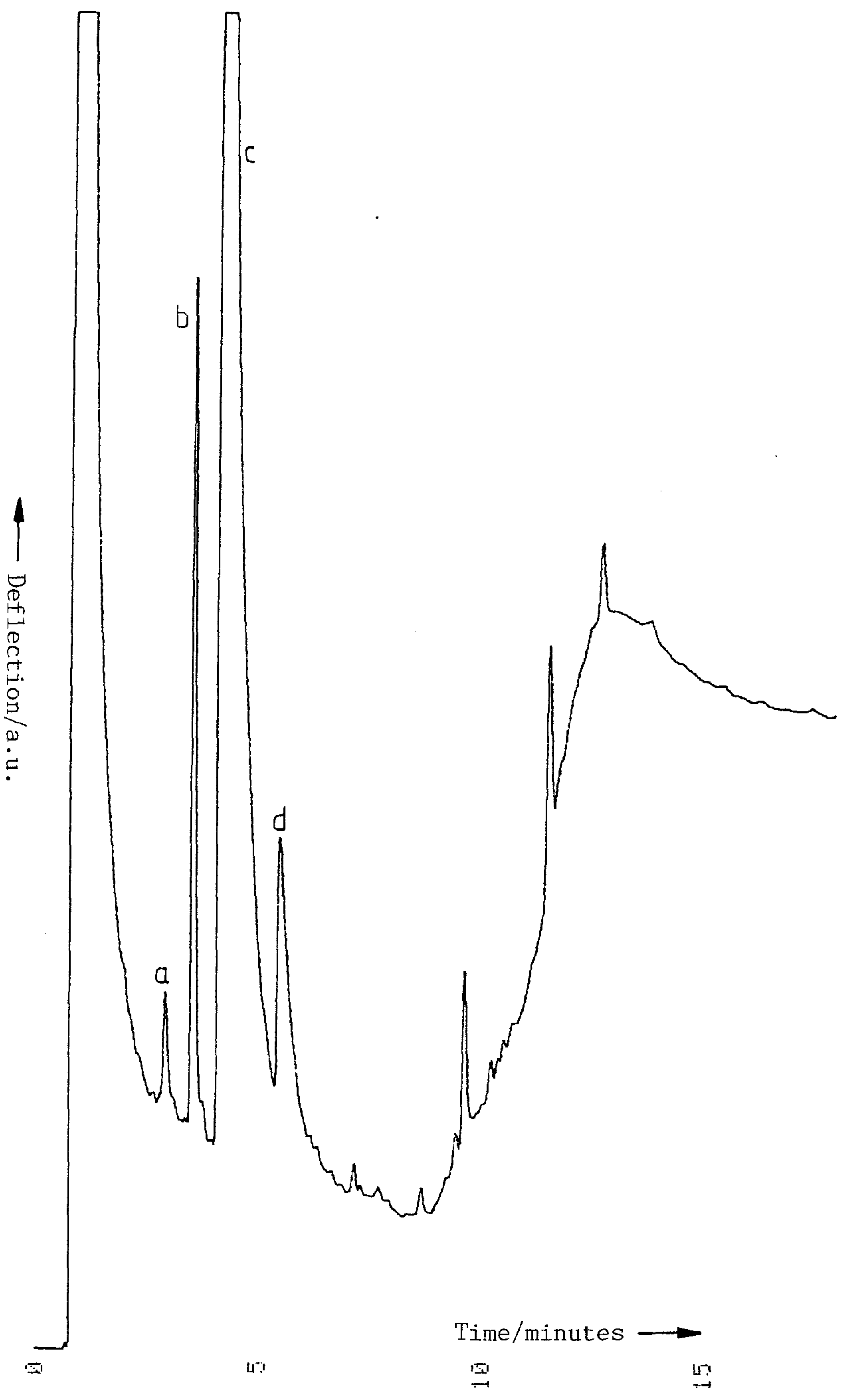
Table 3. GC/MS data from the concentrated male *Dacus cucurbitae* aeration extract (5% FFAP, 80-6-240°C).

Peak	RT/min.	$M^{\oplus}$(%)	*m/z* (r.a., %)			
(a)	8:00	122(65)	42(100),	122(65),	40(25),	81(20)
			39(20),	44(15),	32(15),	49(10)
standard		122(100)	122(100),	42(99),	81(14),	39(13)
spectrum			40(10),	54(10),	123(9),	53(6)
(b)	9:50	136(60)	54(100),	136(60),	42(50),	32(20)
			53(20),	39(15),	51(5),	69(5)
standard		136(98)	54(100),	136(98),	42(87),	53(30)
spectrum			39(23),	137(16),	52(11),	135(10)
(c)	17.00	129(8)	43(100),	73(70),	72(65),	44(65)
			60(35),	86(25),	41(25),	55(25)
standard		129(15)	72(100),	73(75),	43(70),	44(55)
spectrum			60(50),	86(40),	55(20),	114(15)
(d)	17.10	159(1)	43(100),	71(80),	45(80),	129(75)
			103(40),	102(35),	73(55),	114(30)
standard		159(2)	43(100),	45(74),	71(50),	129(25)
spectrum			73(25),	55(25),	114(15),	103(15)

where peak (a) is 2,3,5-trimethylpyrazine (8),
peak (b) is tetramethylpyrazine (6),
peak (c) is N-(3-methylbutyl)acetamide (4) and
peak (d) is 2-methoxy-N-(3-methylbutyl)acetamide (3).

(see Figure 1)

Figure 1. Male *D. cucurbitae* aeration extract (5% OV101, 100-16-290°C).



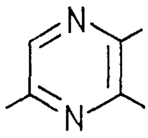
(a) 2,3,5-trimethylpyrazine (8)

(b) tetramethylpyrazine (6)

(c) N-(3-methylbutyl)acetamide (4)

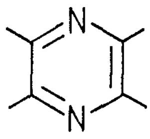
(d) 2-methoxy-N-(3-methylbutyl)acetamide (3)

(a)



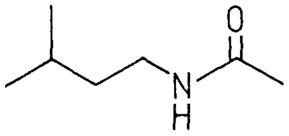
(8)

(b)



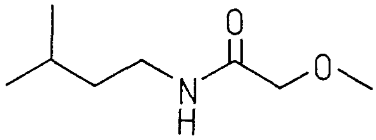
(6)

(c)



(4)

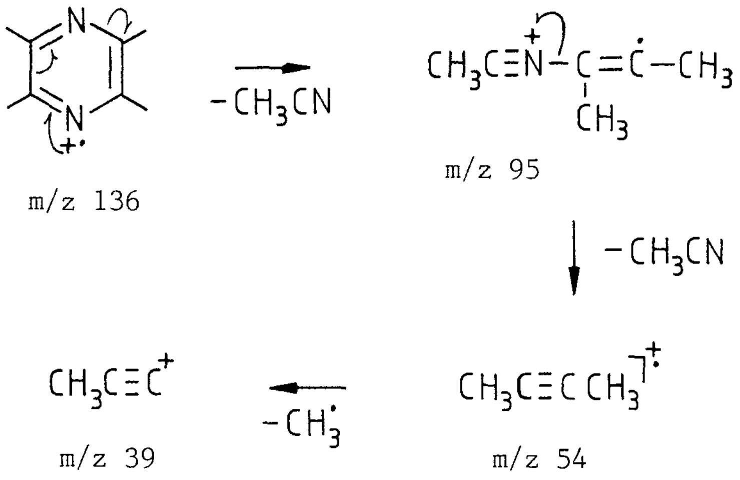
(d)



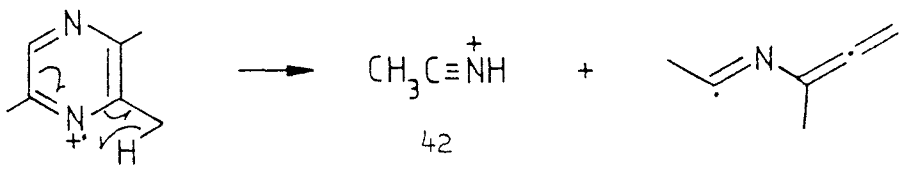
(3)

Tetramethylpyrazine (6), the major pyrazine component (100 ng/insect/week), and 2,3,5-trimethylpyrazine (8) (20 ng/ insect/week) were identified by comparison of mass spectral data[46-49] and gas chromatographic behaviour of the natural products with authentic samples. The mass spectrum of tetramethylpyrazine arises from an unusual fragmentation of the aromatic ring (Scheme 1), since there are no C_3 alkyl substituents to permit the McLafferty rearrangement process.[45]

Scheme 1. The mass spectral fragmentation of tetramethylpyrazine.



2,3,5-Trimethylpyrazine (8) was identified as a minor component. The base peak at m/z 42 arises partly from a rearrangement involving a methyl group,



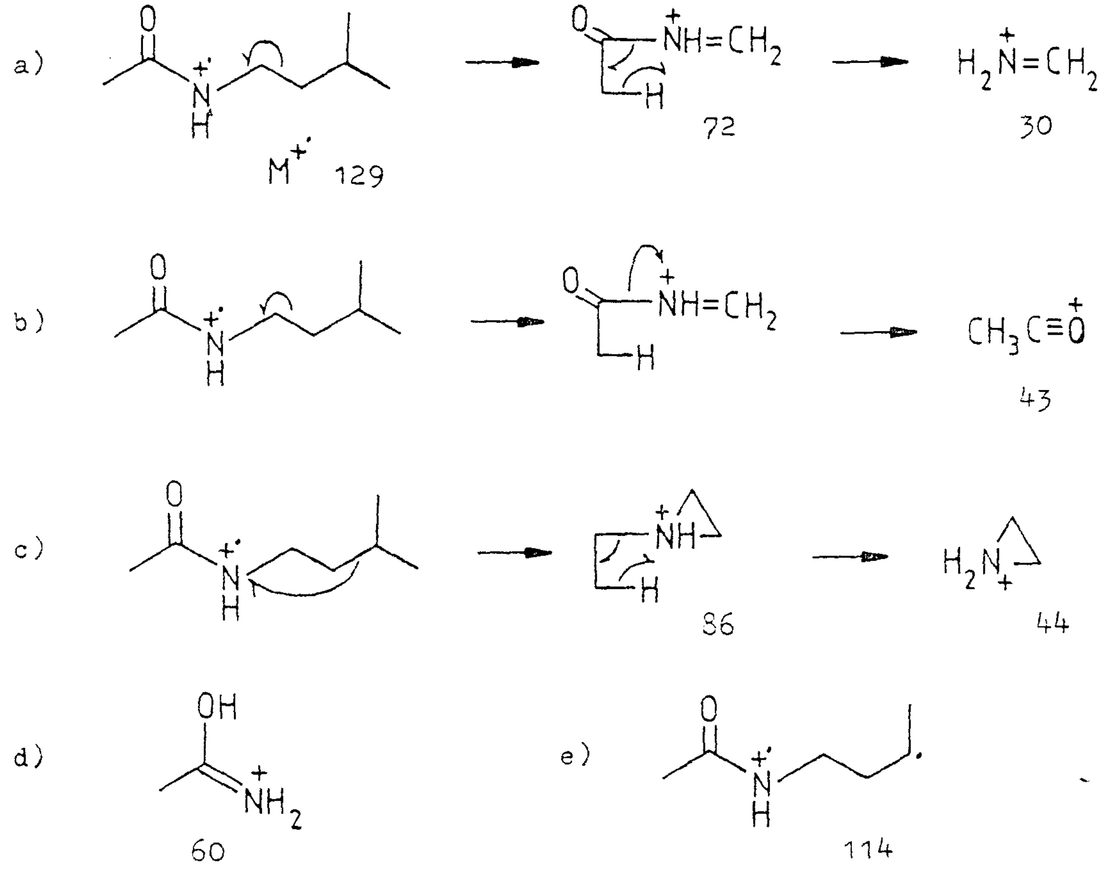
Both of these pyrazines have been found in the rectal gland of male *D. cucurbitae* using solid-sample GC/MS analysis.[13] The observations reported here make it likely that the pyrazines are genuine products of the insect metabolism, rather than decomposition products formed at the GC inlet as a result of using the solid-sample GC/MS technique.

Several pyrazines have been identified as trail pheromones of various ant species. For example, the trail pheromone of the ant, *Tetramorium caespitum*, has recently been identified as a 70:30 mixture of 2,5-dimethylpyrazine and 3-ethyl-2,5-dimethylpyrazine, respectively.[50] Pyrazines have also been reported as marking volatiles in wasps[51] and may perform a similar function for *D. cucurbitae*.

No ethyl esters were found in the aeration extract, in marked contrast to the solid-sample GC/MS results previously obtained.[13] Thus, it seems that ethyl esters are stored in the rectal glands of male *D. cucurbitae*, but are not involved in the insect communication system.

However, both aeration studies and solid-sample GC/MS analysis of the rectal glands revealed the presence of N-(3-methylbutyl)acetamide (4). This compound was the major component of the aeration extract from both male and female *D. cucurbitae* (200ng/fly/week) and has been shown to elicit activation and increased flight activity of female *D. cucurbitae*.[12]

Scheme 2. Mass spectral fragmentation of N-(3-methylbutyl)acetamide.



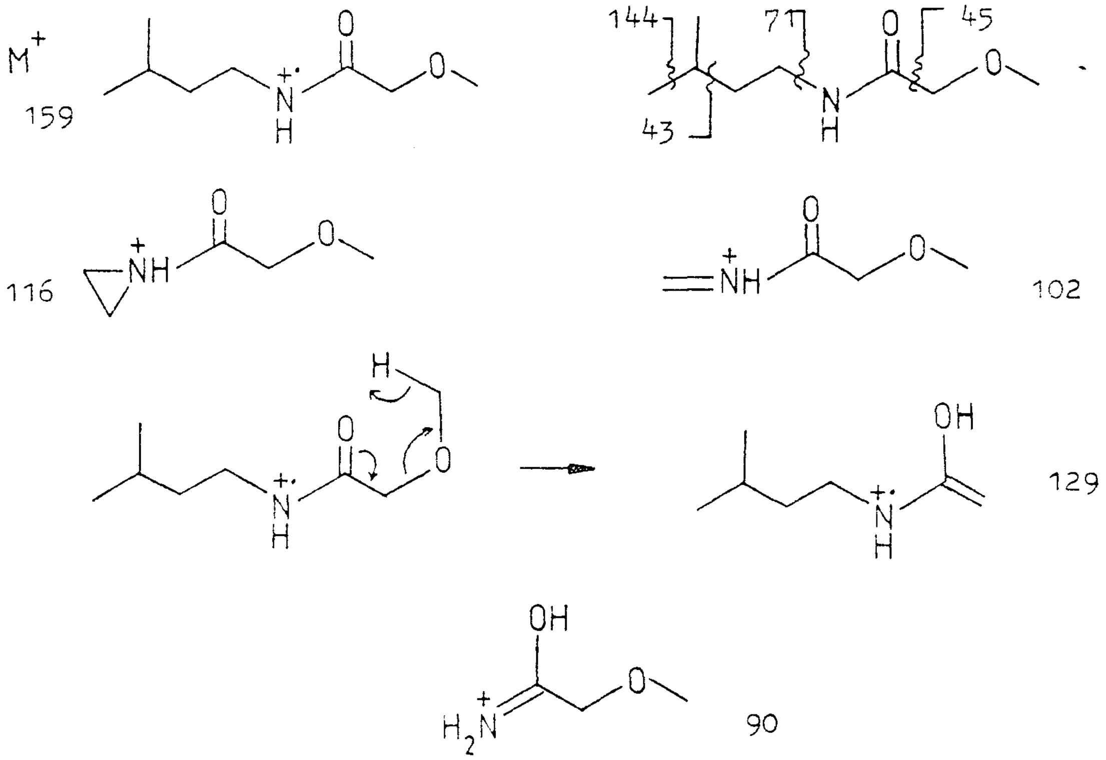
The mass spectral fragmentation of N-(3-methylbutyl)acetamide (4) (Scheme 2) showed several characteristic ions. The ion *m/z* 60 was attributed to a protonated acetamide, C_2H_6NO, leaving a C_5 amine fragment, and the ion *m/z* 114 suggested the loss of a methyl radical, possibly from an N-(3-methylbutyl)- group. The absence of an ion at *m/z* 100 (corresponding to the loss of an ethyl radical) eliminated the possibility of an N-(pentyl) group, an N-(2-methylbutyl) group and an N-(1-ethylpropyl) group.[11] The absence of an ion at *m/z* 58, and the presence of an *m/z* 44 base peak excluded the possibility of an N-(1-methylbutyl) group,[11] thereby leaving the proposed N-(3-methylbutyl) group as the most likely structure.

The structure assigned from the mass spectrum was confirmed by unambiguous synthesis. The mass spectral and gas chromatographic properties of the synthetic compound were found to be identical to those of the natural product.

It has been suggested that the amine residues are ultimately derived from the amino acids leucine and isoleucine, though whether the insect accomplishes the decarboxylation, or derives the amines from a food source is unknown.[11]

The structure of a second amide component, 2-methoxy-N-(3-methylbutyl)acetamide (3) (100ng/insect/week) was similarly assigned initially from mass spectral data (Scheme 3) and then confirmed by synthesis. This amide has been reported as a component of the rectal gland secretion of male *D. cucurbitae.*[12]

Scheme 3. Mass spectral fragmentation of 2-methoxy-N-(3-methylbutyl)acetamide.



The male *D. cucurbitae* chamber extract was analysed by GC and electron impact mass spectrometry (Table 4). Mass chromatography for *m/z* 180 did not reveal the lactone (1) reported by Jacobson *et al*[10] as a key component of *D. cucurbitae* "smoke". Methyl stearate was identified as a major component of the chamber extract, together with branched C_{36} and C_{40} hydrocarbons. Both the male and female *D. cucurbitae* chamber extracts were shown to contain the same chemical constituents.

Table 4. Analysis of male *D. cucurbitae* chamber extract (EI probe, temperature programme, 150-350°C).

	Compound	*m/z* (r.a., %)				$M^{\oplus}$(%)
a)	Methyl stearate	74(100),	87(70),	43(35),	41(30)	298(10)
		55(25),	57(25),	69(20),	298(10)	
	standard spectrum	74(100),	87(65),	43(27),	41(22)	298(7)
		75(19),	55(17),	143(16)		
b)	Branched $C_{36}H_{74}$	97(55),	99(45),	113(45),	83(45)	506(1)
c)	Branched $C_{40}H_{82}$	97(100),	57(98),	69(90),	85(90)	562(2)
		111(75),	83(65),	99(60),	43(55)	

1.2.4 Female *Dacus cucurbitae*

Aeration of female melon fruit flies followed by GC and GC/MS analysis indicated a mixture containing four main components (Figure 2); the components were identified as (E,E)-2,8-dimethyl-1,7-dioxaspiro-[5.5]undecane (31a), (Z,E)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (31b) and N-(3-methylbutyl)acetamide (4); the fourth component may be a C_5 alcohol (Table 5).

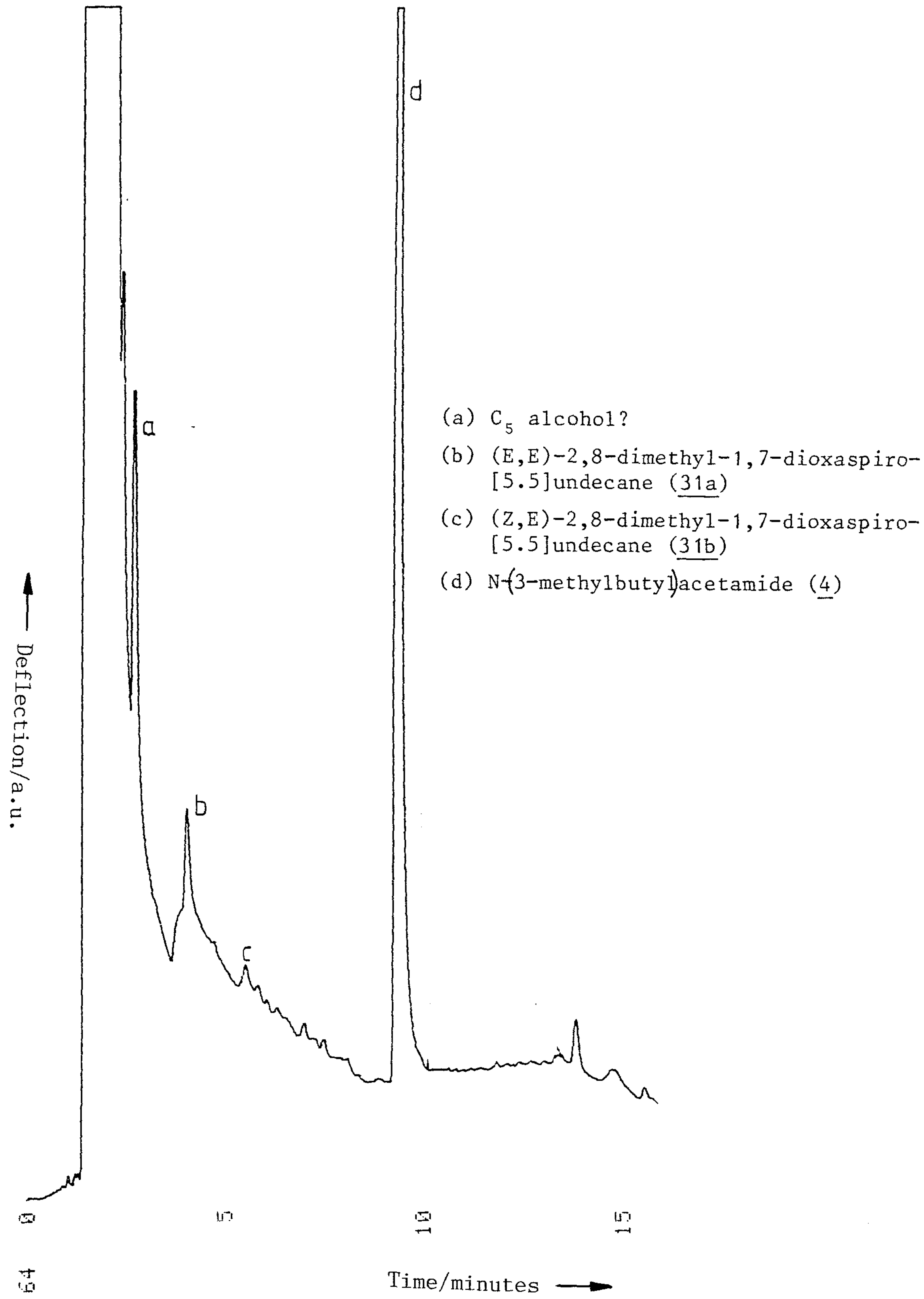
Table 5. GC/MS data from the concentrated female *Dacus cucurbitae* aeration extract (5% Carbowax 20M, 80-6-220°C).

Peak	RT/min	$M^{\oplus}$(%)	*m/z* (r.a., %)			
(a)	8:00	106?(10)	55(100),	70(85),	32(85),	42(80)
			41(60),	43(55),	57(50),	31(50)
(b)	10:00	184(10)	112(100),	115(95),	97(50),	55(40)
			43(40),	114(30),	69(40),	140(25)
standard spectrum		184(10)	115(100),	55(65),	97(60),	69(55)
			112(50),	43(50),	114(40),	73(30)
(c)	13:50	184(5)	32(100),	115(70),	97(40),	112(35)
			69(30),	43(30),	55(25),	114(20)
standard spectrum		184(5)	115(100),	97(70),	112(60),	69(50)
			43(50),	55(50),	43(40),	114(40)
(d)	22:00	129(10)	43(100),	72(75),	73(70),	44(50)
			60(35),	86(25),	55(20),	41(20)
standard spectrum		129(10)	72(100),	73(75),	43(70),	44(55)
			60(50),	86(40),	55(20),	41(20)

where peak (a) is probably a C_5 alcohol,
peak (b) is (E,E)-2,8-dimethyl-1,7-dioxaspiro(5.5)undecane (31a),
peak (c) is (Z,E)-2,8-dimethyl-1,7-dioxaspiro(5.5)undecane (31b),
peak (d) is N-(3-methylbutyl)acetamide (4).

(see Figure 2, overleaf)

Figure 2. Female *D. cucurbitae* aeration extract (5% OV101, 40-12-290°C).



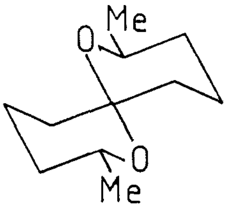
(a) C_5 alcohol?

(b) (E,E)-2,8-dimethyl-1,7-dioxaspiro-[5.5]undecane (31a)

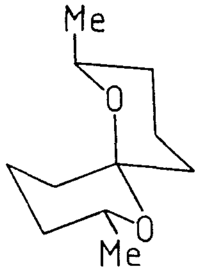
(c) (Z,E)-2,8-dimethyl-1,7-dioxaspiro-[5.5]undecane (31b)

(d) N-(3-methylbutyl)acetamide (4)

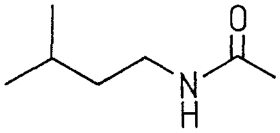
The following structures were assigned on the basis of a comparison between the mass spectral and gas chromatographic properties of the natural products and synthetic standards:



(31a)



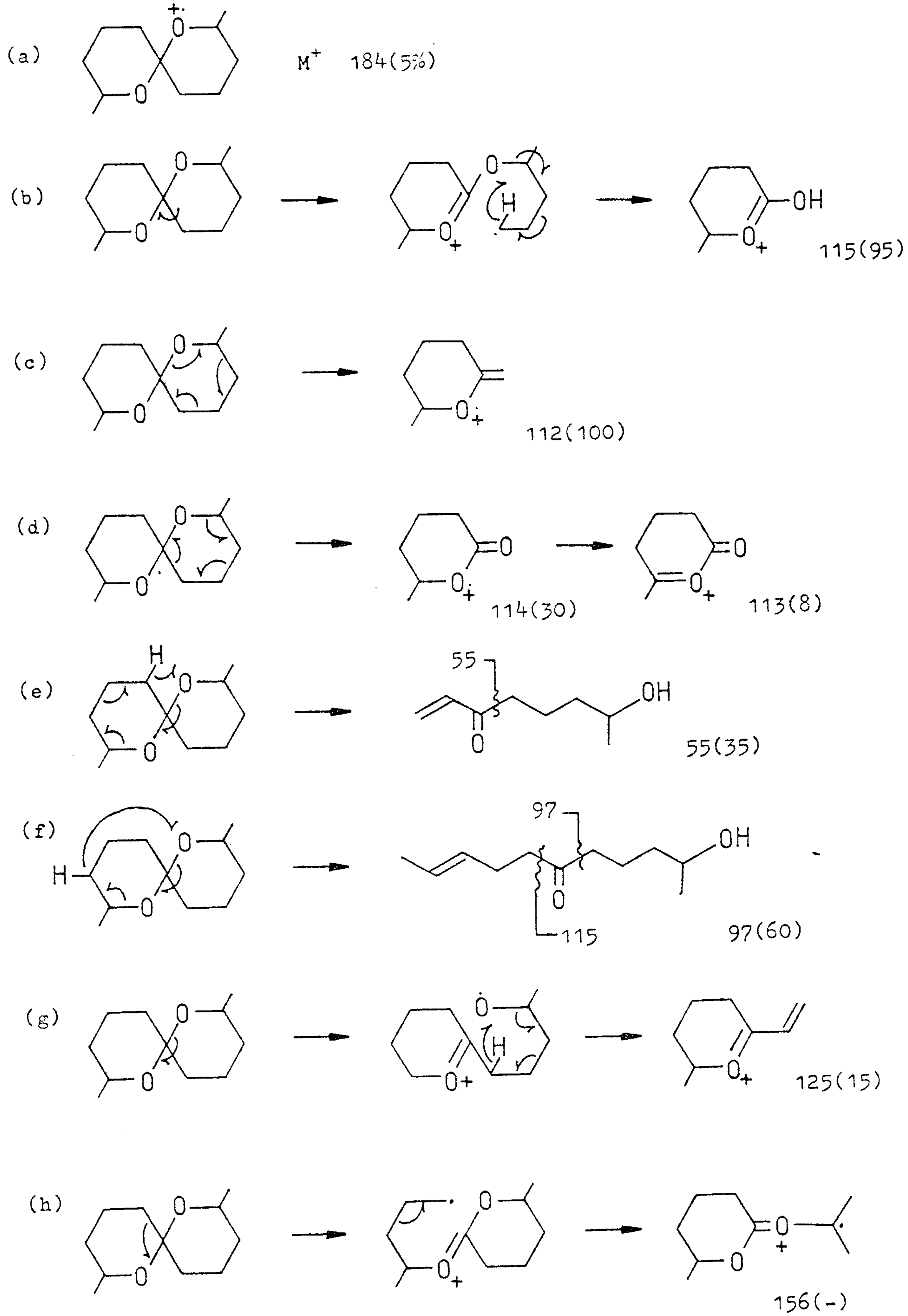
(31b)



(4)

2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane (31) (100ng/insect/week) is a novel component for *Dacus* species and does not occur in the rectal gland,[13] however, this spiroacetal has been reported as a component of the mandibular gland secretion in three species of *Andrena* bees[38] and the three possible stereoisomers of (31) have been synthesised by Mori *et al.*[42] (E,E)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane (31a) and (Z,E)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (31b) were synthesised from 1,5-hexanolide by the technique of Erdmann.[52] The mass spectral and gas chromatographic behaviour of the natural product and the synthetic sample were found to be identical in each case.[35] The relative stereochemistry of (31a) and (31b) was assigned on the basis of ^{1}H and ^{13}C NMR spectra by comparison with literature data.[43] The (E,E)-isomer (31a) is thermodynamically the most stable of the three possible stereoisomers, because this conformation minimises the steric interaction between the two methyl groups in an axial-equatorial conformation. These spiroacetals are thought to be biosynthesised from unbranched oxoundecanediols, presumably initially derived from sucrose in the insect diet.[35]

Scheme 4. Mass spectral fragmentation of 2,8-dimethyl-1,7-dioxaspiro-[5.5]undecanes.



The mass spectrum of 2,8-dimethyl-1,7-dioxaspiro[5.5]undecanes is typical of this type of compound, especially the ions at *m/z* 112 and *m/z* 115 (Scheme 4).[34,37] The (E,E)- and (Z,E)- isomers showed very similar mass spectra.

The female *Dacus cucurbitae* chamber extract appeared to be identical to the corresponding male extract by GC and temperature-programmed electron impact probe mass spectrometry (150-350°C) (Table 4).

1.2.5 *Dacus cucurbitae* summary

Both male and female *Dacus cucurbitae* aeration extracts were shown to contain N-(3-methylbutyl)acetamide (4) as a major component, but only the male extract contained 2-methoxy-N-(3-methylbutyl)acetamide (3) and tetramethylpyrazine (6) as major components. The female aeration extracts contained (E,E)- and (Z,E)-2,8-dimethyl-1,7-dioxaspiro[5.5]-undecanes (31a) and (31b) as female-specific major and minor components, respectively.

Neither the male nor the female chamber extract contained the lactone (1) reported by Jacobson *et al.*[10]

1.2.6 Male *Dacus dorsalis*

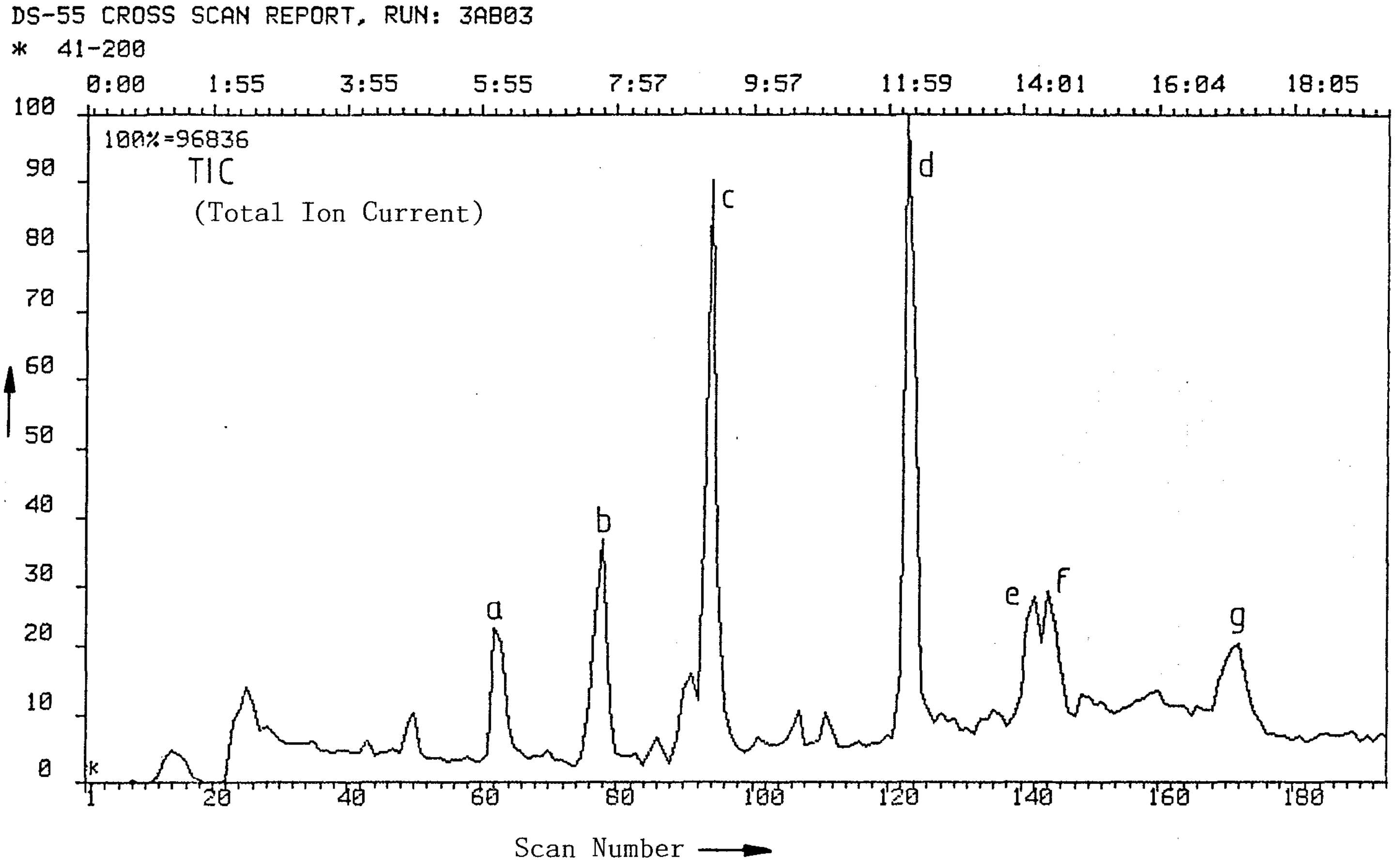
A combination of GC and GC/MS analysis of the concentrated aeration extract revealed the presence of a multi-component mixture (Figure 3, Table 6). The major components of the aeration extract were N-(3-methylbutyl)acetamide (4) (1μg/insect/week), ethyl dodecanoate (32) (200ng/insect/week), ethyl tetradecanoate (33) (200ng/insect/week) and 2,3,5-trimethylpyrazine (8) (50ng/insect/week). Several other (minor) components were present in such small quantities that interpretable mass spectra could not be obtained.

Analysis of both the male and female chamber extracts by temperature-programmed electron impact probe mass spectrometry (150-300°C) revealed the presence of a saturated straight-chain hydrocarbon, $C_{30}H_{62}$; *m/z* 422 ($M^{\oplus}$, 1%), 141(5), 127(5), 113(7), 99(10), 97(10), 85(35), 71(55), 57(100), 43(80).

Table 6. GC/MS analysis of male *Dacus dorsalis* aeration extract (combined data from 5% FFAP, 5% Dexsil 410 and 5% OV101 columns).

	Compound	*m/z* (r.a., %)				$M^{\oplus}$(%)
a)	2,3,5-Trimethylpyrazine (8)	42(100),	122(90),	40(20),	39(20)	122(90)
		81(10),	54(5),	123(5),	53(4)	
	standard spectrum	122(100),	42(99),	81(14),	39(13)	122(100)
		40(10),	54(10),	123(9),	53(6)	
b)	Branched C_9H_{20}?	57(100),	85(30),	99(20),	71(20)	128(2)
		43(20),	41(20)			
c)	N-(3-Methylbutyl)acetamide (4)	73(100),	72(90),	43(90),	44(50)	129(10)
		60(40),	86(30),	55(15),	114(10)	
	standard spectrum	72(100),	73(75),	43(70),	44(55)	129(15)
		60(50),	86(40),	55(20),	114(15)	
d)	Ethyl laurate (32)	88(100),	101(45),	43(30),	73(20)	228(2)
	$C_{11}H_{23}CO_2Et$	55(20),	183(10),	157(5)		
	standard spectrum	88(100),	101(50),	43(25),	73(20)	228(1)
		70(20),	55(20),	69(10),	89(10)	
e)	Unknown, possibly	60(100),	43(80),	73(60),	102(50)	229(10)
	an amide	85(25),	228(15),	129(15)		
f)	Ethyl myristate (33)	88(100),	101(50),	43(35),	55(25)	256(3)
	$C_{13}H_{27}CO_2Et$	73(20),	157(10),	211(10)		
	standard spectrum	88(100),	101(55),	43(30),	41(20)	256(5)
		55(20),	57(20),	73(15),	157(10)	

Figure 3. Male *D. dorsalis* aeration extract (5% FFAP, 80-12-240°C).



(a) 2,3,5-Trimethylpyrazine (8)

(b) Nonane

(c) N-(3-Methylbutyl)acetamide (4)

(d) Ethyl dodecanoate (32)

(e) Unknown

(f) Ethyl tetradecanoate (33)

(g) Unknown

The mass spectral fragmentations of N-(3-methylbutyl)acetamide, 2,3,5-trimethylpyrazine and the straight-chain saturated ethyl esters (all found in the rectal gland) have been discussed.[13]

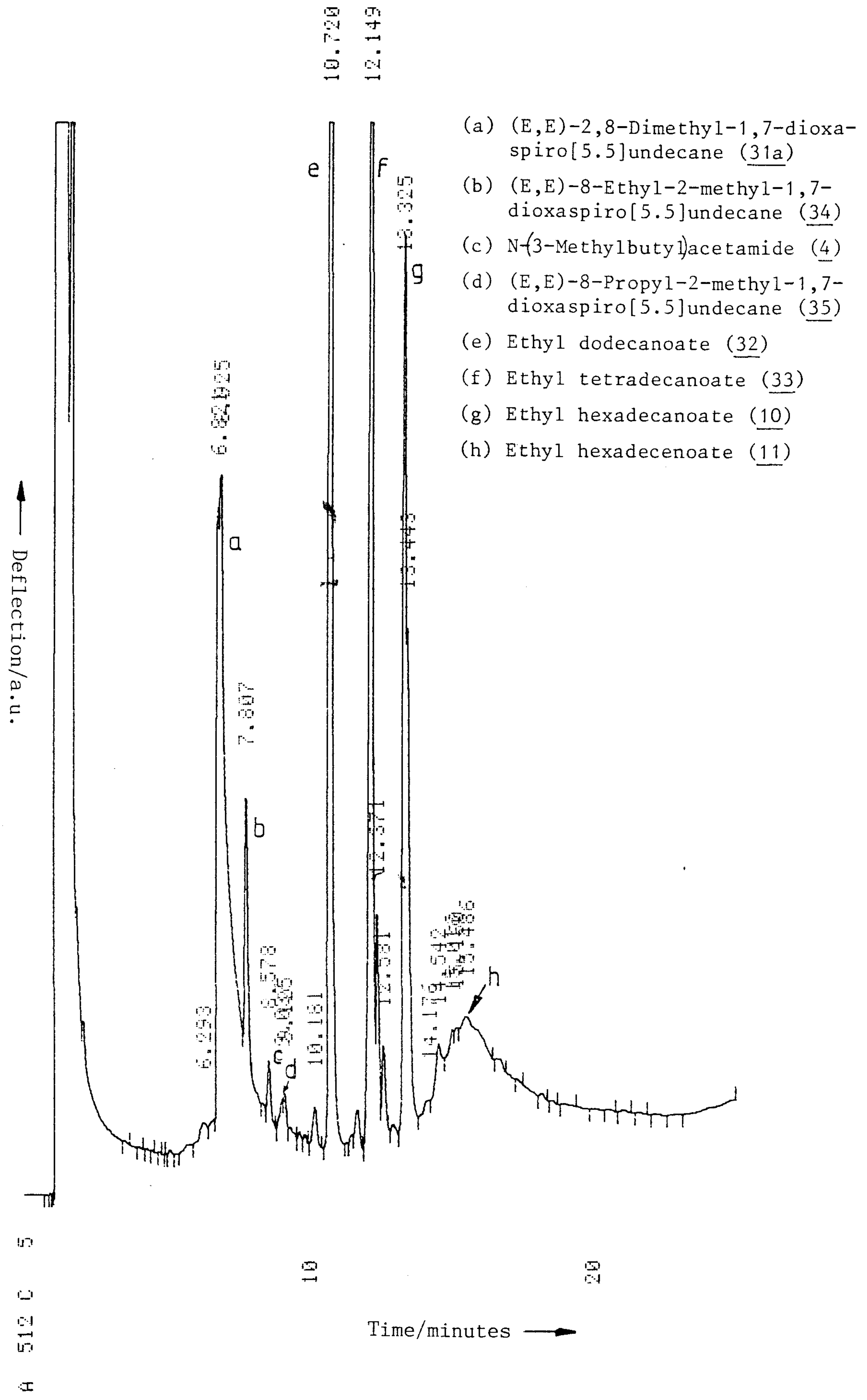
It is interesting that 2-methoxy-N-(3-methylbutyl)acetamide (3), a major component of the male *D. cucurbitae* aeration extract, was not identified from male *D. dorsalis*. Ethyl hexadecanoate (10) and ethyl hexadecenoate (11) were identified from the rectal gland,[13] but were not found by aeration. The converse was true of ethyl dodecanoate (32) and ethyl tetradecanoate (33), which were only present in the aeration extract. N-(3-Methylbutyl)acetamide (4) and 2,3,5-trimethylpyrazine (8) were found in the aeration extracts from both male *D. dorsalis* and *D. cucurbitae*.

1.2.7 Female *Dacus dorsalis*

The concentrated aeration extract was analysed by GC and GC/MS (Figure 4, Table 7). The major components of the aeration extract were identified as N-(3-methylbutyl)acetamide (200ng/insect/week) (4), (E,E)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (100ng/insect/week) (31a), the novel compound (E,E)-8-ethyl-2-methyl-1,7-dioxaspiro[5.5]undecane (100ng/insect/week) (34), (E,E)-8-propyl-2-methyl-1,7-dioxaspiro[5.5]-undecane (20ng/insect/week) (35), together with four ethyl esters: ethyl dodecanoate (200ng/insect/week) (32), ethyl tetradecanoate (100ng/insect/week) (33), ethyl hexadecanoate (50ng/insect;week) (10) and ethyl hexadecenoate (20ng/insect/week) (11).[53]

It is highly significant that no spiroacetals are found in male *D. dorsalis* extracts. The spiroacetals were initially identified on the basis of their mass spectra as 8-alkyl-2-methyl-1,7-dioxaspiro-[5.5]undecanes, particularly characteristic were the fragment ions at *m/z* 112, 115[54] (see Scheme 4). For example, 8-ethyl-2-methyl-1,7-dioxaspiro[5.5]undecane showed prominent ions at *m/z* 112, 115 in the mass spectrum, the molecular ion appeared at *m/z* 198 (10%), and the ion at *m/z* 169 (M-29) arising from loss of an ethyl radical indicated the alkyl substitution of the spiroacetal.[54] (E,E)- and (Z,E)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, (31a) and (31b) respectively, were synthesised by the method of Francke *et al*[43] and the natural product was shown to be the (E,E)- isomer by comparison of GC retention time

Figure 4. Female *D. dorsalis* aeration extract (5% OV101, 50-16-290°C).



(a) (E,E)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane (31a)

(b) (E,E)-8-Ethyl-2-methyl-1,7-dioxaspiro[5.5]undecane (34)

(c) N-(3-Methylbutyl)acetamide (4)

(d) (E,E)-8-Propyl-2-methyl-1,7-dioxaspiro[5.5]undecane (35)

(e) Ethyl dodecanoate (32)

(f) Ethyl tetradecanoate (33)

(g) Ethyl hexadecanoate (10)

(h) Ethyl hexadecenoate (11)

Table 7. GC/MS Analysis of female *Dacus dorsalis* aeration extract (combined data from 5% FFAP, 5% Dexsil 410 and 5% OV101 columns).

	Compound	*m/z* (r.a., %)				$M^{\oplus}$(%)
a)	(E,E)-2,8-Dimethyl-1,7-dioxaspiro-[5.5]undecane (31a)	112(100), 55(40),	115(95), 43(40),	97(50), 114(30),	69(40) 140(25)	184(10)
	standard spectrum	112(100), 43(50),	115(95), 69(45),	97(60), 114(35),	55(55) 140(20)	184(10)
b)	(E,E)-8-Ethyl-2-methyl-1,7-dioxaspiro-[5.5]undecane (34)	115(100), 55(25),	112(95), 169(20),	129(60), 140(20),	97(45) 154(10)	198(10)
	standard spectrum	115(100), 55(35),	112(95), 169(25),	97(50), 140(20),	129(40) 154(20)	198(10)
c)	N-(3-Methylbutyl)acetamide (4)	73(100), 60(40),	72(90), 86(30),	43(90), 55(15),	44(50) 114(10)	129(10)
	standard spectrum	72(100), 60(50),	73(75), 86(40),	43(70), 55(20),	44(55) 114(15)	129(15)
d)	(E,E)-8-Propyl-2-methyl-1,7-dioxa-spiro[5.5]undecane (35)	112(100), 143(55),	115(75), 83(45),	125(10), 183(45),	97(55) 140(40)	212(5)
	standard spectrum	115(100), 112(65),	43(80), 97(50),	55(80), 69(40),	83(70) 140(30)	212(5)
e)	Ethyl laurate, $C_{11}H_{23}CO_2Et$ (32)	88(100), 183(10),	101(43), 89(10),	73(17), 70(9),	69(12) 83(7)	228(4)
	standard spectrum	88(100), 70(20),	101(50), 55(20),	43(25), 69(10),	73(20) 89(10)	228(1)
f)	Ethyl myristate, $C_{13}H_{27}CO_2Et$ (33)	88(100), 55(38),	43(65), 57(27),	41(55), 73(20),	101(49) 157(10)	256(4)
	standard spectrum	88(100), 55(20),	101(55), 57(20),	43(30), 73(15),	41(20) 157(10)	256(5)
g)	Ethyl palmitate, $C_{15}H_{31}CO_2Et$ (10)	88(100), 69(15),	43(55), 147(10),	101(30), 241(5)	57(20)	284(10)
	standard spectrum	88(100), 55(32),	101(54), 57(30),	43(45), 91(22),	41(33) 157(15)	284(10)
h)	$C_{15}H_{29}CO_2Et$ (11)	88(100), 41(35),	101(70), 73(25),	55(65), 157(20),	70(60) 237(15)	282(10)
	standard spectrum	88(100), 236(46)	101(93),	81(71),	237(55)	282(10)

and mass spectral data. Determination of the relative configuration of the spiroacetals has been carried out on the basis of solvent-dependent shifts in ^{1}H-NMR and γ-effects in ^{13}C-NMR spectra.[43] (E,E)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane (31a) has been previously reported in scent marks from the mandibular gland of the bees, *Andrena wilkella*,[35] *A. ocreata* and *A. ovatula*,[38] and may fulfil a similar role in fruit fly ecology. It would appear that these spiroacetals are not produced in the rectal gland of female *D. dorsalis* (or *D. cucurbitae*), because no spiroacetals were identified by Baker *et al*[12] as components of the rectal gland secretions. Synthetic samples of (E,E)- and (Z,E)-8-alkyl-2-methyl-1,7-dioxaspiro[5.5]undecanes were prepared for comparison with the natural products as shown in Scheme 5.

Finally, a bishomologous series of the ethyl esters of C_{12}, C_{14} and C_{16} saturated alkanoic acids was identified from *D. dorsalis*, together with the ethyl ester of the C_{16} monounsaturated alkanoic acid, all of which have been reported for *D. cucurbitae*[12] and *D. dorsalis*[13] from female rectal gland secretions.

1.2.8 *Dacus dorsalis* summary

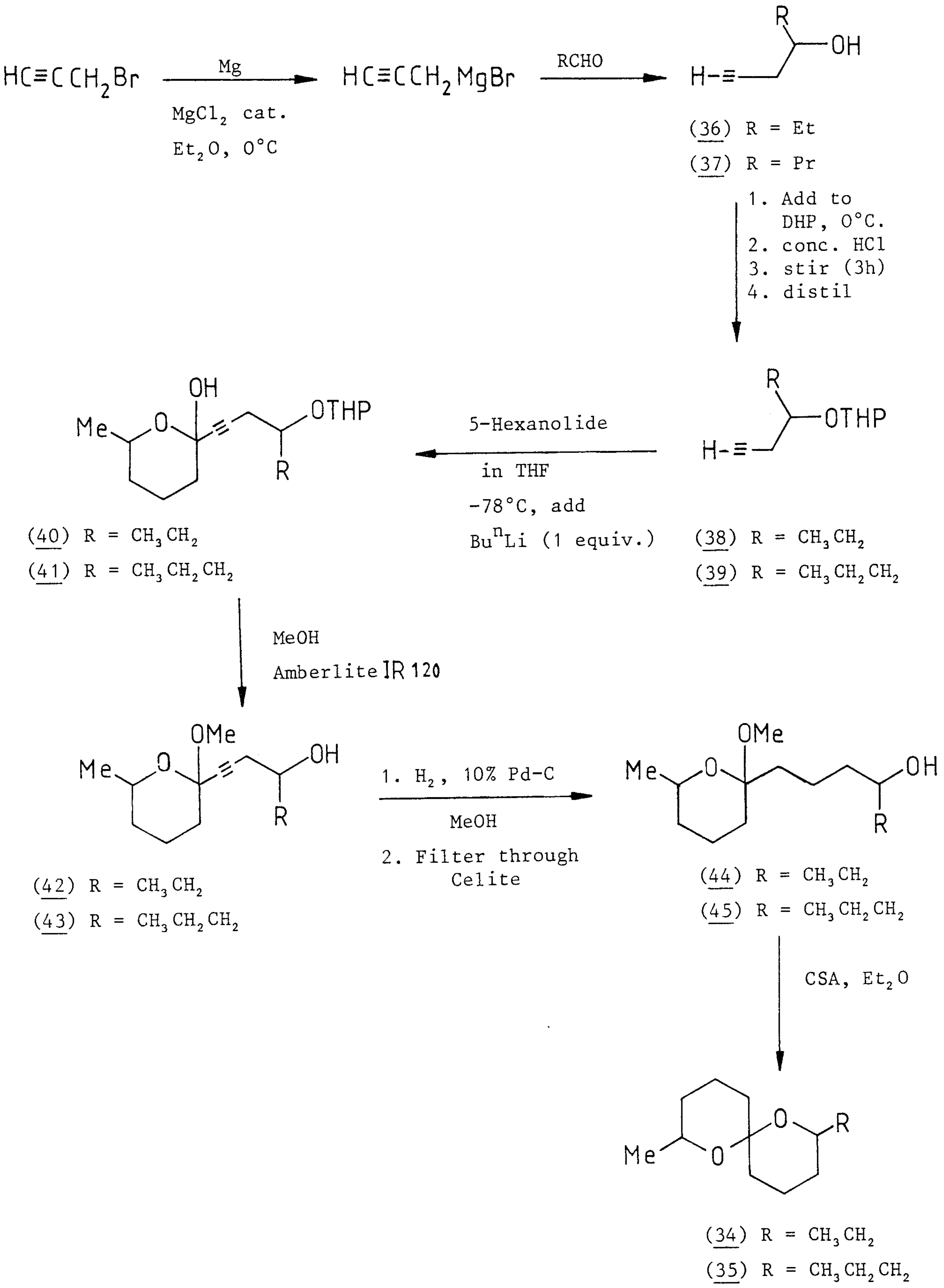
N-(3-Methylbutyl)acetamide (4), ethyl dodecanoate (32) and ethyl tetradecanoate (33) were identified from both the male and female *Dacus dorsalis* aeration extracts. The only compound to be identified as a male-specific aeration component was 2,3,5-trimethylpyrazine (8). Five female-specific compounds were identified by aeration, including three 2,8-dialkyl-1,7-dioxaspiro[5.5]undecanes, ethyl hexadecanoate (10) and ethyl hexadecenoate (11). The spiroacetal components were identified as (E,E)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (31a), (E,E)-8-ethyl-2-methyl-1,7-dioxaspiro[5.5]undecane (34) and (E,E)-8-methyl-2-propyl-1,7-dioxaspiro[5.5]undecane (35). The role of the spiroacetals in the female fruit fly biology is as yet unknown.

1.3 Experimental

1.3.1 Instrumentation

See Section 2.4 for IR, GC, GC/MS and NMR procedures.

Scheme 5. General reaction scheme for preparation of 8-alkyl-2-methyl-1,7-dioxaspiro[5.5]undecanes



1.3.2 Methods and Materials

All glassware used for insect extracts was washed in chromic acid, rinsed with water, washed with aqueous sodium hydrogen carbonate followed by glass distilled water and finally baked overnight in an oven. All solvents were redistilled before use in an all glass apparatus. Pentane (99%) was obtained from BDH Chemicals Ltd., dichloromethane (puriss) from May and Baker, methanol, ethyl acetate and tetrachloromethane, 99.9% pure (Gold Label), were obtained from Aldrich Limited.

Solvents were dried and purified as described in Section 2.4.1. 3-Methylbutylamine, acetyl chloride, 5-ketohexanoic acid, propanal, dihydropyran and butanal (all from Aldrich) were distilled prior to use. 3-Bromopropyne (Aldrich) was dried by stirring over calcium hydride (24h).

The titration method described by Winkle *et al.*[55] was used to check the concentration of *n*-butyl-lithium (Aldrich) in hexane solutions.

1.3.3 General Procedures

See Section 2.4.2.

1.3.4 The Aeration Experiment (See Figure 5).

D. dorsalis and *D. cucurbitae* were taken from a laboratory culture originally obtained from U.S.D.A., Honolulu. The sexes were segregated within one day of emergence and kept at 25 ± 1°C, 70 ± 5% relative humidity and a light intensity of *ca.* 1000 lx maintained on a 13h light : 11h dark cycle.

The volatile secretions produced by sexually mature (10 day old) male or female fruit flies (50) were obtained by passing filtered air (25ml min^{-1}) through a glass chamber (40cm x 6cm O.D.) containing live flies and absorbing the volatile compounds on an activated charcoal filter (5cm x 1cm O.D.). After seven days and nights the emitted volatiles were extracted from the charcoal filter by solvent desorption with dichloromethane (10ml). The aeration extract was concentrated (100μl) by distillation at atmospheric pressure and analysed by GC and GC/MS. Typically, the extract was analysed by GC (5% Carbowax 20M on 100-120 Diatomite AAW-DMCS, 5% OV101 on 100-120 Chromosorb W-HP) and subsequently by GC/MS.

A blank aeration (without flies) was carried out to ensure that artefacts were not present in the extract. The interior surface of the aeration chamber was extracted with dichloromethane (2 x 10ml) and concentrated as previously described to enable the chamber extract to be analysed.

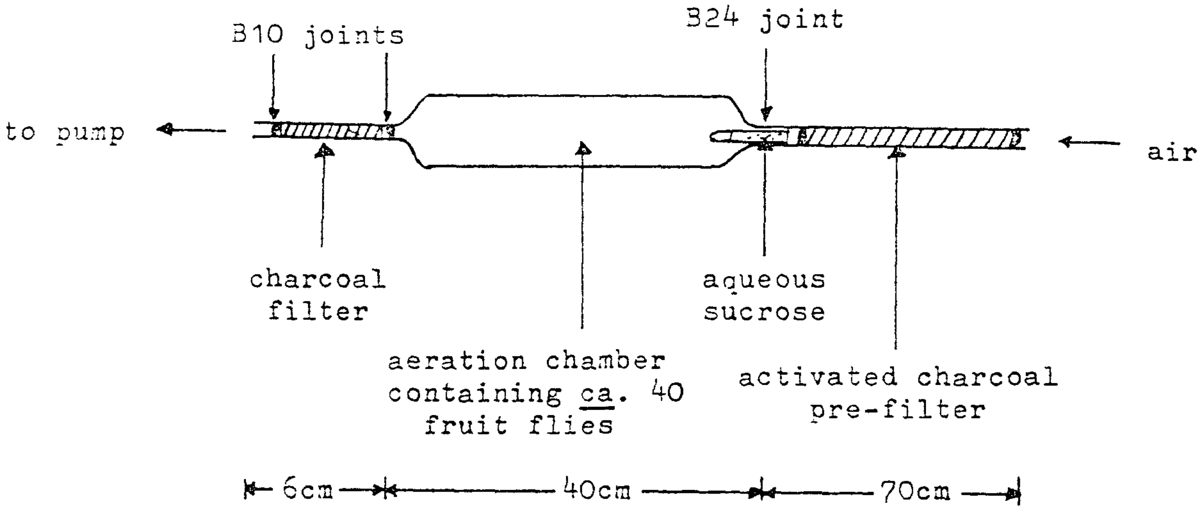


Figure 5. A typical aeration experiment.

1.3.5 Experimental Procedure

1.3.5.1 N-(3-Methylbutyl)-acetamide (4)

N-(3-Methylbutyl)-acetamide was prepared in 58% yield as a colourless oil by the method of Dehn,[56] b.p. 64-66°C at 0.06 mmHg (lit.,[13] 78-80°C at 0.2 mmHg). High resolution mass spectrometry gave $C_7H_{15}NO$ for *m/z* 129.1148 (calc. *m/z* 129.1153). ν_{max} (thin film) 3300 (m, NH, secondary amide), 1660 (m, amide C=0 stretch) and 1560 cm^{-1} (m, amide NH bend); δ_H (60 MHz, $CDCl_3$) 0.95 (6H, d, *J* 7 Hz, 2 x $C\underline{H}_3CH$), 1.45 (3H, m, $C\underline{H}_3CO$), 2.0 (3H, m, $-C\underline{H}_2CH_2NH-$ and $Me_2C\underline{H}-$), 3.25 (2H, m, $-C\underline{H}_2NH$) and 6.6 (1H, br s, $N\underline{H}$); *m/z* 129 ($M^{\oplus}$, 15%), 114 ($M-CH_3$, 12), 86(40), 73(75), 72(100), 60(50), 55(22), 44(55) and 43(72).

1.3.5.2 2-Chloro-N-(3-methylbutyl)-acetamide

2-Chloro-N-(3-methylbutyl)-acetamide was synthesised in 62% yield by the method of Braun and Münch,[57] b.p. 126-127°C at 15 mmHg (lit.,[55] 134-135°C at 13 mmHg). High resolution mass spectrometry gave $C_7H_{14}NOCl$ for *m/z* 163.0603 (calc. *m/z* 163.0760); ν_{max} (liquid film) 3300 (m, amide NH), 1660 (m, amide C=O) and 1550 cm^{-1} (m, amide NH bend); δ_H (60 MHz, $CDCl_3$) 0.9-1.1 (6H, d, *J* 7 Hz, 2 x $C\underline{H}_3CH$), 1.3-1.8 (3H, m, $C-C\underline{H}_2-C$), 3.1-3.6 (2H, q, *J* 3 Hz, $C-C\underline{H}_2N$), 4.0 (2H, s, $ClC\underline{H}_2C=O$) and 6.3-6.7 (1H, m, $N\underline{H}$); *m/z* 163 ($M^{\oplus}$, 5%), 148 ($M-CH_3$, 5), 128(10), 120(30), 107(85), 106(90), 94(50), 72(50), 70(50), 55(70) and 43(100).

1.3.5.3 2-Methoxy-N-(3-methylbutyl)-acetamide (3)

2-Methoxy-N-(3-methylbutyl)-acetamide was prepared in 75% yield from 2-chloro-N-(3-methylbutyl)-acetamide by the method of Herbert,[13] b.p. 71-72°C at 0.1 mmHg (lit.,[13] 78-80°C at 0.2 mmHg). High resolution mass spectrometry gave $C_8H_{17}NO_2$ for *m/z* 159.1262 (calc. *m/z* 159.1251). ν_{max} (thin film) 3300 (m, secondary amide NH), 1670 (m, amide C=O) and 1530 cm^{-1} (m, amide NH bend); δ_H (60 MHz, $CDCl_3$) 0.9, 1.0 (6H, d, *J* 7 Hz, 2 x $C\underline{H}_3CH$), 1.2-1.9 (3H, m, $Me_2C\underline{H}-$ and $Me_2CHC\underline{H}_2-$), 2.9-3.3 (2H, m, $CH_2C\underline{H}_2NH$), 3.4 (3H, s, $OC\underline{H}_3$) and 6.9-7.2 (1H, br s, $N\underline{H}$); *m/z* 159 ($M^{\oplus}$, 5%), 129(5), 91(100), 71(20), 45(25) and 43(30).

1.3.5.4 5-Hexanolide (5-hexanolactone, 5-caprolactone)

5-Hexanolide was synthesised from 5-ketohexanoic acid by the method of Taub *et al*[58] in 68% yield, b.p. 108-110°C at 14 mmHg (lit.,[56] 112-113°C at 21 mmHg). ν_{max} (thin film) 1730 (s, C=O lactone); δ_H (60 MHz, $CDCl_3$) 1.3, 1.5 (3H, d, *J* 10 Hz, $C\underline{H}_3CH-$), 1.6-2.2 (4H, m, $C-C\underline{H}_2-C$), 2.3-2.7 (2H, m, $C-C\underline{H}_2C=O$) and 4.1-4.6 (1H, m, $CH_3C\underline{H}$); *m/z* 114 ($M^{\oplus}$, 5%), 99 ($M-CH_3$, 15), 70($M-CO_2$, 70), 60(10), 55(35) and 42(100).

1.3.5.5 (±)-(E,E)- and (±)-(Z,E)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane, (31a) and (31b).

(31a) and (31b) were prepared by the method of Francke *et al*.[43] Flash column chromatography with ether-hexane (5:95) as eluant yielded (±)-(E,E)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (31a) (0.19g, 5%) and (±)-(Z,E)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (31b) (0.11g, 3%) as colourless oils.

(±)-(E,E)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane (31a) gave $C_{11}H_{20}O_2$ for *m/z* 184.1536 (calc. *m/z* 184.1463) from high resolution mass spectrometry and showed a GC retention time of 8.44 minutes (5% OV101, 100-12-290°C). δ_H (100MHz, $CDCl_3$) 1.1-1.2 (6H, 2d, *J* 7 Hz, 2 x $C\underline{H}_3CH$-), 1.5-1.8 (12H, m, C-$C\underline{H}_2$-C) and 3.70 (2H, m, $C\underline{H}O$ diequatorial); δ_C (25.15 MHz, $CDCl_3$) 96.1 (s), 65.1 (d), 35.7 (t), 33.3(t), and 22.2 (q); *m/z* 184 ($M^{\oplus}$, 10%), 140(5), 125(10), 115(100), 114(40), 112(50), 97(60), 83(10), 73(30), 69(55), 55(65) and 43(50).

(±)-(Z,E)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane (31b) gave $C_{11}H_{20}O_2$ for *m/z* 184.1464 (calc. *m/z* 184.1463) and showed a GC retention time of 5.18 minutes (5% OV101, 100-12-290°C). δ_H (360 MHz, $CDCl_3$) 1.1-1.2 (6H, 2d, *J* 7 Hz, 2 x $C\underline{H}_3CH$-), 1.5-1.8 (12H, m, C-$C\underline{H}_2$-C), 3.6 (1H, m, $C\underline{H}O$ equatorial) and 4.1 (1H, m, $C\underline{H}O$ axial); δ_C (25.15 MHz, $CDCl_3$) 97.1 (s), 68.6 (d), 65.9 (d), 36.6 (t), 33.6 (t), 32.6 (t), 30.6 (t), 22.3 (q), 22.1 (q), and 19.1 (t); *m/z* 184 ($M^{\oplus}$, 5%), 140(10), 125(10), 115(100), 114(40), 112(60), 97(70), 83(10), 69(50), 55(50) and 43(40).

1.3.5.6 4-Tetrahydropyranoxyhex-1-yne (38)

4-Tetrahydropyranoxyhex-1-yne was synthesised by the method of Deslongchamps *et al*[44] in 21% yield (from propanal), b.p. 90-93°C at 20 mmHg. High resolution mass spectrometry gave $C_{11}H_{18}O_2$ for *m/z* 182.1202 (calc. *m/z* 182.1408). The GC retention time was 7.76 minutes (5% OV101, 50-16-280°C); ν_{max} (thin film) 3310, 1120, 1065, 1020 and 960 cm^{-1}; δ_H (60 MHz, $CDCl_3$) 0.8-1.2 (3H, d, *J* 6 Hz, $C\underline{H}_3$-C), 1.3-2.1 (9H, m, C-$C\underline{H}_2$-C and $\underline{H}C{\equiv}C$), 2.20-2.60 (2H, m, $C\underline{H}_2C{\equiv}C$), 3.30-4.20 (3H, m, $C\underline{H}O$) and 4.5-5.0 (1H, m, $OC\underline{H}O$); *m/z* 182 ($M^{\oplus}$, 2%), 101(5), 85(100), 81(10), 79(10), 67(15), 57(35) and 41(60).

1.3.5.7 6-Methyl-2-methoxy-2(4-hydroxy-1-hexynyl)tetrahydropyran (42)

To a stirred solution of 4-tetrahydropyranoxyhex-1-yne (38) (5g, 28 mmol) in THF (20ml) at -78°C, was slowly added *n*-butyl-lithium (26 mmol of 1.8 mol dm^{-3} solution in hexane). After stirring (0.5h), a solution of 1,5-hexanolide (2.8g, 28 mmol) in THF (40ml) was rapidly added and the solution stirred (1h). The reaction was quenched with aqueous sodium dihydrogen phosphate (50ml) at -60°C, allowed to warm to room temperature overnight (12h), the organic phase was removed and the aqueous layer was extracted into ether (3 x 50ml). The combined organic phases were dried (Na_2SO_4) and the solvent removed to yield a brown, oily residue (7.71g).

The crude residue (7.71g) was stirred overnight (12h) with a trace of Amberlyst in methanol (25ml). The reaction mixture was filtered through Celite and the solvent removed to afford crude 6-methyl-2-methoxy-2(4-hydroxy-1-hexynyl)tetrahydropyran (42) (7.60g). Flash column chromatography with ether eluant yielded (42) (3.78g, 60%) as a yellow oil. High resolution mass spectrometry gave $C_{12}H_{19}O_2$ (M - OCH_3) for *m/z* 195.1406 (calc. *m/z* = 195.1385); ν_{max} (thin film) 3450 (hydrogen-bonded OH), 2250 (C≡C), 1250, 1040 and 930 cm^{-1}; δ_H (60 MHz, $CDCl_3$) 1.4-0.9 (6H, m, $C\underline{H}_3$-C), 1.5-2.0 (8H, m, C-$C\underline{H}_2$-C), 2.4-2.6 (2H, m, $C\underline{H}_2$C≡C), 3.4 (3H, s, $OC\underline{H}_3$) and 3.5-3.9 (2H, m, C$\underline{H}$O); *m/z*, 195 (M - OCH_3, 80%), 153(45), 139(55), 125(100), 107(50), 96(70), 79(50), 67(65), 59(85), and 55(60).

1.3.5.8 (±)(E,E)- and(±)(Z,E)-8-Ethyl-2-methyl-1,7-dioxaspiro[5.5]undecane (34a) and (34b).

6-Methyl-2-methoxy-2(4-hydroxy-1-hexynyl)tetrahydropyran (42) (1.0g, 4.42 mmol) was dissolved in methanol (50ml) and stirred overnight (12h) under an atmosphere of hydrogen in the presence of 10% palladium on charcoal (0.1g). The reaction mixture was filtered through Celite and the solvent removed to afford crude 6-methyl-2-methoxy-2(4-hydroxy-hexanyl)tetrahydropyran (44) (0.82g, 80%) as a brown oil.

Crude 6-methyl-2-methoxy-2(4-hydroxyhexanyl)tetrahydropyran (0.82g) was stirred with(R)(+)-10-camphorsulphonic acid (0.1g) in ether (50ml) for 15 minutes. The reaction mixture was washed with aqueous sodium hydrogen carbonate (3 x 50ml), dried (Na_2SO_4) and the solvent removed to afford the novel 8-ethyl-2-methyl-1,7-dioxaspiro[5.5]undecanes (34a) and (34b). Flash column chromatography with ether-petrol (5:95) as eluant yielded(±)(E,E)-8-ethyl-2-methyl-1,7-dioxaspiro[5.5]undecane b.p. 96-98°C at 25 mmHg (Kugelrohr) (34a) (0.37g, 53%) and(±)(Z,E)-8-ethyl-2-methyl-1,7-dioxaspiro[5.5]undecane (34b) b.p. 116-118°C at 25 mmHg (Kugelrohr) (0.25g, 35%) as colourless oils.

(±)-(E,E)-8-Ethyl-2-methyl-1,7-dioxaspiro[5.5.]undecane (34a); high resolution mass spectrometry gave $C_{12}H_{22}O_2$ for m/z 198.1618 (calc. m/z = 198.1619). (Found: C, 72.48; H, 10.90. $C_{12}H_{22}O_2$ requires C, 72.73; H, 11.11%); (34a) showed a GC retention time of 4.65 minutes, 5% OV101 100-16-280°C; δ_C (25.15 MHz, $CDCl_3$) 96.0 (s), 70.4 (d), 65.1 (d), 35.6 (t), 34.8 (t), 33.1 (t), 31.2 (t), 29.4 (t), 21.9 (q), 19.2 (t), 19.1 (t) and 10.3 (d); δ_H (360 MHz, $CDCl_3$) 0.95 (3H, d, J 7 Hz, CH_3CH-), 1.1 (3H, t, J 3 Hz, CH_3CH_2-), 1.25-1.7 (14H, m, $C-CH_2-C$), 3.4 (1H, m, HCO) and 3.7 (1H, m, HCO); m/z 198 ($M^{\oplus}$, 10%), 184 ($M - CH_3$, 5), 116(85), 115(25), 112(25), 97(30), 88(25), 85(30), 83(30), 69(90), 55(80) and 44(100).

(±)-(Z,E)-8-Ethyl-2-methyl-1,7-dioxaspiro[5.5]undecane (34b); high mass spectrometry gave $C_{12}H_{22}O_2$ for m/z 198.1640 (calc. m/z = 198.1619). (Found: C, 72.64; H, 11.07. $C_{12}H_{22}O_2$ requires C, 72.73; H, 11.11%); (34b) showed a GC retention time of 5.19 minutes, 5% OV101 100-16-280°C; δ_C (25.15 MHz, $CDCl_3$) 97.5 (s), 74.1 (d), 66.2(d), 36.5 (t), 33.3 (t), 32.2 (t), 30.6 (t), 29.3 (t), 22.2 (q), 20.2 (t), 18.4 (t) and 10.5 (d); δ_H (360 MHz, $CDCl_3$) 1.0 (3H, d, J 7 Hz, CH_3CH-), 1.1 (3H, m, CH_3CH_2-), 1.4 - 1.8 (14H, m, $C-CH_2-C$), 3.3 (1H, m, -CHO equatorial) and 4.2 (1H, m, -CHO axial); m/z 198 ($M^{\oplus}$, 10%), 169 ($M - C_2H_5$, 20), 129(80), 115(35), 112(30), 111(70), 99(20), 83(55), 69(60), 55(100) and 41(70).

1.3.5.9 4-Tetrahydropyranoxyhept-1-yne (39)

4-Tetrahydropyranoxyhept-1-yne (39) was prepared from butanal by the method of Deslongchamps *et al*[44] (Section 1.5.5.6) as a green-yellow oil (40% yield), b.p. 92-95°C at 25 mmHg. The GC retention time of (39) was 8.49 minutes (5% OV101, 50-16-280°C). High resolution mass spectrometry gave $C_9H_{17}O_2$ ($M - C_3H_3$) for m/z 157.1175 (calc. m/z = 157.1228); ν_{max} (thin film) 3310, 1120, 1065, 1020 and 950 cm^{-1}; δ_H (60 MHz, $CDCl_3$) 0.9-1.2 (3H, d, J 7 Hz, CH_3-C), 1.3-2.1 (11H, m, $C-CH_2-C$ and $HC\equiv C-$), 2.20-2.60 (2H, m, $CH_2C\equiv C$), 3.30-4.20 (3H, m, CHO) and 4.5-5.0 (1H, m, OCHO); m/z 196 ($M^{\oplus}$, not seen), 157 ($M - C_3H_3$, 2%), 101(5), 95(7), 85(100), 67(12), 56(25) and 41(10).

1.3.5.10 6-Methyl-2-methoxy-2-(4-hydroxy-1-heptynyl)tetrahydropyran (43)

6-Methyl-2-methoxy-2-(4-hydroxy-1-heptynyl)tetrahydropyran (43) was prepared from 4-tetrahydropyranoxyhept-1-yne (39) in 60% yield by the method described in Section 1.5.5.8; high resolution mass spectrometry gave $C_{13}H_{21}O_2$ (M - OCH_3) for *m/z* 209.1489 (calc. *m/z* = 209.1541); ν_{max} (thin film) 3400, 2120, 1465 and 1020 cm^{-1}; δ_H (60 MHz, $CDCl_3$) 1.0-1.2 (6H, m, $C\underline{H}_3$-C), 1.3-2.0 (10H, m, C-$C\underline{H}_2$-C), 2.2-2.6 (2H, m, $C\underline{H}_2C{\equiv}C$), 3.2 (3H, s, $OC\underline{H}_3$) and 3.5-3.9 (2H, m, $C\underline{H}O$); *m/z* 240 ($M^{\oplus}$, not seen), 208 (M - CH_3OH, 10%), 136(25), 121(10), 107(10), 94(35), 85(15), 73(50), 69(55), 55(100) and 43(60).

1.3.5.11 (±)-(E,E)- and(±)(Z,E)-8-Propyl-2-methyl-1,7-dioxaspiro[5.5]undecane (35a) and (35b)

6-Methyl-2-methoxy-2-(4-hydroxy-1-heptynyl)tetrahydropyran (43) (0.5g, 2.08 mmol) was stirred overnight in methanol (25ml) under an atmosphere of hydrogen in the presence of 10% palladium on charcoal (50mg). The reaction mixture was filtered through Celite and the solvent removed. ^{1}H NMR and GC analysis revealed that cyclisation had occurred *in situ* to yield a mixture of 8-propyl-2-methyl-1,7-dioxaspiro[5.5]-undecanes. Flash column chromatogrpahy with ether-petrol (5:95) as eluant afforded(±)(E,E)-8-propyl-2-methyl-1,7-dioxaspiro[5.5]undecane (35a) (56mg, 13%) and(±)(Z,E)-8-propyl-2-methyl-1,7-dioxaspiro[5.5]-undecane (35b) (0.30g, 68%) as colourless oils.

(±)-(E,E)-8-Propyl-2-methyl-1,7-dioxaspiro[5.5]undecane (35a); high resolution mass spectrometry gave $C_{13}H_{24}O_2$ for *m/z* 212.1778 (calc. *m/z* = 212.1776); the GC retention time of (35a) was 7.20 minutes, (5% OV101 100(2)-16-280°C); δ_C (25.15 MHz, $CDCl_3$) 96.2 (s), 68.6 (d), 65.0 (d), 38.8 (t), 35.6 (t), 35.4 (t), 33.0 (t), 31.4 (t), 21.8 (q), 19.1 (t), 19.05 (t), 19.0 (t), and 14.2 (q); δ_H (360 MHz, $CDCl_3$) 0.90 (3H, d, *J* 7 Hz $C\underline{H}_3CH$-), 1.10 (3H, t, $C\underline{H}_3CH_2$-), 1.2-2.0 (16H, m, C-$C\underline{H}_2$-C), 3.5 (1H, m, $\underline{H}CO$) and 3.65 (1H, m, $\underline{H}CO$); *m/z* 212 ($M^{\oplus}$, 5%), 169 (M - C_3H_7, 10), 140(30), 125(25), 115(100), 112(65), 97(50), 83(70), 69(40), 55(80) and 43(80).

(±)-(Z,E)-8-Propyl-2-methyl-1,7-dioxaspiro[5.5]undecane (35b); high resolution mass spectrometry gave $C_{13}H_{24}O_2$ for *m/z* 212.1883 (calc. *m/z* = 212.1776); the GC retention time of (35b) was 7.80 minutes, 5% OV101 100(2)-16-280°C; δ_C (25.15 MHz, $CDCl_3$) 97.6 (s), 72.4 (d), 66.3 (d), 38.8 (t), 36.2 (t), 32.2 (t), 31.2 (t), 28.4 (t), 22.0 (q), 19.2 (t), 19.1 (t), 18.6 (t) and 14.1 (q); δ_H (360 MHz, $CDCl_3$)

0.90 (3H, t, $C\underline{H}_3CH_2$-), 1.10 (3H d, *J* 6 Hz, $C\underline{H}_3CH$-), 1.2-2.0 (16H, m, C-$C\underline{H}_2$-C), 3.45 (1H, m, $\underline{H}$CO equatorial) and 4.20 (1H, m, $\underline{H}$CO axial); *m/z* 212 ($M^{\oplus}$, 5%), 169 (M - C_3H_7, 10), 143(35), 125(30), 115(20), 112(15), 97(25), 85(80), 83(100), 55(70) and 43(70).

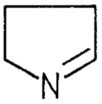
CHAPTER TWO

Synthesis of (E,E)-α-Farnesene, a Biologically-active Component from the Aeration Extract of Male *Ceratitis capitata* (Wiedmann), Diptera: Tephritidae

2.1 Introduction

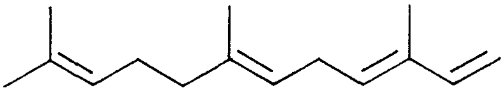
2.1.1 Chemical Communication in Medfly

The Mediterranean fruit fly, *Ceratitis capitata* (Wiedmann) is a pest of many deciduous and subtropical fruits, and occurs throughout Southern Europe, the Near East, Africa, Central and South America, Hawaii and South West Australia.[2] Sexually mature *Ceratitis capitata* are known to release a volatile chemical substance from their anal ampoules which attracts virgin females.[3] (E)-6-Nonen-1-ol (19) and methyl (E)-6-nonenoate (20) have been isolated by aeration/cold-trapping of the volatiles produced by male *C. capitata*, and the latter shown to be a male attractant in the field.[21] Recently Baker *et al*[20] have demonstrated that the mixture of volatiles produced by male *C. capitata* consists of nine components and that one of these, 3,4-dihydro-2H-pyrrole (Δ^1-pyrroline) (18), appears to be the key constituent in relation to the biological activity of the volatile emissions of the male fly.



(18)

(E,E)-α-Farnesene (46) is a major component not only of the aeration extract from male *C. capitata*, but also in solid sample GC/MS of the anal ampoules of male flies[13] (*ca.* 200ng/insect). It is significant that analysis of aeration products from female *C. capitata* failed to show any evidence of the nine components isolated from the male flies.



(46)

The biological activity of these nine male specific components was studied using a double tube olfactometer.[59,60] An aqueous solution of 3,4-dihydro-2H-pyrrole (18) was found to be highly attractive to virgin female *C. capitata*, and compared favourably with the response of females to live males. No other compound was active singly, although several of the other components (among them E,E-α-farnesene) displayed activity in mixtures approximating to the ratios found in aeration experiments.

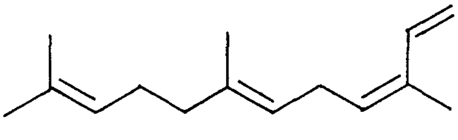
Thus, it was decided to attempt an efficient synthesis of (E,E)-α-farnesene to provide enough pure material for biological testing to investigate the role of (E,E)-α-farnesene in the communication system of *C. capitata*, thereby aiding the development of an effective pheromone-based control system.

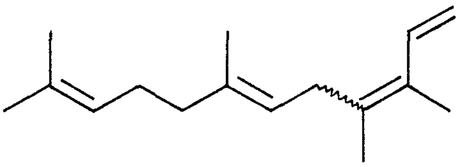
2.1.2 Natural Occurrence of (E,E)-α-Farnesene

(E,E)-α-Farnesene (3,7,11-trimethyl-1,3,6,10-dodecatetraene) (46) has been reported as virtually the only sesquiterpene present in the cuticle of Granny Smith apples.[61,62] It has also been found in the coatings of Delicious and Crofton apples,[63] pears (Packham, Winter Cole), and quinces, while Meigh and Filmer[64] have reported it in English apples. Its occurrence in these coatings, all of which have triterpene acids as major constituents, suggests an origin from a farnesyl or nerolidyl intermediate in the biosynthesis of these C_{30} acids. The rapid increase of (E,E)-α-farnesene during storage[62] coupled with the relatively constant content of the triterpene acids suggests that the coupling of the C_{15} units has been inhibited and (E,E)-α-farnesene results from the accumulation of the C_{15} intermediate.

(E,E)-α-Farnesene has been reported as the sole constituent in the Dufour's (accessory venom) gland of a myrmicine ant, *Aphaenogaster longiceps*,[65] and as a component of the trail pheromone of the red imported fire ant, *Solenopsis invicta*.[66] The other components of the trail pheromone were (Z,E)-α-farnesene (47) and two homofarnesenes (48).

(47)





(48)

α-Farnesenes have been reported in other ant species, as have some *mono*, *bis* and *tris*-homofarnesenes. In these other species, however, their biological functions are unknown.[67]

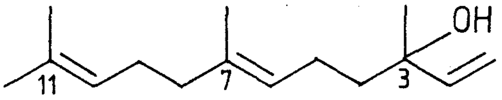
(E,E)-α-Farnesene has been identified as an attractant for codling moth, *Laspeyresia pomonella*, larvae[67] and has also been isolated from the Dufour's gland of andrenid bees,[69] from the osmeterial gland of

Papilio (swallowtail) larvae,[70] and as a component from the dorsal gland of the springbok, *Antidorcas marsupialis*.[71]

(E,E)-α-Farnesene is rapidly autoxidised at 1°C, the primary monomeric products being conjugated triene hydroperoxides,[72] probably *via* free radicals at the conjugated 1,3-diene system.

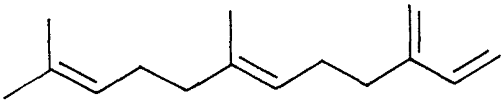
2.1.3 Previous Synthetic Approaches to (E,E)-α-Farnesene

The preparation of (E,E)-α-farnesene (46) was first reported by Anet,[73] who dehydrated 3-hydroxy-3,7,11-trimethyl-1,6E,10-dodecatriene (E-nerolidol) (49) with phosphoryl chloride in the presence of excess pyridine to yield a mixture of farnesenes. Anet removed any polar impurities by column chromatography on Florisil (most other adsorbents tend to decompose farnesenes), and then purified the mixture further by repeated preparative GC on Apiezon L and Carbowax 20M columns.

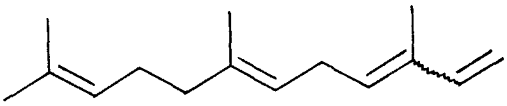


(49)

Subsequently Bowers *et al*[74] synthesised (E,E)-α-farnesene (46) by dehydration of (E)-nerolidol (49) (1.2g) affording a mixture of hydrocarbons which was chromatographed on Florisil impregnated with a 10% loading of silver nitrate using an ether-chloroform gradient elution. From the 50% ether in chloroform fractions (E)-7,11-dimethyl-3-methylene-1,6,10-dodecatriene (E-β-farnesene) (50) (80mg) was obtained, whilst (E,E)-α-farnesene (46) (22mg) and mixed (E)-α-farnesenes (51) (28mg) were obtained from the 75% to 100% ether in chloroform fractions.



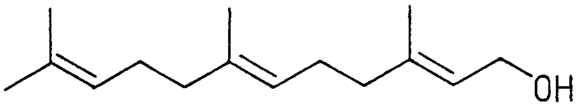
(50)



(51)

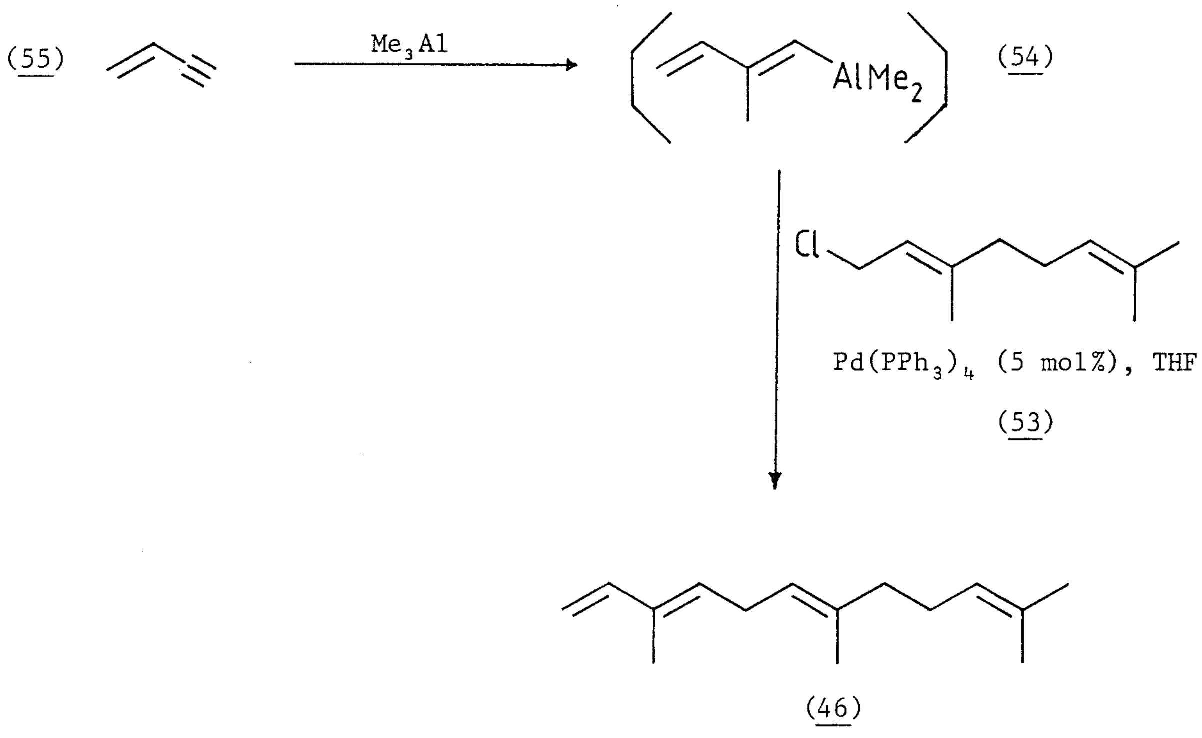
Brieger *et al*[75] prepared (E,E)-α-farnesene (46) *via* rhodium (III) chloride catalysed isomerisation of (E)-β-farnesene (50). Conjugated farnesene isomers were reported as by-products of the isomerisation.

A synthesis of (E,E)-α-farnesene (46) from (E,E)-farnesol (52) has been reported which depends on the effectiveness of organoaluminium reagents for oxirane ring opening, as an example of a general method for converting allylic alcohols to 1,3-dienes.[76] Another route to



(52)

(E,E)-α-farnesene (46) using organoaluminium chemistry has been reported in the literature by Negishi and Matsushita.[77] This approach utilised the cross-coupling reaction of (E)-1-chloro-3,7-dimethyl-2,6-octadiene (geranyl chloride) (53) with (1E)-(2-methyl-1,3-butadienyl)dimethylalane (54) in the presence of 5 mol per cent tetrakis(triphenylphosphine)-palladium (0) as catalyst; an 86% yield of (E,E)-α-farnesene (46) from 1-buten-3-yne (55) was reported (Scheme 6).



Scheme 6. Preparation of (E,E)-α-farnesene (46) by the palladium-catalysed stereo- and regiospecific coupling of an allylic derivative with an alkenylmetal.

2.2 Results and Discussion

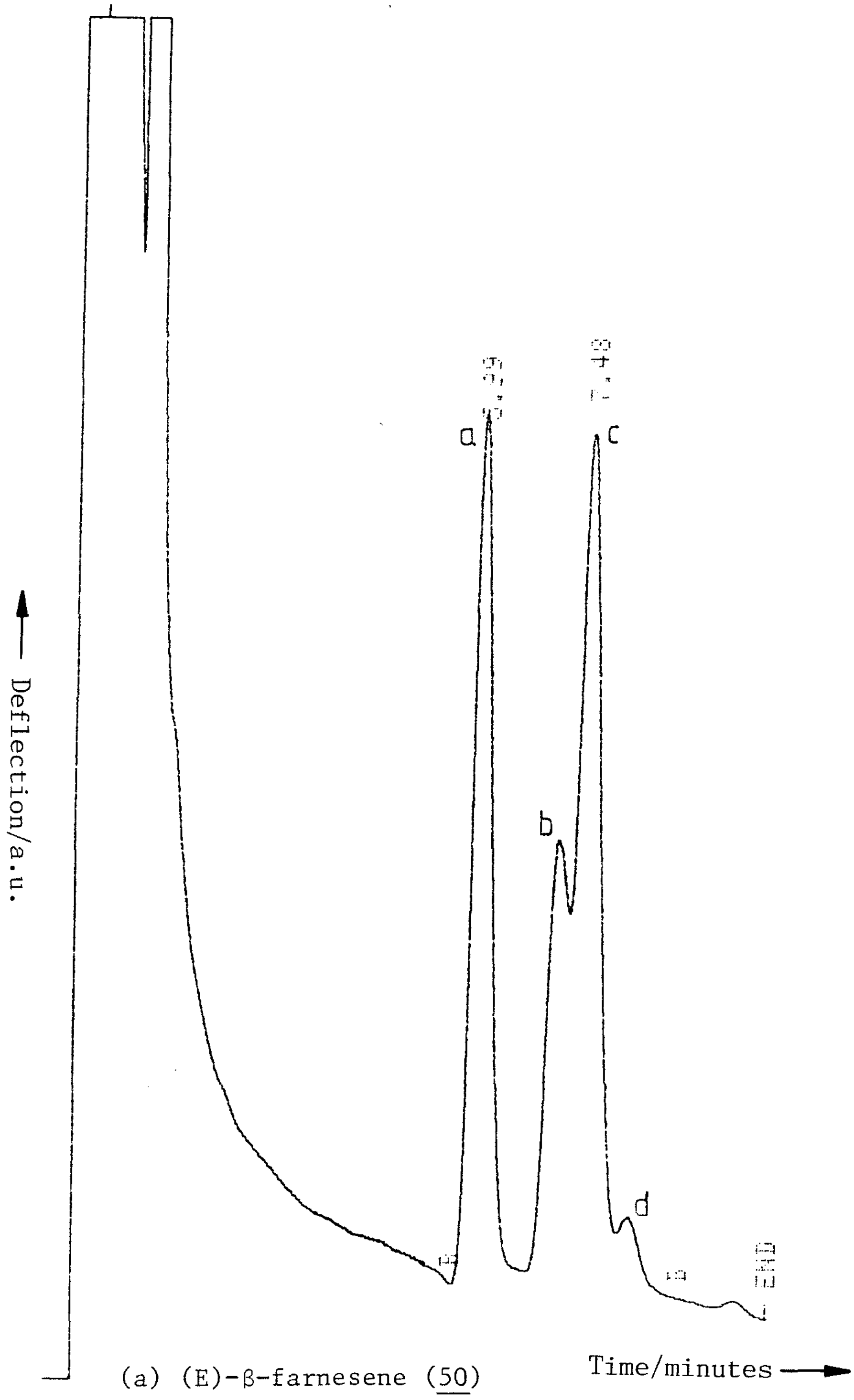
Initially it was decided to prepare (E,E)-α-farnesene by the method of Bowers *et al.*[74] Thus (E)-nerolidol (49) was dehydrated with phosphoryl chloride in the presence of an excess of pyridine (as proton-acceptor) to give a mixture of four hydrocarbons. This farnesene mixture was analysed by GC/MS and found to comprise (E)-β-farnesene (50), (Z,E)-α-farnesene (47) and (E,E)-α-farnesene (46) together with another (unidentified) farnesene isomer (Figure 6). The mass spectral data of these farnesenes is shown in Section 2.4.6.1. The dehydration products were identified by comparing the mass spectra with literature data.[46,73]

In an attempt to optimise the yield of (E,E)-α-farnesene (46) from this reaction, a variety of conditions were tried. The results are shown in Section 2.4.7.1 (Table 8) and it may be seen that none of the methods tried affords a particularly high yield of (E,E)-α-farnesene (46). However, stirring the dehydration reaction mixture for 48 hours at room temperature (method A) gave a better yield of the desired product than heating at 70°C for five minutes (method B) or stirring for five minutes at room temperature (method C).

Despite repeated attempts to separate the farnesene isomers by chromatography on Florisil loaded with 25% silver nitrate using gradient elution (pentane-ether; 100% pentane to 100% ether with 10% steps), it was not possible to obtain (E,E)-α-farnesene (46) free of (E)-β-farnesene (50). Separation by HPLC was also attempted using a reversed phase Zorbax ODS 25cm x 4.6mm column, refractive index detection, and solvent systems of 10% water, 15% water and 20% water in methanol, but once again no viable separation was achieved. The separation of farnesene isomers by reversed-phase argentation HPLC has been reported in the literature,[78,79] but it was thought impracticable to attempt this separation technique due to the inherent risk of damaging the HPLC column by using silver ions in the mobile phase.

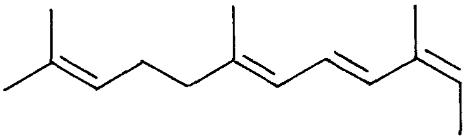
After attempting several other methods, it was decided to dehydrate (E)-nerolidol (49) as previously described to afford a mixture of farnesenes, isomerise the (E)-β-farnesene (50) in the mixture to (E,E)-α-farnesene (46) using the rhodium (III) chloride-catalysed isomerisation reported by Brieger,[75] selectively form the sulpholene of (E,E)-α-farnesene (56) by the method of Pickett *et al*,[80] isolate

Figure 6. GC/MS analysis of products from the dehydration of (6E)-nerolidol (49) (5% FFAP, 130-4-170°C).



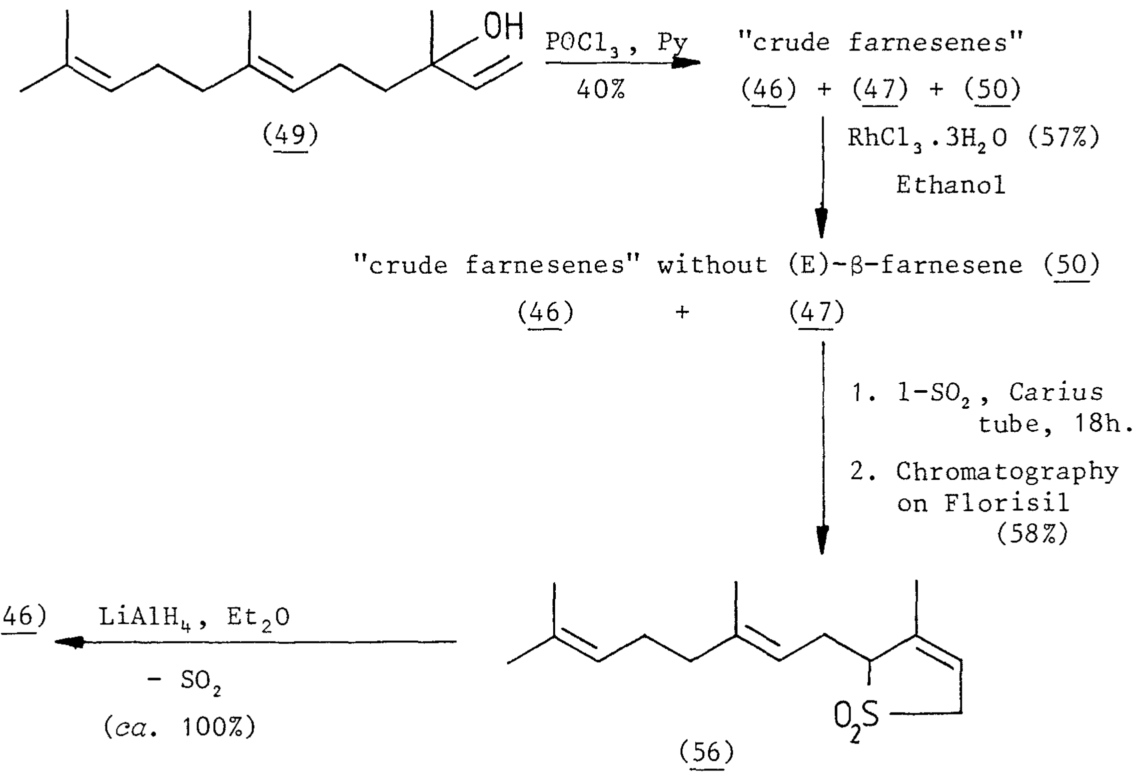
(a) (E)-β-farnesene (50)
(b) (Z,E)-α-farnesene (47)
(c) (E,E)-α-farnesene (46)
(d) unidentified farnesene isomer

the pure sulpholene, 2-(3,7-dimethyl-2,6-octadienyl)-3-methyl-2,5-dihydrothiophene-1,1-dioxide (56) by column chromatography on Florisil, and finally regenerate the desired (E,E)-α-farnesene (46) from the sulpholene (56) by one of the literature methods, i.e. heating to 180°C at 1 mmHg for a few minutes,[80] lithium aluminium hydride-promoted extrusion of sulphur dioxide,[81] or refluxing in octane[82] (Scheme 7). This method was successful and afforded a 5% overall yield of 92% pure (E,E)-α-farnesene (46). Unfortunately, it was not possible to purify this product further by reversed-phase HPLC (Zorbax ODS, 15% water in methanol). The impurities (8% by GC) in the final product are presumably due to the formation of sulpholenes from by-products of the rhodium-catalysed isomerization, probably allofarnesenes,[75] for example (57):



(57)

Scheme 7. Preparation of (E,E)-α-farnesene *via* the sulpholene.



2.3 Conclusion

(E,E)-α-Farnesene was synthesised in *ca.* 5% overall yield from (E)-nerolidol. The synthetic material and the natural product had identical mass spectra and co-eluted on three different types of GC column (capillary BP10, 5% OV101 packed, and 5% FFAP packed).

2.4 Experimental

2.4.1 Purification of Reagents and Solvents

The purity of (±)-(6E)-3,7,11-trimethyl-1,6,10-dodecatrien-3-ol (E-nerolidol), a gift of the Takasago Chemical Company Limited, Japan, was found to be greater than 98% the 6E isomer by GC (5% FFAP, 200°C) and was used as supplied. Pyridine (BDH) was dried over potassium hydroxide and then distilled from calcium hydride. Phosphoryl chloride (BDH), acetyl chloride (Aldrich) were freshly distilled prior to use.

Ether was dried by treating with sodium wire, left to stand for 24h, and then distilled from calcium hydride; tetrahydrofuran was pre-dried over potassium hydroxide and then refluxed over sodium/benzophenone; ethanol (absolute) was refluxed with magnesium turnings in the presence of iodine (catalytic quantity) and then distilled from the resulting Grignard reagent.

All solvents used for purification by chromatography were distilled prior to use.

'Ether' in all cases refers to diethyl ether and petroleum ether corresponds to the fraction boiling between 40°-60°C.

2.4.2 General Procedures

'Flash' column chromatography was performed according to the procedure of Still *et al.*[83] using Macherey-Nagal Kieselgel 60 (230-400 mesh) with the solvents described. Analytical TLC was carried out on pre-coated silica gel plates [Merck Kieselgel 60F, 254] and were visualised by UV fluorescence, standing in iodine vapour, or spraying with an aqueous solution of potassium permanganate, methanolic 2,4-dinitrophenylhydrazine or vanillin in methanolic sulphuric acid.

Removal of solvents was carried out by evaporation at reduced pressure (*ca.* 15mm Hg) using a rotary evaporator.

All apparatus was flame dried under a positive pressure of dry nitrogen prior to use, and all reactions were performed under an atmosphere of dry nitrogen unless otherwise specified.

2.4.3 Gas Chromatography

Gas-liquid chromatography (GC) was conducted using Pye 104 and series D gas chromatographs (Pye Unicam, Cambridge) fitted with flame ionisation detectors (FID) using hydrogen and air pressures of 0.85 and 0.5 kg cm^{-2} respectively. Packed column chromatography was performed using glass analytical columns 3.1m x 2mm containing stationary phases coated on Diatomite C (CLQ for OV phases), acid/alkali washed, DMCS treated, 100-120 mesh (J.J. Chromatography Ltd., Kings Lynn). The stationary phases used included Carbowax 20M 5%, OV101 5% and FFAP 5%. The nitrogen gas flow rate was 25 $cm^3 min^{-1}$.

Capillary GC utilised a 50m x 0.2mm SCOT glass capillary column coated with CW 20M. Helium (2-3 $cm^3 min^{-1}$) was used as the carrier gas; 25 $cm^3 min^{-1}$ of nitrogen make-up gas was used. An injector system similar to that described by Grob[84] (but without a septum purge) was used.

2.4.4 Gas Chromatography - Mass Spectrometry

High resolution electron impact (EI) mass spectra were obtained on a Kratos MS-30 spectrometer interfaced *via* a Ryhage all-glass jet separator to a Pye 204 chromatograph. The GC/MS interface was maintained at 250°C with a source temperature of 100°C using the above GC columns. The data was analysed using a NOVA-3 computer (Data General, Mass., USA) equipped with a DS-55 data system (Kratos, Manchester). Helium was used as the carrier gas (30 $cm^3 min^{-1}$).

2.4.5 High Performance Liquid Chromatography

HPLC was carried out on a Du Pont 8800 instrument using a variable wavelength ultra-violet (UV) detector, or on a Waters Associates ALC 202 system using a refractive index detector. A pre-packed Zorbax ODS column (25cm x 4.6mm) was used for reversed-phase HPLC and a pre-packed Zorbax SIL column (25cm x 4.6mm) was used for normal-phase HPLC. The flowrate was 1.2 ml min^{-1} unless otherwise stated and the solvent systems used for particular separations are as stated in the text. All HPLC solvents were obtained from Fisons plc, filtered through a Sartorius membrane filter (0.45µm pore size) and thoroughly degassed prior to use. HPLC was performed at ambient temperature unless otherwise stated.

2.4.6 Instrumentation

Infra-red spectra (IR) were recorded using a Perkin-Elmer 157G grating spectrophotometer as thin films on sodium chloride plates or as 10% solutions in chloroform or carbon tetrachloride in sodium chloride solution cells (cell thickness = 0.1mm). The absorption bands are given in wavenumbers (cm^{-1}) relative to a polystyrene standard and are described with the following abbreviations; s = strong, m = medium, w = weak, b = broad.

^1H NMR spectra were obtained at 60MHz using a Hitachi-Perkin-Elmer R-24B high resolution spectrometer; 360MHz ^1H NMR spectra were recorded using a Bruker AM360 spectrometer. Tetramethylsilane was used as an internal standard. Peak positions are quoted on the δ scale relative to tetramethylsilane (zero) using the following abbreviations: s = singlet, d = doublet, t = triplet, dd = double doublet, q = quartet, dt = doublet of triplets, ddd = double doublet of doublets, m = multiplet, b = broad. Coupling constants, J, are expressed in hertz.

Melting points were determined using an Electrothermal electrically heated block or Reichert Koffler hot stage melting point apparatus and are uncorrected.

Elemental analyses were carried out at the micro-analytical laboratory, University College, London.

2.4.7 Experimental Procedures

2.4.7.1 Dehydration of (E)-nerolidol (49) by phosphoryl chloride in pyridine.[72]

Method A

(E)-Nerolidol (5ml, 20mmol) (49) in pyridine (25ml) was added to phosphoryl chloride (10ml) in pyridine (25ml) and the mixture was stirred for 48h at room temperature. The solution was poured into cold water (50 ml) and extracted with pentane (5 x 20ml). The extract was washed with water (2 x 20ml), dried (Na_2SO_4) and concentrated to *ca.* 4ml. Chromatography on Florisil with pentane eluant yielded a mixture of crude farnesenes (1.4g, 6.86 mmol, 30%). The crude farnesene mixture was analysed by GC/MS (Table 9) and the products identified by comparing the mass spectra with literature data.[73]

Method B

Similar to method A with the difference being that initially phosphoryl chloride (0.5ml) was added to (E)-nerolidol (1ml, 4 mmol) in pyridine (2ml) at 70°C. The mixture was stirred at 70°C for 5 minutes and then allowed to cool to room temperature. Repeating the work-up procedure of Method A afforded a mixture of crude farnesenes (330mg, 40%).

Method C

Similar to method B, except that the reaction was performed at room temperature throughout. A mixture of crude farnesenes (290mg, 35%) was obtained.

The following farnesene isomer ratios, calculated on the basis of GC peak areas, were obtained by dehydration of (E)-nerolidol(49) using methods A, B and C (Table 8):-

Farnesene isomer	Percentage of given farnesene isomer		
	Method A	Method B	Method C
(E)-β-Farnesene (50)	41	48	47
(Z,E)-α-Farnesene (47)	21	20	20
(E,E)-α-Farnesene (46)	35	30	31
Unidentified farnesene isomer	3	2	2
Overall percentage yield of crude farnesenes	30	40	35

2.4.7.2 Isomerisation of (E)-β-farnesene (50)

(E)-β-Farnesene was isomerised using the method of Brieger *et al.*[75] Rhodium (III) chloride trihydrate (6mg) was added to a stirred solution of crude farnesenes (100mg, 0.49 mmol) in ethanol (1ml). The mixture was stirred at 70°C and the reaction followed by GC (5% FFAP, 125°C) until the peak due to (E)-β-farnesene (50) disappeared (*ca.* 30 minutes). The mixture was cooled, taken up in ether (2ml), washed with aqueous

Table 9. GC/MS analysis of products from the dehydration of (E)-nerolidol (49) by phosphoryl chloride in pyridine (5% FFAP, 140°C).

Identification	RT/min.	$M^{\oplus}$(%)	Base peak *m/z*	*m/z* (%)			
(E)-β-Farnesene (50)	4.0	204(2)	69	93(45),	41(45),	133(15),	81(15),
				79(15),	55(15),	120(10),	53(10).
(Z,E)-α-Farnesene (47)	5.2	204(12)	69	93(70),	41(60),	107(35),	135(30),
				109(20),	79(20),	67(20),	55(20).
(E,E)-α-Farnesene (46)	6.0	204(1)	93	55(85),	41(80),	69(70),	79(50),
				107(45),	123(40),	119(35),	80(35).
An unidentified farnesene isomer	6.2	204(15)	93	121(30),	109(30),	80(30),	119(25),
				204(15),	94(15),	92(15),	79(15).

sodium hydrogen carbonate (5ml) and water (3 x 5ml) and dried (Na_2SO_4). Removal of solvent afforded a quantitative recovery of a mixture of (E,E)-α-farnesene (46), (Z,E)-α-farnesene (47) and an unidentified farnesene isomer with mass spectra identical to that reported in the literature.[73]

2.4.7.3 2-(3,7-Dimethyl-2,6-octadienyl)-3-methyl-2,5-dihydrothiophene-1,1-dioxide (56)

2-(3,7-Dimethyl-2,6-octadienyl)-3-methyl-2,5-dihydrothiophene-1,1-dioxide (56) was prepared by the general method of Pickett *et al.*[80] Crude farnesene mixture after isomerisation (170mg, 0.83 mmol) and liquid sulphur dioxide (*ca.* 200mg) were sealed in a Carius tube at room temperature for 18h. The ampoule was opened after cooling and excess sulphur dioxide was allowed to evaporate. The residue was chromatographed on Florisil using a gradient elution (pentane-ether, 100% pentane to 100% ether, 10% steps). Removal of solvent afforded (56) as a straw-coloured liquid (129mg, 58%). High resolution mass spectrometry gave $C_{15}H_{24}SO_2$ for *m/z* 268.1406 (calc. *m/z* = 268.1497). ν_{max} (CCl_4) 1310 (s, SO_2) and 1110 (s, ring); δ_H (60 MHz; $CDCl_3$) 1.70 (12H, S, 4 x $C\underline{H}_3$-C), 2.08 (6H, m, 3 x $C\underline{H}_2$-C=), 3.70 (3H, m, ring), 5.16 (2H, m, C=C$\underline{H}$) and 5.78 (1H, m, C=C$\underline{H}$ ring); *m/z* 268 (M^+, 1%), 225(5), 203(8), 161(5), 147(5), 135(20), 134(20), 123(15), 119(20), 107(15), 93(40), 81(20), 69(100), 55(25) and 41(35).

2.4.7.4 Regeneration of (E,E)-α-farnesene (46) from (56)

(E,E)-α-Farnesene (46) was regenerated from (56) using the method of Gaoni.[81] 2-(3,7-Dimethyl-2,6-octadienyl)-3-methyl-2,5-dihydrothiophene-1,1-dioxide (56) (15mg, 0.06 mmol) was added to a stirred suspension (1:1 by mass) of lithium aluminium hydride in ether (5ml) and refluxed for 1h. The excess lithium aluminium hydride was destroyed with excess ethyl acetate (10ml) and the ether layer was washed with aqueous sodium sulphate, dried (Na_2SO_4) and the solvent removed to afford (E,E)-α-farnesene (46) (12mg, 100%). High resolution mass spectrometry gave $C_{15}H_{24}$ for *m/z* 204.1945 (calc. *m/z* = 204.1878). ν_{max} (CCl_4) 3080 (w), 2960 (s), 2900 (s), 1660 (w), 1640 (m), 1600 (m), 980 (m) and 880 (s); δ_H (100MHz, $CDCl_3$) 1.60 (3H, s, $C\underline{H}_3$-C=), 1.63 (3H, s, $C\underline{H}_3$-C=), 1.66 (3H, s, $C\underline{H}_3$-C=), 1.75 (3H, s, $C\underline{H}_3$-C=), 2.03 (4H, m, C-$C\underline{H}_2$-C=) and 2.80 (2H, t, *J* 6 Hz, =C-$C\underline{H}_2$-CH=); *m/z* 204 (1%), 161(5), 135(10), 123(30), 119(35), 107(40), 93(100), 79(30), 69(50), 55(40) and 41(85).

CHAPTER THREE

Attempted Identification of an Aggregation Pheromone in the Oriental Cockroach, *Blatta orientalis* (Linnaeus), Blattodea: Blattidae

3.1 Introduction

Cockroaches have been pests in human dwellings for centuries and are very hard to eradicate, largely as a result of their nocturnal, secretive habits. They may be found wherever there is a food supply, water and warmth and are noticeable by their distinctive musty odour. Cockroaches can also carry viruses and bacteria that cause such diseases as hepatitis, polio, typhoid, plague and salmonella, which they may track from one place to another.[85,86] The Oriental cockroach or 'black beetle', *Blatta orientalis*, is the most common species in Britain.[87] It is usually just over 30mm long when fully grown and dark brown in colour. Male and female adult *Blatta orientalis* can be distinguished readily, because the female has vestigial wings while the male's wings are a little shorter than the body of the insect. Both sexes can move very fast although neither can fly. Other common cockroach species include the German cockroach, *Blattella germanica*, and the American cockroach, *Periplaneta americana*. A broad range of insecticidal formulations is available for cockroach control, but resistance to organophosphates (e.g. diazinon) and chlorinated hydrocarbons (e.g. DDT) has been reported for *B. germanica*.[87]

3.1.1 Chemical Communication in Cockroaches

Chemical communication is known to be very important in cockroaches,[88] and the research reported here was initially aimed at isolating and identifying a sesquiterpenoid previously reported as an aggregation pheromone for *Blatta orientalis*.[89] Ritter and Persoons have suggested that aggregation pheromones, although they act only at short distances, might be used to increase the active area of, for example, a trap containing insecticide.[90]

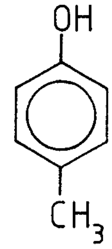
The analysis of cockroach secretions has revealed various allomones, aphrodisiacs, sex and aggregation pheromones.[91] The function of the cephalic glands of cockroaches is unknown, with the notable exception of *Blaberus craniifer* and *Eublaberus distanti* which secrete a 1:1 mixture of undecane and tetradecane from their mandibular glands as an aggregation pheromone.[92] The abdominal exocrine glandular system consists of tergal glands, sternal glands and glands associated with the second abdominal spiracle. The tergal glands usually only occur in adult males and their secretions act as aphrodisiacs. Other glands, including the sternal gland, have a defensive function. The allomonal secretions in 10 species of cockroaches from 7 genera have recently been analysed by Brossut[91] who identified 43 compounds, among them acids, alcohols, aldehydes, hydrocarbons, ketones, lactones, phenols and quinones; these compounds may function as repellents. The sternal gland of the Malayan cockroach, *Archiblatta hoeveni*, is known to produce a mixture of *p*-cresol (major component) (58) and phenol (minor) (59) as a defensive spray when attacked.[93] A mixture of 1,4-benzoquinone (10%) (60), *p*-cresol (25%), 2-ethylphenol (15%) (61), 4-ethylphenol (15%) (62), (E)-2-hexenal (63), gluconic acid (64) and gluconolactone (65) have been identified in the sternal gland of *B. orientalis*. 2-Heptanone (66) and undecane (67) are produced in the tergal glands.[91]

3.1.2 Aggregation Pheromones

Cockroach aggregation pheromones have rarely been reported in the literature, however, most cockroach species are known to be gregarious and tend to aggregate in response to chemical stimuli.[94-96] Several attempts have been made to isolate and identify aggregation pheromones of *B. germanica*, but the behavioural activity could not be attributed to any single component found in the faeces.[90] Ritter and Persoons have reported that the aggregation pheromone is a complex mixture, and also concluded that free carboxylic acids played an important role in the aggregation-activity.[90] Such acids have been reported in nature as being feeding stimulants and attractants for *B. germanica*.[97] However, the volatile fatty acids present in the frass of *B. germanica*, particularly propionic, isovaleric and valeric acids, have also recently been shown to repel two to twelve-day-old larvae.[98]

3.1.3 Sex Pheromones and Sex Pheromone Mimics

Long-range and contact pheromones mediate courtship behaviour and mating in many cockroach species. Two sex pheromones, (3S,11S)-(+)-3,11-dimethylnonacosan-2-one (68) and (3S,11S)-(+)-29-hydroxy-3,11-dimethylnonacosan-2-one (69), have been identified from body washes of *B. germanica* females.[99-101] These compounds elicit sexual response in males by direct chemoreception. Pheromonal compounds present in the cuticular wax of females are thought to be passed on to the male during fencing of the antennae prior to copulation. Both antipodes of synthetic (68) elicit behavioural responses.[102]



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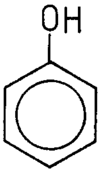

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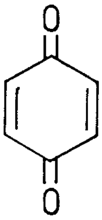

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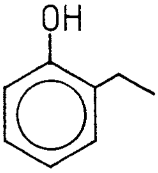

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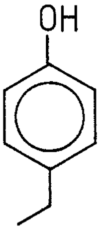

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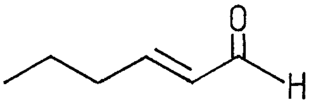

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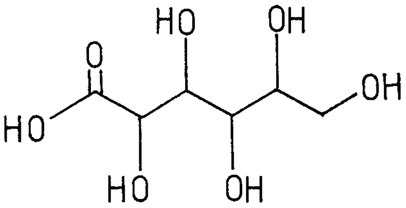

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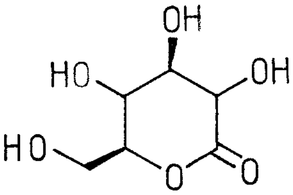

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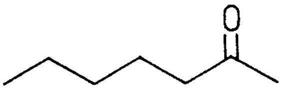

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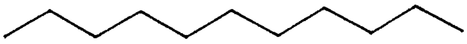

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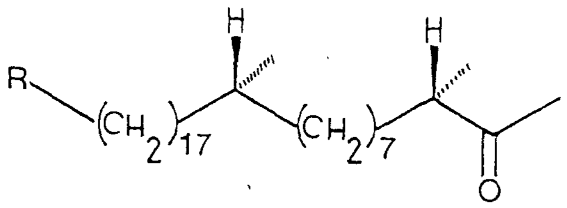
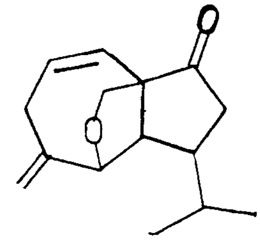


R = Me (68)
R = CH$_2$OH (69)

'Periplanone-A' (70) and 'periplanone-B', (71a), two independently active sex pheromones, have been isolated from faeces of the American cockroach, together with four unidentified minor components, after a massive scale rearing and extraction programme involving *ca.* 100 000 virgin females.[103,104] Only 20μg of periplanone-A and 200μg of periplanone-B were obtained (1:10 ratio). Nishino *et al.* have recently isolated the same compounds in a 9:2 ratio.[105] Periplanone-A was found to be unstable and isomerised to give (72), a more stable but biologically inactive compound. Periplanone-A has been identified as 7-methylene-4-isopropyl-12-oxatricyclo[4.4.2.0^{1,5}]-9-dodecen-2-one (70) ($C_{15}H_{20}O_2$) by comparing its NMR, MS, UV and IR data with those of its stable rearrangement product.[106] A germacranoid structure has been suggested for periplanone-B; (1Z,5E)-1,10(14)-diepoxy-4(15),5-germacradien-9-one (71a).[107] Three of the four possible diastereoisomers, one of which was identical to the natural pheromone, have been synthesised stereoselectively by Still.[108] Several other syntheses of periplanone-B have appeared recently in the literature.[109] The absolute configuration of periplanone-B was established as (1R, 2R, 5E, 7S, 10R) (71b) by an X-ray crystallographic study of periplanol-B (73).[110] Both the natural and synthetic (-)-enantiomer elicited activity with a threshold of 10^{-6} to 10^{-7} μg.[110]

The behavioural response of male *P. americana* to the natural sex pheromone can be mimicked by several compounds. These include germacrene-D (74),[111] (+)-verbenyl acetate (75)[112] and (+)-(E)-verbenyl propanoate (76)[113] in addition to (+)- and (-)-bornyl acetate (77).[114] These substances are thought to stimulate the sex pheromone receptor sites.[113] Recently, Nishino *et al* have demonstrated that (-)-enantiomers do not stimulae the sex pheromone receptors, which only respond to (+)-enantiomers.[115]



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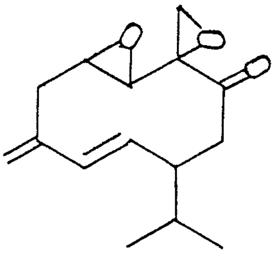

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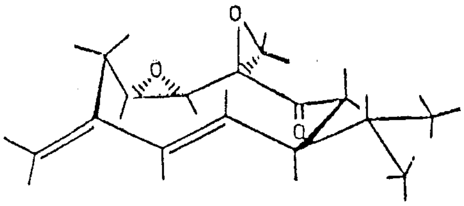

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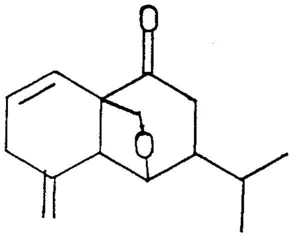

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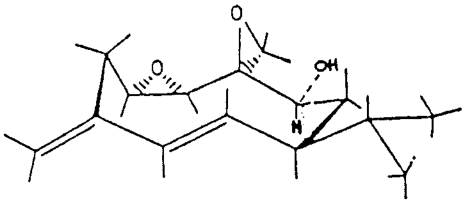

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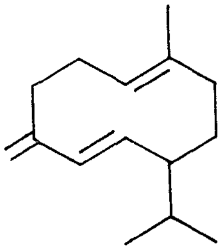

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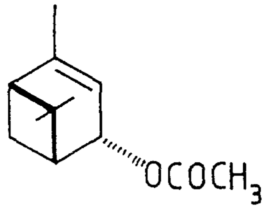

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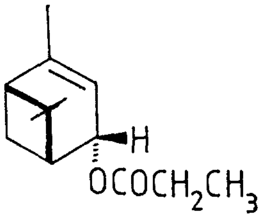

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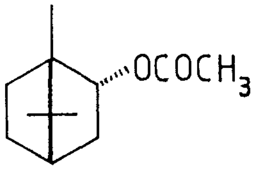


(77)

Warthen *et al* have recently isolated a *B. orientalis* sex pheromone from the faeces of adult virgin females.[116] The sex pheromone is reported to have a relative molecular mass of 232 and a mass spectrum closely resembling that of periplanone-B; it elicited a sexual response at 10pg and 1ng with *B. orientalis* and *P. americana* adult males, respectively. Only 200ng of the pheromone was obtained from three collections over a six-week period from *ca.* 15 000 female cockroaches, using an extraction and isolation procedure similar to that employed by Persoons.[103] The chemical structure of the *B. orientalis* sex pheromone is still unknown.

3.2 Results and Discussion

3.2.1 Isolation of a Proposed Aggregation Pheromone

Previous to this study the most likely aggregation pheromone components for *B. orientalis* were thought to be two monoöxygenated sesquiterpenes.[89] The major component was tentatively identified as a secondary alcohol, but the structural formula of this component could not be determined. The minor component was identified as an alcohol, Scheme 8. Extraction of the *Blatta* sesquiterpenoid.

Faeces (5kg)

- Extracted 3x dichloromethane
- Filtered (glass wool)
- Concentrated
- Dissolved residue in methanol
- Filtered and concentrated

↓

Methanol-soluble extract

- Removed methanol, redissolved residue in petroleum ether
- Dry flash column chromatography, gradient elution (50ml fractions)
- Concentrated fractions, GC/MS (mass chromatography for *m/z* 93, 133, 189, 220)

↓

10% ethyl acetate/petroleum ether fraction

- Sephadex LH20 column chromatography
- Elution with dichloromethane (50ml fractions)

↓

Fraction 6

- Concentrated
- Dry flash column chromatography, gradient elution
- 100% petroleum ether - ether 100%, 10% steps
- 50ml fractions

↓

30% ether - petroleum ether fraction

- HPLC, reversed-phase
- 20% water-methanol

↓

Pure sesquiterpenoid (10mg)

but no other structural information was obtained. During this study a total of *ca.* 1.5mg 80% pure sesquiterpenoid (major) and *ca.* 200ng of the minor sesquiterpenoid was obtained from 5kg of *B. orientalis* faeces. Thus, it was decided to improve the existing extraction method in order to obtain larger quantities of the sesquiterpenoids to facilitate more detailed structural analysis, probably by means of X-ray crystallography.

Originally, the separation procedure of Neequaye[89] was followed, but this was later optimised as shown in Scheme 8 to yield *ca.* 10mg of the major sesquiterpenoid from each 5kg of faeces extracted (see Section 3.4.2 for experimental details). The mass spectrum and GC retention time of this sesquiterpenoid were identical to those previously observed; the GC retention time was 10.5 minutes (5% OV101, 100-12-290°C) and the mass spectrum is shown in Figure 7. Only the major sesquiterpenoid reported by Neequaye[89] was isolated in this study as mass chromatography for the characteristic *m/z* 93, 133, 189 and 220 ions revealed the absence of the minor sesquiterpenoid component. Similarly, mass chromatography showed that neither of the sesquiterpenoids were present in the faeces of the German cockroach, *B. germanica*.

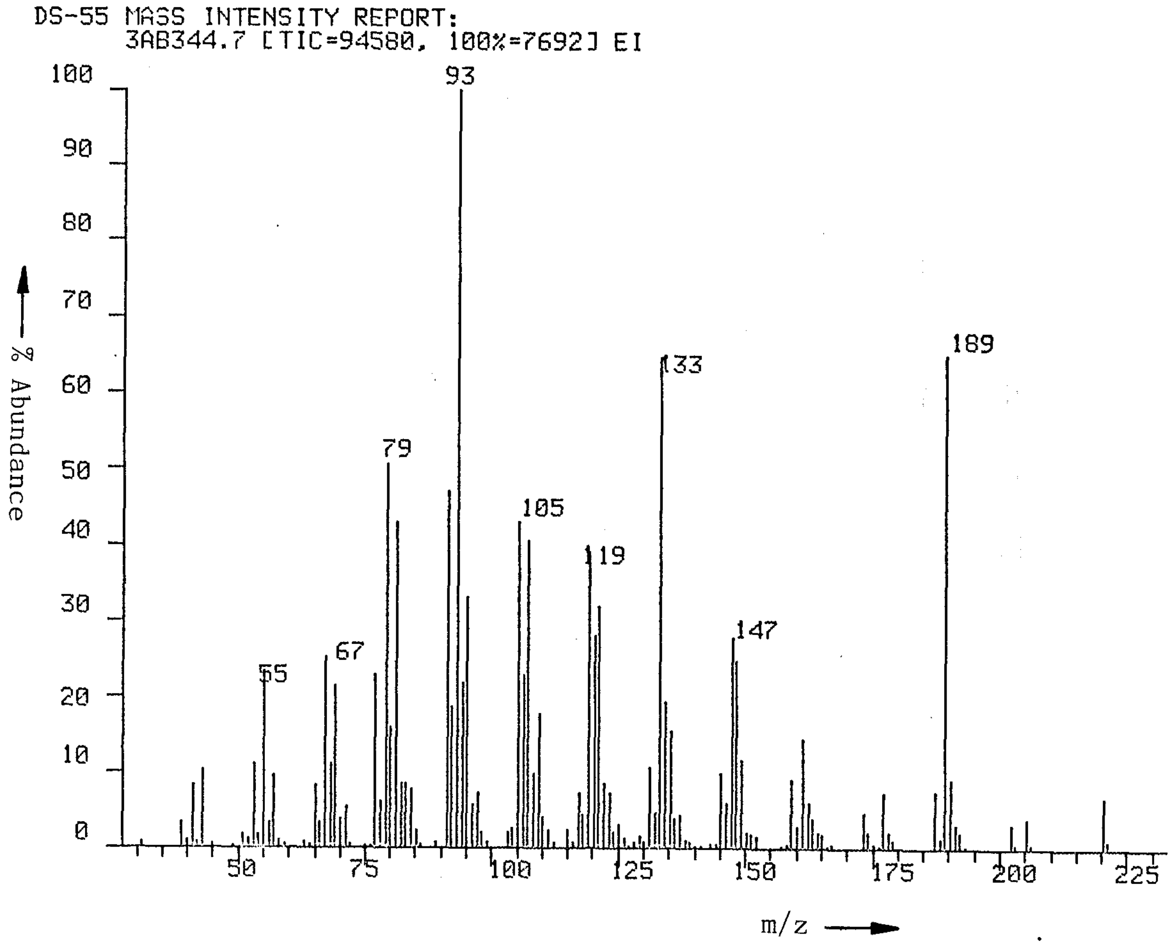
The major sesquiterpenoid was analysed by standard IR, ^{1}H and ^{13}C NMR, and mass spectral techniques (Section 3.2.2). Unfortunately, the pure sesquiterpenoid did not crystallise readily even by slow evaporation from hexane at -20°C in the freezer; any crystals that did form melted below room temperature. Eventually several crystals were obtained by slow evaporation from carbon tetrachloride at 0°C, but these crystals were found to be vitreous by X-ray analysis. Hence the structure of the sesquiterpenoid could not be determined directly by X-ray crystallography.

The major sesquiterpenoid was shown to be biologically inactive singly as an aggregation pheromone, although its activity may be synergised by other components in the pheromone blend (see Section 3.4.5 for bioassay results).

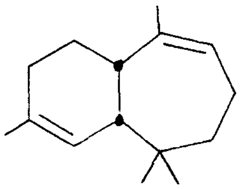
3.2.2 Structural Analysis of the Sesquiterpenoid

High resolution mass spectrometry gave a structural formula of $C_{15}H_{24}O$ for the sesquiterpenoid (M^{+} *m/z* 220.1797; calculated *m/z* 220.1827). The mass spectrum (Figure 7) also indicated a bicyclic,

Figure 7. Electron impact mass spectrum of the *Blatta* sesquiterpenoid.



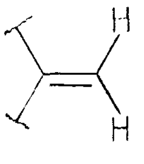
four-rings-plus-double bonds system. The high relative intensity of the fragment ions at m/z 93 (C_7H_9), 133 ($C_{10}H_{13}$) and 119 (C_9H_{11}), combined with the relatively low intensity of the m/z 161 ($C_{12}H_{17}$) ion, indicated a bicyclic carbon skeleton with different numbers of carbon atoms in each ring.[117] The loss of m/z 18 (H_2O) from the molecular ion to give an ion at m/z 202 indicated the likely presence of an alcohol functionality in the natural product, while the prominent ion at m/z 189 (M-31, 70%), presumably due to loss of methanol from the molecular ion, indicated that the unknown was probably a primary alcohol.[47] The mass spectrum of the sesquiterpenoid did not correspond exactly to any of the mass spectra stored on the NIH database (National Institute of Health, Washington D.C., U.S.A.), but closely resembled the mass spectrum of gamma-himachalene (78) previously reported in the literature.[117]



(78)

The infra-red spectrum of the unknown sesquiterpenoid clearly indicated the presence of an exocyclic methylene group (signals at 1635 cm^{-1} and 900 cm^{-1}) and confirmed the likely presence of an hydroxyl moiety (broad signals around 3500 cm^{-1} and 1030 cm^{-1}). The infra-red spectrum also suggested the probable absence of carbonyl, conjugated diene and isopropyl groups.

The ^1H NMR data of the natural product is shown in Table 10 and Figure 8. This data indicates the presence of an exocyclic methylene group (signals at 5.0, 4.9 ppm) (79), a vinyl proton (5.3-5.4 ppm) (80), two protons situated alpha- to an oxygen atom (at 3.4 ppm) and probably in a primary alcohol (81), a methyl group attached to a double bond (1.3-1.4 ppm) (82) and an ordinary methyl group (1.10 ppm).



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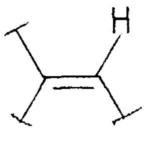
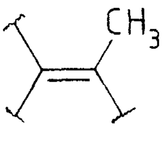


(80)

$-C\underline{H}_2OH$ (81)



(82)

Figure 8. ^1H NMR spectrum of the *B. orientalis* sesquiterpenoid.

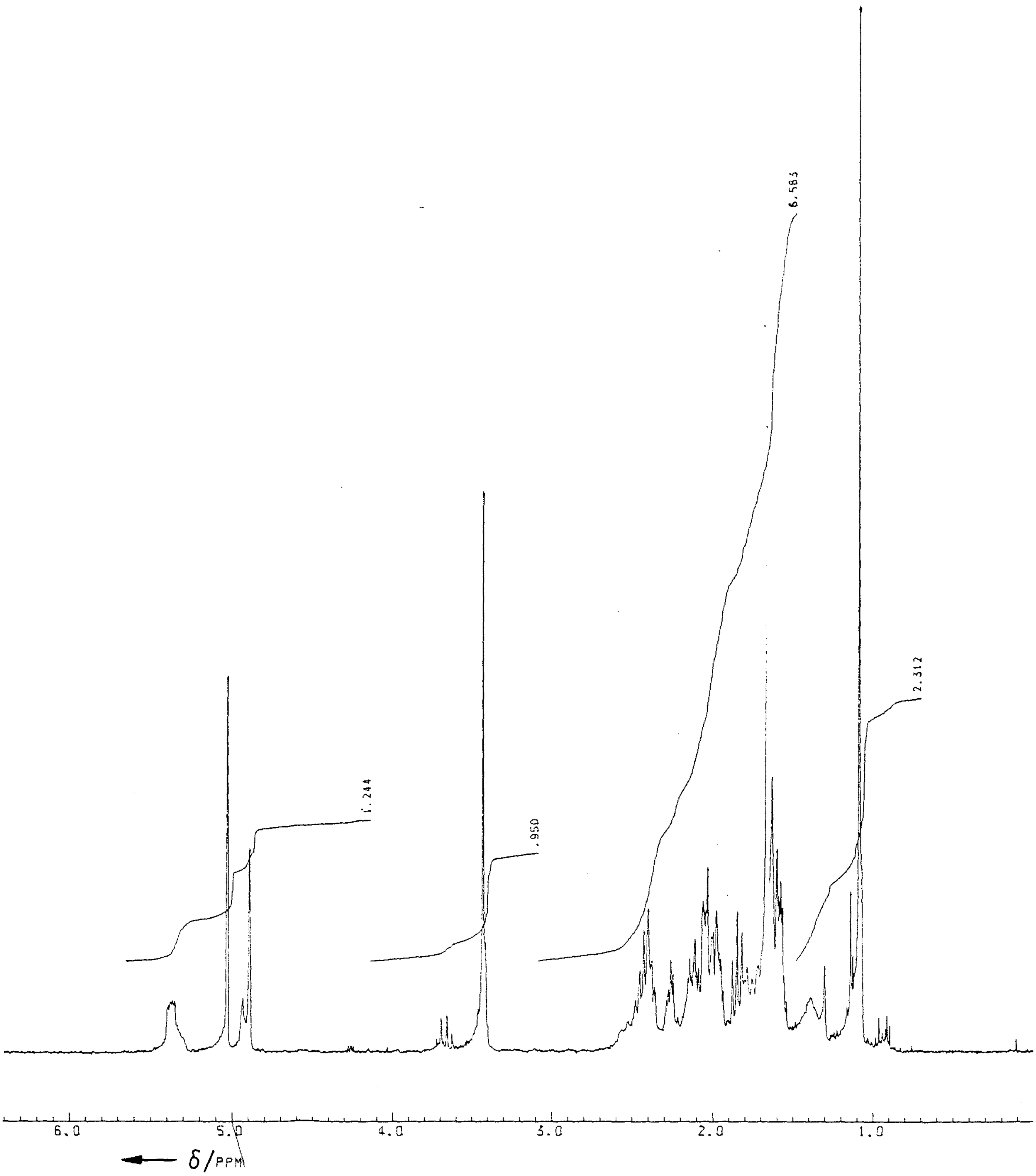


Table 10. ^1H NMR data for the unknown sesquiterpenoid.

δ_H/ppm		Number of protons
5.3-5.4	Vinyl proton	1 (m)
5.0	*Exo*-methylene proton	1 (s)
4.9	" " "	1 (m)
3.4	Protons α- to oxygen	2 (s)
2.6-1.5	C-$C\underline{H}_2$-C protons	13 (m)
1.3-1.4	$C\underline{H}_3C=$	3 (s)
1.10	$C\underline{H}_3$-C	3 (s)

The ^{13}C NMR spectrum was obtained by scanning the sample (10mg) overnight (12h) and the resulting data is shown in Table 11 and Figure 9. The signals at 124.5 and 112.0 ppm are presumably due to double bond carbons (79) and (80) while those at 71.6 ppm confirm the presence of a $-CH_2OH$ group, the signals at 48.5 and 48.0 represent tertiary carbon atoms (probably at a ring junction), while the five signals at 39.9, 35.1, 35.0, 30.0 and 28.1 ppm represent five secondary carbon atoms, and the final two signals at 17.9 and 16.3 ppm are due to two methyl groups. Only 12 signals were observed in the ^{13}C NMR spectrum, as insufficient material was available to detect the three quaternary carbon atoms.

Figure 9. ^{13}C NMR spectrum of the *B. orientalis* sesquiterpenoid.

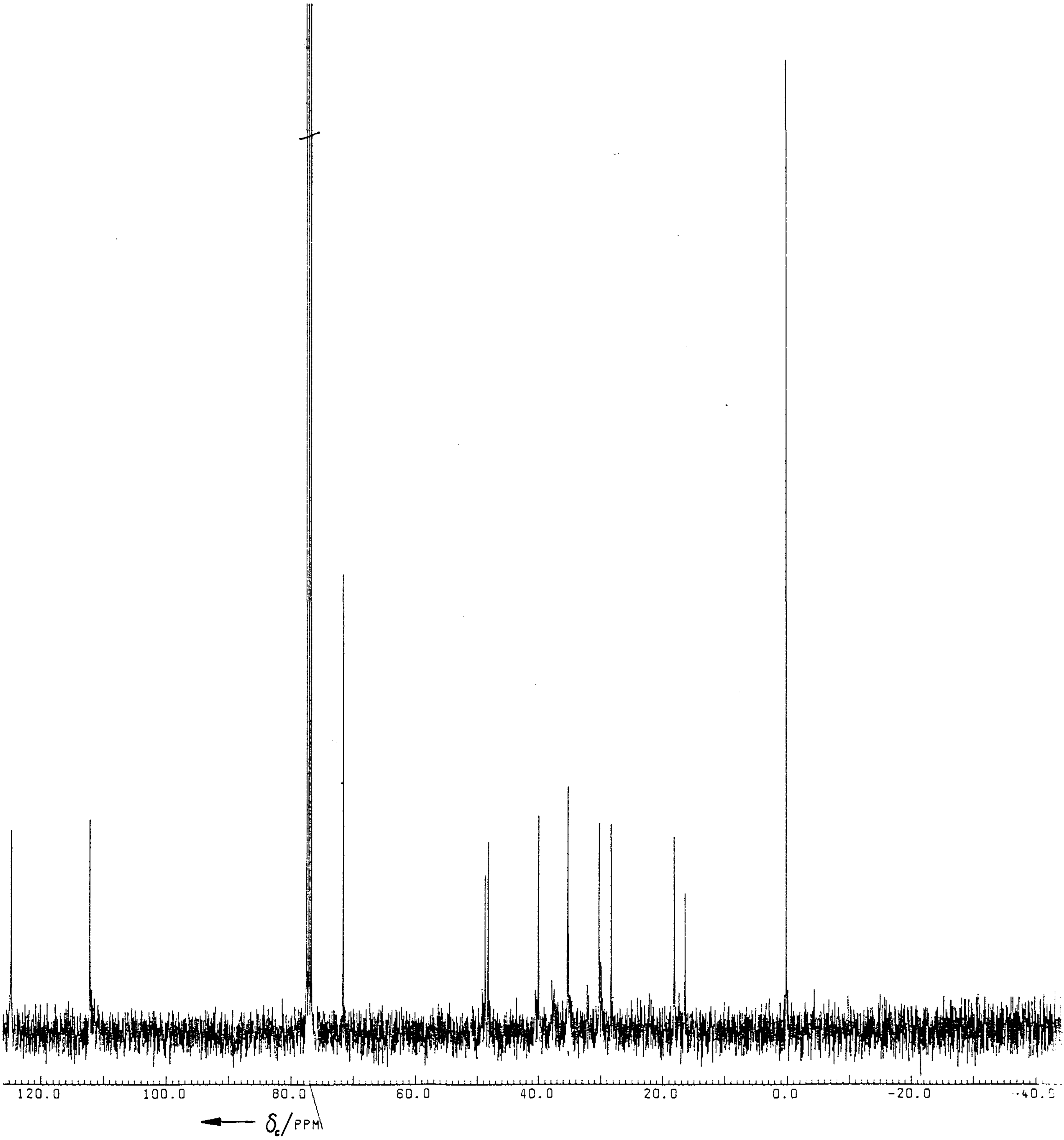


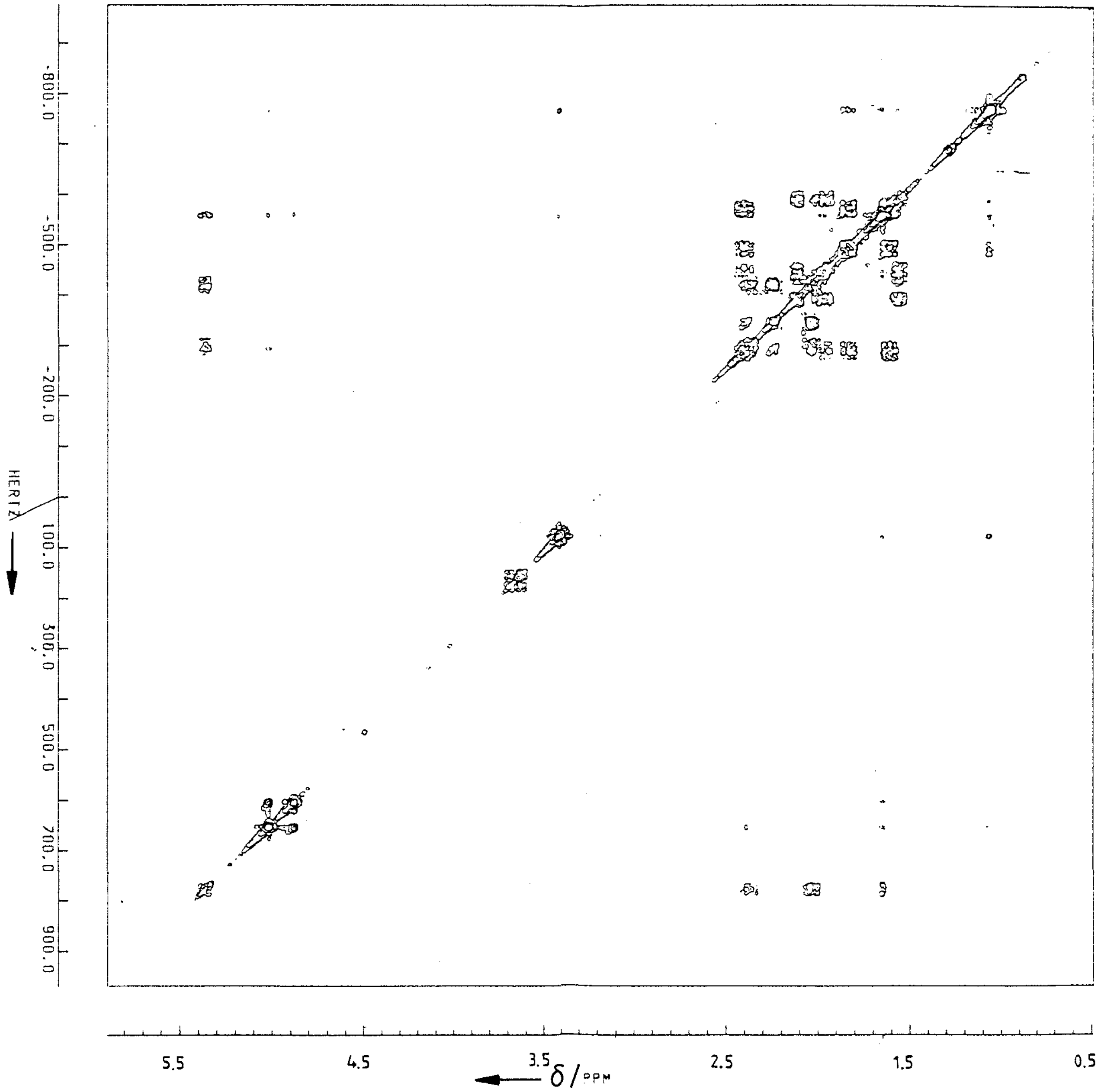
Table 11. ^{13}C NMR data for the unknown sesquiterpenoid.

δ_C/ppm	Inference
124.51 112.00	Double bond carbons (2)
71.58	$-CH_2OH$ carbon
48.52 48.04	Tertiary carbons (probably at a ring junction) (2), $-\overset{\backslash}{\underset{/}{C}}H$
39.86 35.13 35.05 30.05 28.14	Secondary carbons (5), $\overset{\backslash}{\underset{/}{C}}H_2$
17.98 16.28	Primary carbons, methyl groups (2), CH_3-

The chemical structure of the *Blatta* sesquiterpenoid was investigated further by means of Proton Homonuclear Correlation ^1H NMR Spectroscopy (COSY) (see Figure 10 and Table 12). From this data it may be deduced that the double bond proton at δ 5.35 is coupled to the signals at δ 1.68, 2.04 and 2.40 in the $C-C\underline{H}_2-C$ region of the spectrum. Similarly, the exocyclic methylene proton at δ 5.00 is coupled to the signals at δ 1.65 and 1.90, while the protons α- to the hydroxy group are coupled to the methylene protons at δ 1.68 and also to the methyl protons at δ 1.10. The couplings in the region from δ 2.40 to δ 1.54 are shown in Table 12, but it appears that little additional structural information can be deduced from this data.

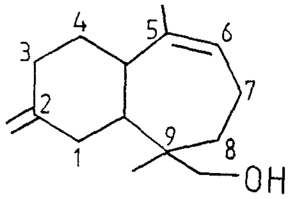
The J-resolved ^1H NMR data is shown in Figure 11 and Table 13. The signals at δ 2.62 and δ 1.83 appear as triplets, those at δ 1.65 and δ 1.46 as doublets, while the signals at δ 1.93 and δ 1.96 may be tentatively assigned as doublets of double doublets. The $C-C\underline{H}_2-C$ region (δ 1.5 to δ 2.5) of the sesquiterpenoid ^1H NMR spectrum contains a number of complex multiplets which are difficult to assign, even after J-resolved ^1H NMR analysis.

Figure 10. COSY ^1H NMR spectrum of the sesquiterpenoid isolated from faeces of *B. orientalis*.



The ^1H NOE (Nuclear Overhauser Effect) difference spectrum showed no conclusive evidence of coupling between the methyl groups at δ 1.05 and the protons α- to oxygen (i.e. $C\underline{H_2}OH$).

In conclusion, the COSY and J-resolved ^1H NMR data of the *Blatta* sesquiterpenoid appear compatible with the novel isoprenoid structure (83); 5,9-dimethyl-2-methylidene-9-hydroxymethyl-3,4,4a,7,8,9a-hexahydro-1H-benzocycloheptene. A comparison of the spectroscopic data for (83) can be made with the three known structurally-related himachalene stereoisomers.



(83)

Table 12. COSY ^1H NMR Data for the unknown sesquiterpenoid (see Figure 10).

Proton Chemical shift/δ	Chemical Shifts of Coupled Protons/δ					
1.54	1.96,	2.12				
1.63	1.83,	2.40				
1.68	5.35					
1.83	1.60,	2.40				
1.96	1.54,	2.40				
2.04	2.25,	2.40,	5.35			
2.25	2.04,	2.40				
2.40	1.60,	1.83,	1.96,	2.04,	2.25,	5.35
3.40	1.10,	1.68				
5.00	1.65,	1.90				
5.35	1.68,	2.04,	2.40			

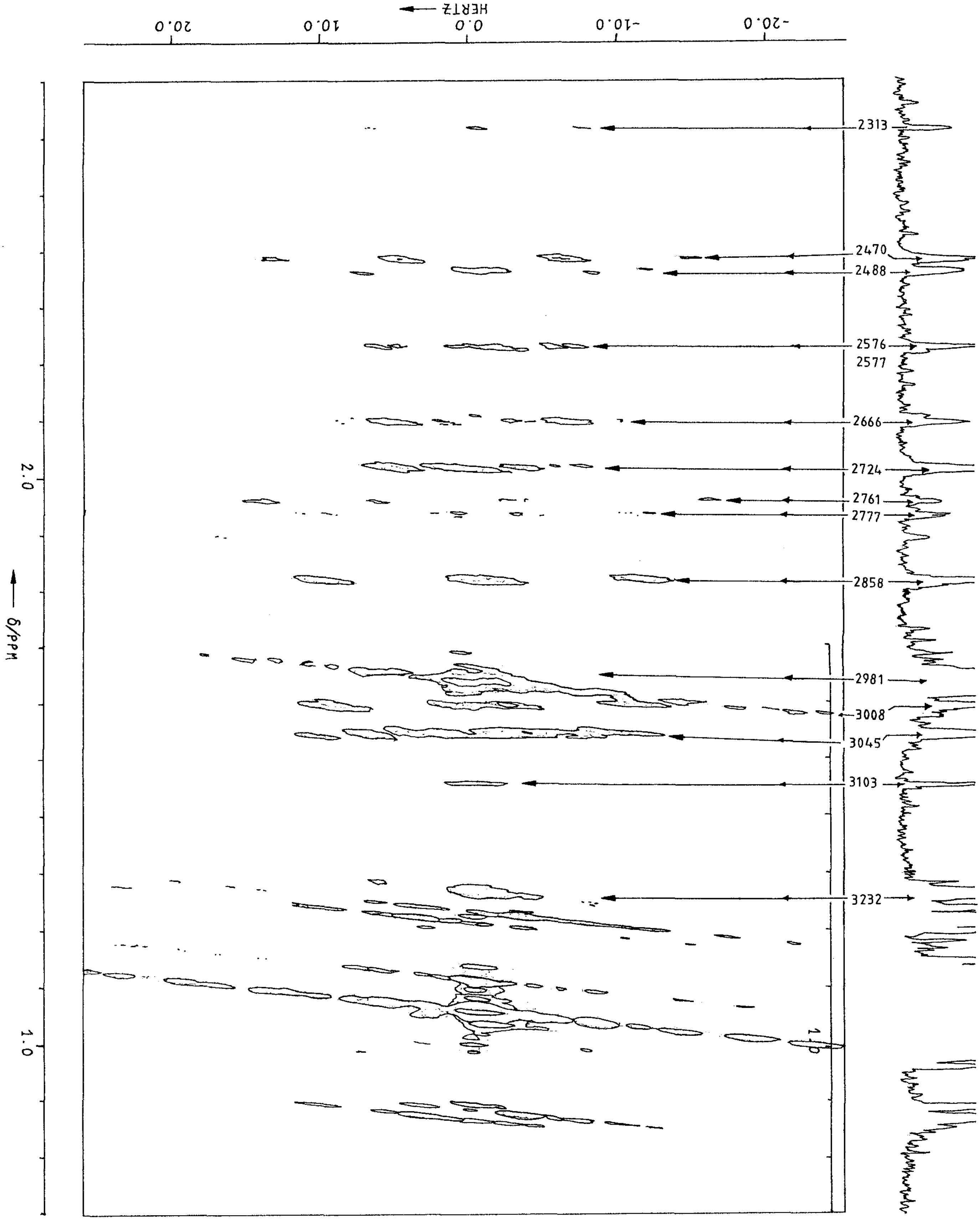
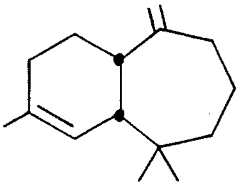


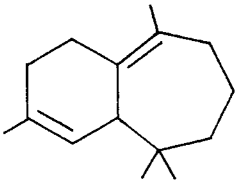
Figure 11. J-resolved ^{1}H NMR spectrum of the <u>Blatta</u> sesquiterpenoid.

The ^{1}H NMR spectrum of a synthetic sample of racemic alpha-himachalene (84) (kindly provided by Professor W. Oppolzer, Département de Chimie Organique, Université de Genève, Genève, Switzerland)[118] appeared to be almost identical to the proton NMR spectrum of the major sesquiterpenoid isolated from the faeces of B. orientalis. The ^{1}H NMR spectrum of the authentic sample of racemic alpha-himachalene (84) showed two sharp singlets due to two discrete methyl groups at 1.0 and 1.1 ppm, an additional vinylic methyl group at a chemical shift of 1.4 ppm, twelve methylene protons in the region between 1.1 and 2.4 ppm, two exocyclic methylene protons at 4.8-5.0 ppm (complex multiplet) and one vinylic proton at 5.4 ppm (broad singlet).[119] The only apparent difference between the two NMR spectra concerned the -C$\underline{H}_2$OH singlet observed for the sesquiterpenoid at a chemical shift of 3.4 ppm and the corresponding extra methyl group in the case of the synthetic alpha-himachalene at 1.10 ppm.



(84)

The proton NMR spectrum of racemic beta-himachalene (85)[119] and racemic gamma-himachalene (78)[117] reported in the literature did not show the exocyclic methylene signals in the region between 4.9 and 5.0 ppm characteristic of the natural product, and were thus distinctively different from the ^{1}H NMR spectra of either racemic alpha-himachalene (84) or the Blatta sesquiterpenoid.



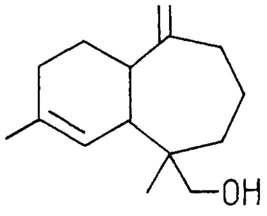
(85)

The ^{13}C NMR spectra of racemic alpha- and beta-himachalenes have not been reported in the literature, however, the ^{13}C NMR spectrum of racemic gamma-himachalene (78)[117] has been recorded and seemed to be essentially similar to that of the natural product from Blatta orientalis, especially as carbon-13 chemical shift values are known to depend appreciably on the conformation of the molecule involved.[120]

The ^{13}C literature NMR data for natural gamma-himachalene[117] obtained from the essential oil of Turkish aniseed, Pimpinella anisum L. (= Anisum vulgare Gartner), showed signals at 138.0, 134.0, 125.4, 47.5, 43.0, 39.5, 36.5, 30.4, 29.5, 29.0, 26.2, 25.4, 24.6, 24.3 and 23.8 ppm; this literature data was recorded using a Varian CFT-20 (20 MHz) instrument.

Both the mass spectral and infra-red data for the three known himachalene stereoisomers are very similar and bear a strong resemblance to the corresponding data obtained for the natural sesquiterpenoid, particularly the prominent ions at m/z 189 ($M-CH_3OH$), 161 ($C_{12}H_{17}$), 133 ($C_{10}H_{13}$), 119 (C_9H_{11}) and 93 (C_7H_9) in the mass spectrum.

Structure (83) has been proposed for the primary sesquiterpene alcohol isolated from the faeces of B. orientalis on the basis of a comparison between the available ^{13}C and ^{1}H NMR spectra of alpha-himachalene (84), gamma-himachalene (78) and the Blatta sesquiterpenoid. A structure based on either the alpha- or gamma-himachalene skeletons should be theoretically possible from the available spectral information, but structure (83) was preferred to structure (86) on the strength of the marked similarity between the ^{13}C NMR data previously recorded for gamma-himachalene[117] and the corresponding data for the Blatta sesquiterpenoid.



(86)

Table 13. J-Resolved ^1H NMR Data for the unknown sesquiterpenoid (see Figure 11).

Scan Number	Multiplicity (probable)	Chemical shift/δ
2313	t	2.62
2761	ddd	1.96
2777	ddd	1.93
2858	t	1.83
2981	d	1.65
3105	d	1.46

3.2.3 Derivatisation and X-Ray Analysis

In order to assign an unambiguous structure to the *Blatta* sesquiterpenoid it was decided to attempt to form a crystal for X-ray analysis. Since it was not possible to crystallise the pure sesquiterpenoid, it was decided to derivatise the natural product before attempting crystallisation.

The (*R*)(+)-10-camphorsulphonate derivative was readily formed and crystallised easily. However, none of the resulting crystals were suitable for X-ray analysis. Moreover, the (*R*)(+)-10-camphorsulphonate decomposed within two weeks (half-life *ca.* one week) to afford an unidentifiable hydrocarbon polymer, presumably *via* initial elimination of (*R*)-(+)-10-camphorsulphonic acid followed by extensive Wagner-Meerwein skeletal rearrangement.

The 3,5-dinitrobenzoate appeared relatively stable and crystallised from hexane at -20°C. Unfortunately, again the crystals proved unsatisfactory for X-ray analysis.

Inspired by the work of Butenandt *et al* on the sex pheromone system of the silkworm moth, *Bombyx mori*,[121] it was decided to prepare the 4(4'-nitrophenylazo)benzoate derivative. This derivative was readily prepared, but, despite many attempts, did not crystallise in a suitable form.

It seems likely that the sesquiterpenoid derivatives prepared during this study would not pack together in a non-vitreous crystalline form due to the molecular shape of the derivatives themselves. The one remaining option for a structural proof of the *B. orientalis* sesquiterpenoid is to resort to total synthesis followed by spectroscopic comparison of the natural and synthetic products.

3.2.4 Filter Paper Extract

Filter paper (Whatman No. 1), which had been used to line the living quarters of mixed male and female *B. orientalis*, was washed with solvent (100ml) and the resulting extract was concentrated and analysed by GC and GC/MS (see Figures 12 and 13, Tables 14 and 15).

Behavioural bioassay using the method of Ishii and Kuwahara[122,123] (Section 3.4.4) showed that both the excrement-soiled filter paper and the methanol extract elicited strong aggregation responses from mixed male and female *B. orientalis* of all ages, but especially in the case of young nymphs (first and second instars) (Section 3.4.5). Dichloromethane did not extract the aggregation-active principle from the filter paper, indicating that the aggregation pheromone probably contains relatively polar components.

The aggregation response decreased rapidly with time (half-life *ca.* one week), indicating that the aggregation pheromone is probably unstable (c.f. periplanone A).

The compounds extracted were identified by comparison of their mass spectral fragmentation patterns with those of known compounds and included a variety of branched and straight-chain alkanes in the range C_{14} to C_{30}, together with several compounds which could not be identified on the basis of their mass spectra alone. The *Blatta* sesquiterpenoid was significantly absent from the filter paper extract.

Treatment of the active filter paper extract with diazomethane greatly reduced the biological activity (see Section 3.4.5). Thus, it appears that carboxylic acids may play an important role in the aggregation of *B. orientalis*. Ritter and Persoons[90] also concluded that the aggregation of *B. germanica* is partially due to certain free carboxylic acids present in the faeces, but the activity could not be attributed to any single compound. A synthetic mixture of the compounds identified, pooled in the same ratio as they occur in the

Figure 12. Capillary GC/MS analysis of fresh *Blatta* filter paper extract (25m BP5 column, 50,2-8-275°C, 1µl splitless injection).

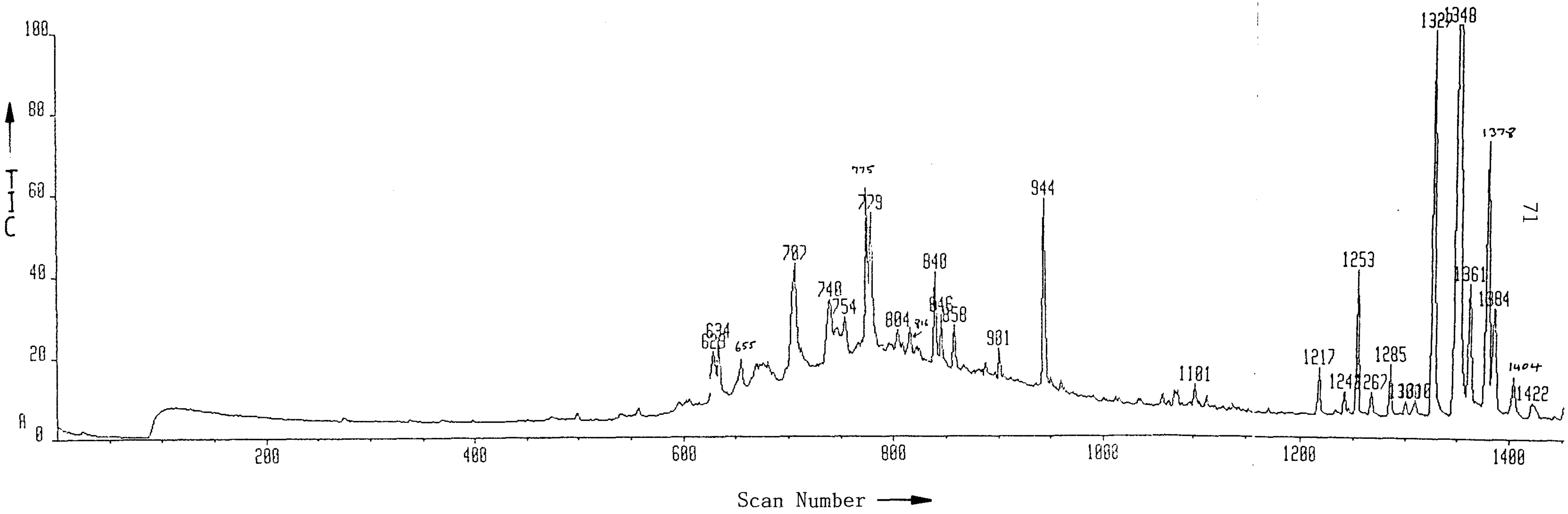
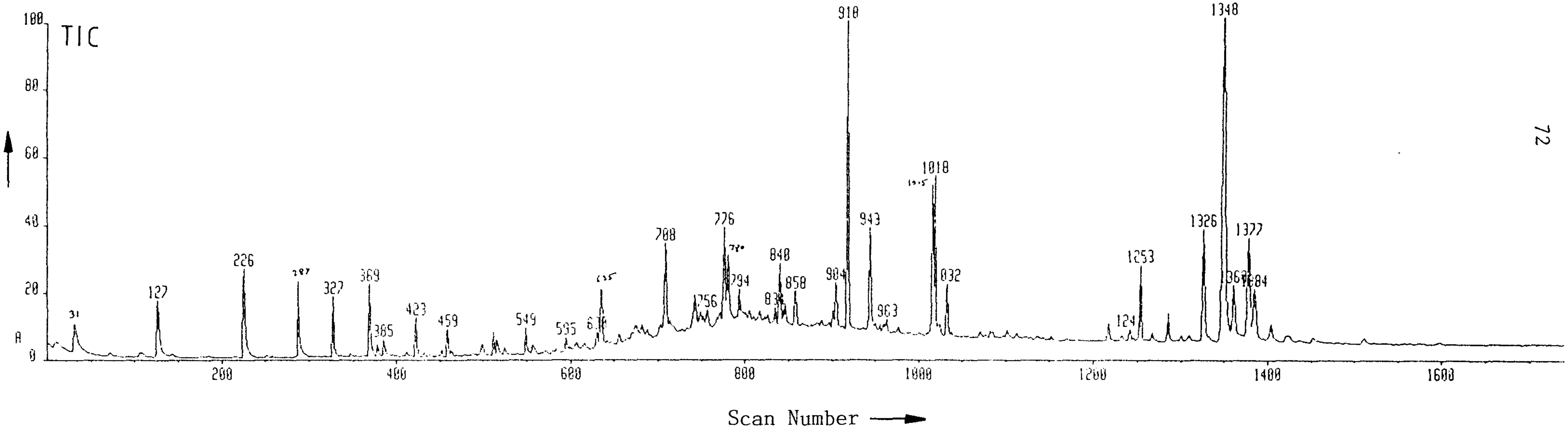


Figure 13. Capillary GC/MS analysis of fresh *Blatta* filter paper extract after addition of diazomethane (25m BP5 column, 50,2-8-275°C, 1µl splitless injection).



faeces, possessed about 50% of the activity of a *B. germanica* dichloromethane faeces extract.[88] Tsuji has attributed feeding-stimulant activity to palmitic, linoleic, stearic and oleic acids for starved *B. germanica*, *P. americana* and *P. fuliginosa*.[97]

After treatment with diazomethane the *B. orientalis* filter paper extract was analysed by GC and GC/MS, which showed that the following carboxylic acids were probably present in the aggregation-active extract: benzoic, cinnamic, hexadecenoic, linoleic, oleic, palmitic, pentadecanoic, phenylacetic and stearic, in addition to a variety of alkanes in the range C_{12} to C_{30}.[46] All these acids were bioassayed both singly and in the same ratio as they occur in the faeces, but no significant biological activity was observed.

The crude filter paper extract was purified by flash column chromatography, preparative GC and preparative HPLC, but, unfortunately, each of these procedures resulted in a complete loss of biological activity. None of the fractions obtained by these methods elicited any aggregation response, either singly or in combination with other fractions.

It is noteworthy that neither "fresh" faeces (from BASF, Limburgerhof, West Germany) on filter paper nor a solvent extract of these faeces ever elicited an aggregation response from *B. orientalis* in Southampton, possibly because the aggregation pheromone had decomposed in transit.

3.3 Conclusion

The sesquiterpenoid suggested by Neequaye[89] as an aggregation pheromone was isolated using an improved procedure and shown to be biologically inactive. The compound was identified as a primary sesquiterpene alcohol on the basis of ^{13}C NMR, ^{1}H NMR, J-resolved ^{1}H NMR, COSY ^{1}H NMR, IR and mass spectra. The optical rotation was also recorded. A tentative structure for the sesquiterpenoid (83) has been proposed.

It appears that the *B. orientalis* aggregation pheromone is unstable under normal conditions (half-life *ca.* one week), but is likely to comprise a complex mixture in which certain carboxylic acids appear to play an important role.

3.4 Experimental

3.4.1 Instrumentation

See Section 2.4 for details of GC, GC/MS, HPLC, IR and NMR analysis together with General Procedures.

An all-glass splitter system similar to that described by Baker *et al*[124] was used for preparative GC. The splitter was connected to the GC column with a 6mm stainless steel Swagelok union bored out to 7mm and fitted with graphite ferrules (SGE, Melbourne, Australia). A trapping system similar to that recently described by Morgan and Attygalle[125] was employed. The splitter outlet was maintained at a temperature 10-20°C above that of the column oven using a Pye-Unicam injection port heater. A fresh capillary tube (6cm x 4mm i.d.) was used for each fraction collected.

3.4.2 Materials and Methods

3,5-Dinitrobenzoyl chloride, (R)-(+)-10-camphorsulphonyl chloride, 4,4'-dimethylaminopyridine and 4(4'-nitrophenylazo)benzoyl chloride were all of derivatisation grade and were obtained from Aldrich Limited (Gillingham, Dorset). The carboxylic acids for behavioural bioassay were either obtained from Aldrich Limited or from Sigma Chemicals Limited (Poole, Dorset). Derivatisation grade pyridine was obtained from Regis Phase Separations Limited (Queensferry, Clwyd).

Diazomethane was generated as described in Section 4.4 and an excess of this reagent was added to fresh filter paper extract (*ca.* 500µl) in a microvial. The mixture was left to stand at room temperature (2h), concentrated by evaporation under a stream of dry nitrogen to *ca.* 100µl, and the mixture analysed by GC and GC/MS as previously described.

X-ray analysis was kindly performed by Dr. Keith Prout at the Chemical Crystallography Laboratory, Oxford University, using an Enraf-Nonius CAD4 diffractometer with Cu-K_α radiation and ω-2θ scans ($2\theta_{max}$ = 140°). GC/MS analysis of the filter paper extract was carried out by Dr. Robin Black at the Chemical Defence Establishment, Porton Down, Salisbury, Wiltshire. COSY, NOE and J-resolved ^{1}H NMR analysis was kindly undertaken by Dr. J.A. Robinson on the Bruker AM-360 spectrometer at Southampton University.

I would like to thank Drs. Wolfgang Krieg and Heinrich Adolphi of BASF Aktiengesellschaft, Limburgerhof, West Germany, for providing copious quantities of eggs and faeces from *B. orientalis* and also for their help and advice during the course of this project.

Filter Paper Extracts

Strips of filter paper (Whatman No. 1) were folded and placed in a cage (aluminium, 30cm x 30cm x 40cm) containing *ca.* 250 *Blatta orientalis* of mixed age and sex in a controlled environment room (25 ± 2°C, 70 ± 5% relative humidity, 12 hour light cycle at a light intensity of 1000 lx). The culture was initiated with eggs (100) from *B. orientalis* kept by BASF, Limburgerhof, West Germany. The cockroaches were provided with plenty of food (bran) and water. Excrement-soiled filter paper was removed after 14 days and suspended overnight in dichloromethane or methanol (100ml). The extract obtained was filtered through glass wool, concentrated and analysed by GC and GC/MS.

Faecal Extracts

(i) Collection (BASF, West Germany)

Twenty thousand cockroaches (50:50, male:female) were housed in plastic boxes (40 x 30 x 25 cm) and provided with water and food [wheat flour (3): fish meal (3): powdered sugar (1): dry yeast (1): skimmed milk powder (1) and mashed carrots]; the temperature was 25±2°C and the relative humidity 60 ± 5%. The boxes had a screen bottom through which the faeces fell and were collected (*ca.* 0.6kg every 3 days).

(ii) Isolation of the Sesquiterpenoid

A batch of faeces (5kg) was suspended in dichloromethane (5l) for 48 hour periods with occasional stirring. The dark brown extracts were decanted, filtered through glass wool, and concentrated to give a gummy residue. Methanol (500ml) was added and immediate precipitation of methanol-insoluble compounds occurred (fats and lipids, etc.). The extract was filtered (Whatman No. 1 filter paper), and the methanol-soluble fraction was stored at 0°C overnight. Further precipitation occurred and repeated precipitation at 0°C and filtration (three times) afforded a fraction which was then concentrated (30ml) and redissolved in petroleum ether (100ml).

This solution was applied to TLC silica (50g) packed into a column (5cm x 7cm O.D.). This dry flash column was eluted with petroleum ether (100ml), 10% ethyl acetate in petroleum ether (200ml), and finally 50% ethyl acetate-petroleum ether (200ml). TLC analysis using petroleum ether-ether (5:3) as eluant showed that the desired sesquiterpenoid was eluted by 10% ethyl acetate-petroleum ether.

The 10% ethyl acetate-petroleum ether fraction was concentrated and applied to a Sephadex LH20 column (50g) equilibrated with dichloromethane. Elution with dichloromethane collecting 50ml fractions with TLC analysis showed that the sesquiterpenoid occurred in fraction 6. Dry flash column chromatography of fraction 6 using a gradient elution (pure petroleum ether to pure ether, 10% steps) afforded the desired sesquiterpenoid (30mg) eluted with 30% ether-petroleum ether. The sesquiterpenoid fraction was purified by preparative HPLC using a Zorbax ODS reversed phase column, refractive index detection, a flowrate of 1.2ml min^{-1} and 20% water in methanol as solvent. The pure sesquiterpenoid (10mg) was partitioned from the methanol-water eluting solvent with pentane (5 x 10ml). High resolution mass spectrometry gave $C_{15}H_{24}O$ for *m/z* 220.1797 (calculated *m/z* 220.1827). $[\alpha]_D^{22}$ + 12° (c, 1.5 in $CHCl_3$); ν_{max} (thin film) 3500 (OH), 1635 ($C=CH_2$ exocyclic), 1030 (primary OH) and 900 ($C=CH_2$ exocyclic); δ_H (360 MHz, $CDCl_3$) 5.3 (1H, m, $C=C\underline{H}$), 5.0 (1H, s, exocyclic $C=C\underline{H_2}$), 4.9 (1H, s, exocyclic $C=C\underline{H_2}$), 3.4 (2H, s, $\underline{CH_2}OH$), 2.6-1.5 (13H, m, $C-C\underline{H_2}-C$), 1.3-1.4 (3H, s, $\underline{CH_3}C=$) and 1.10 (3H, s, $\underline{CH_3}-C$); δ_C (25.15 MHz, $CDCl_3$) 124.51, 112.00, 71.58, 48.52, 48.04, 39.86, 35.13, 35.05, 30.05, 28.14, 17.98 and 16.28; *m/z* 220 ($M^{\oplus}$, 7%), 202 (M-18, 4), 189 (M-31, 65), 161(15), 147(30), 133(65), 119(40), 105(40), 93(100), 79(50), 67(25) and 55(25). The GC retention time of the sesquiterpenoid was 13.2 minutes (5% FFAP, 100-12-240°C) and 10.2 minutes (5% OV101, 100-12-290°C).

Derivatisation of the Sesquiterpenoid

(i) (*R*)-(+)-10-Camphorsulphonate

A solution of the sesquiterpenoid (<u>83</u>) (10mg, 45 mmol) in pyridine (2ml) was treated with(*R*)(+)-10-camphorsulphonyl chloride (15mg) and stirred at room temperature for 4h, when GC analysis (5% OV101, 150-8-300°C) showed that the reaction was complete. The solution was poured into cold water and extracted with dichloromethane. The extract was washed with aqueous copper (II) sulphate and water, dried ($MgSO_4$) and evaporated to dryness. Microscale chromatography on Florisil with first dichloromethane (5ml) and then methanol (5ml) as eluant yielded the desired(R)(+)-10-camphorsulphonate derivative (15mg, 77%) from the dichloromethane layer, m.p. 52-53°C (from pentane). High resolution mass spectrometry gave $C_{25}H_{38}SO_4$ for *m/z* 434.2470 (calc. *m/z* = 434.2496); δ_H (360 MHz, $CDCl_3$) 5.3 (1H, m, C=C$\underline{H}$), 5.0 (1H, m, C=C$\underline{H_2}$ exocyclic), 4.9 (1H, m, C=C$\underline{H_2}$ exocyclic), 3.9-4.1 (2H, m, -C$\underline{H_2}$$SO_2$-), 3.5 (2H, 2s, -C$\underline{H_2}$$OSO_2$-), 1.3-2.6 (19H, complex, C-C$\underline{H_2}$-C), 1.2 (3H, s, C$\underline{H_3}$C=) and 1.1 (9H, m, C$\underline{H_3}$-C); *m/z* 434 ($M^{\oplus}$, 2%), 280(3), 233(10), 202(45), 133(50), 109(50), 93(75), 81(75), 55(85) and 41(100).

The(R)(+)-10-camphorsulphonate ester eliminated(R)-(+)-10-camphorsulphonic acid on standing (10 days) to afford an unidentified hydrocarbon polymer.

(ii) 3,5-Dinitrobenzoate

A solution of the sesquiterpenoid (83) (5mg, 23 mmol) in pyridine (2ml) was treated with fresh 3,5-dinitrobenzoyl chloride (10mg, 43 mmol) in the presence of 4,4'-dimethylaminopyridine (5mg) and stirred at room temperature overnight (12h). The reaction mixture was taken up in dichloromethane (10ml) and washed with water, aqueous hydrochloric acid (0.1 mol dm^{-3}), aqueous copper (II) sulphate and water again, dried ($MgSO_4$) and evaporated to dryness. HPLC on a Zorbax ODS reversed phase column with 10% water in acetonitrile as solvent and UV detection at 254nm afforded the desired 3,5-dinitrobenzoate (8mg, 83%), m.p. 62-64°C (from hexane). High resolution mass spectrometry gave $C_7H_3N_2O_5$ for *m/z* 194.9931 (calc. *m/z* 195.0042); δ_H (360 MHz, $CDCl_3$) 1.1 (3H, s, C$\underline{H_3}$-C), 1.2 (3H, s, C$\underline{H_3}$C=), 1.5-2.6 (12H, complex, C-C$\underline{H_2}$-C), 4.2 (2H, m, -C$\underline{H_2}$OCO-), 4.9 (1H, m, C=C$\underline{H_2}$ exocyclic), 5.0 (1H, m, C=C$\underline{H_2}$ exocyclic), 5.3 (1H, s, C=C$\underline{H}$) and 9.2 (3H, m, aromatic ring); *m/z* 414 ($M^{\oplus}$, not observed), 211 (10%), 195(10), 189(10), 149(30), 133(50), 119(30), 107(30), 105(35), 93(100), 91(65), 69(30), 55(75) and 41(70). The 3,5-dinitrobenzoate was recrystallised from hexane (200µl) in a 5ml round-bottomed flask at -20°C (3 weeks).

(iii) 4(4'-Nitrophenylazo)benzoate

A solution of the sesquiterpenoid (83) (10mg, 45 mmol) in pyridine (2ml) was treated with 4(4'-nitrophenylazo)benzoyl chloride (20mg, 69 mmol) as previously described. HPLC (Zorbax ODS, 10% water in acetonitrile, 254nm detection) afforded the desired 4(4'-nitrophenylazo)-benzoate (20mg, 94%), m.p. 73-75°C (from acetonitrile). High resolution mass spectrometry gave $C_{28}H_{31}N_3O_4$ for *m/z* 473.2332 (calc. *m/z* 473.2314; δ_H (360 MHz, $CDCl_3$) 1.1 (3H, s, $C\underline{H}_3$-C), 1.2 (3H, s, $C\underline{H}_3C$=), 1.5-2.6 (12H, complex, C-$C\underline{H}_2$-C), 4.3 (2H, m, -$C\underline{H}_2OCO$-), 4.9 (1H, m, C=$C\underline{H}_2$ exocyclic), 5.0 (1H, m, C=$C\underline{H}_2$ exocyclic), 5.3 (1H, s, C=C$\underline{H}$), 7.45 (2H, m, aromatic ring), 7.6 (2H, m, aromatic ring), 8.0 (2H, m, aromatic ring) and 8.15 (2H, m, aromatic ring); *m/z* 473 ($M^{\oplus}$, 1%), 255(15), 254(90), 224(41), 202(15), 189(13), 187(34), 161(27), 147(25), 133(40), 120(90), 119(42), 93(100), 91(60), 55(60), 43(50) and 41(60).

3.4.3 Capillary GC/MS Data

Capillary GC/MS analysis was kindly performed by Dr. R.M. Black at the Chemical Defence Establishment, Porton Down, using a VG 7070 instrument. The ionizing conditions were as described previously (Section 2.4).

Table 14. Analysis of the *B. orientalis* filter paper extract after addition of excess diazomethane (BP5, 24m column, 50,2-8-275°C, 1μl splitless injection).

Scan	Compound	*m/z* (r.a., %)	$M^{\oplus}$(%)
31	C_4H_7Cl	77(50), 55(100), 49(20), 41(100).	90(40)
127	C_5H_9Cl	91(30), 75(10), 69(60), 63(10), 55(100), 49(10), 42(30), 41(30).	104(5)
226	Unknown	82(20), 76(20), 69(100), 63(5), 56(20), 49(10), 41(60).	?
287	Methyl benzoate	136(30), 105(100), 77(60), 52(20).	136(30)
327	$C_6H_{12}Cl_2$	141(30), 139(50), 103(40), 90(10), 83(85), 75(20), 67(20), 55(100).	154(-)?
369	Methyl phenylacetate	91(100), 65(15), 59(5), 39(5).	150(-)

Table 14 (cont'd.)

Scan	Compound	*m/z* (r.a., %)				$M^{\oplus}$(%)
423	$C_7H_{14}Cl_2$	141(40), 97(40), 55(100),	139(60), 91(10), 43(100).	116(10), 81(15),	103(40), 67(20),	168(-)?
459	Methyl cinnamate	133(10), 65(10),	104(100), 51(10).	97(70),	77(20),	164(30)
549	Unknown, possibly an amine	143(20),	70(10),	58(100),	56(40).	171(80)?
635	Branched C_{12} alkane	147(30), 71(50),	99(5), 57(100),	85(30), 43(85).	73(70),	168(-)
708	*n*-C_{14} alkane	99(5), 43(90).	85(30),	71(50),	57(100),	198(1)
776	*n*-C_{16} alkane	99(5), 43(85).	85(40),	71(60),	57(100),	226(2)
780	Branched C_{16} alkane	127(5), 71(70),	113(10), 57(100),	97(10), 43(55)	85(20),	226(1)
840	*n*-C_{18} alkane	99(5) 71(60),	87(20), 57(100),	85(35), 43(80).	73(30),	254(2)
858	Methyl pentadecanoate	233(60), 101(10), 69(20),	221(15), 97(60), 55(20),	147(20), 74(100), 43(65).	129(10), 73(75)	256(5)
904	Methyl hexadecenoate	236(10), 123(15), 81(45),	194(10), 110(20), 74(80),	152(10), 97(30), 69(60),	137(10), 97(40), 55(100).	268(2)
918	Methyl palmitate (methyl hexadecanoate)	227(5), 69(10),	143(10), 55(20),	87(60), 43(30).	74(100),	270(2)
943	Di-*n*-butyl Phthalate ester	149(100), 41(20).	104(5),	76(5),	57(5),	278(-)
1015	Methyl linoleate? (methyl Z,Z-9,12-octadecadienoate)	123(10), 67(100),	109(20), 55(70),	95(40), 41(70).	81(70),	294(2)
1018	Methyl oleate? (methyl Z-9-octadecenoate)	264(10), 123(10), 83(40),	222(10), 111(15), 74(65),	180(10), 97(30), 69(70),	137(10), 97(40), 55(100).	296(2)

Table 14 (cont'd.)

Scan	Compound	*m/z* (r.a., %)	$M^{\oplus}$(%)
1032	Methyl stearate (methyl octadecanoate)	255(10), 199(5), 143(15), 129(5), 87(70), 74(100), 69(10), 55(20).	298(2)
1253	n-C_{24} alkane	337(5), 127(5), 113(10), 99(10), 85(30), 71(50), 57(100), 43(60).	338(2)
1326	n-C_{26} alkane	113(10), 99(10), 85(40), 71(60), 57(100), 43(75).	366(1)
1348	n-C_{28} alkane	127(5), 113(5), 99(15), 85(40), 71(65), 57(100), 43(70).	394(1)
1377	Branched C_{30} alkane	127(5), 113(5), 99(10), 85(30), 71(50), 57(100), 43(60).	422(1)

Table 15. Analysis of the *B. orientalis* filter paper extract before addition of diazomethane (same conditions as previous Table).

Scan	Compound	*m/z* (r.a., %)	$M^{\oplus}$(%)
629	Unknown	111(5), 97(20), 85(30), 73(60), 71(60), 57(100), 43(90).	147(30)?
634	Unknown	85(30), 73(40), 71(50), 57(100), 43(80).	147(20)?
655	Unknown	190(60), 175(60), 173(100), 156(10), 145(30), 111(20), 75(30).	192(30)?
707	n-C_{14} alkane	99(5), 85(30), 71(60), 57(100), 43(80).	198(1)
740	n-C_{15}-alkane	113(5), 99(10), 85(30), 71(50), 57(100), 43(60).	212(2)
754	Unknown	147(40), 85(10), 73(100), 71(10), 57(40), 43(20).	221(30)?
775	n-C_{16} alkane	99(10), 85(30), 71(60), 57(100), 43(70).	226(2)

Table 15. (cont'd.)

Scan	Compound	*m/z* (r.a., %)	$M^{\oplus}$(%)
779	Branched C_{16} alkane	183(5), 127(5), 113(15), 99(10), 85(20), 71(80), 57(100), 43(40).	226(2)
804	n-C_{17} alkane	99(10), 85(40), 71(65), 57(100), 43(70).	240(1)
816	Unknown, possibly branched C_{17} alkene	135(10), 97(20), 83(100), 82(70) 71(30), 57(60), 55(80), 43(70).	238?
840	n-C_{18} alkane	113(5), 99(10), 85(40), 71(60), 57(100), 43(80).	256(1)
846	Branched C_{18}	127(10), 113(10), 97(20), 85(40), 71(80), 57(100), 43(55).	256(2)
858	Unknown	233(100), 221(20), 205(10), 147(20), 73(70), 43(70).	248(20)?
901	n-C_{19} alkane	99(10), 85(30), 71(60), 57(100), 43(90).	270(2)
994	Di-n-butyl phthalate plasticiser	223(10), 205(10), 149(100), 104(10), 93(10), 76(10), 65(10), 57(10).	278(-)
1101	Unknown	268(20), 240(15), 227(15), 201(20), 135(60), 133(60), 91(70), 79(50), 67(100).	314(10)?
1217	n-C_{23} alkane	113(5), 99(10), 85(40), 71(60), 57(100), 43(70).	324(-)
1253	n-C_{24} alkane	113(5), 99(10), 85(30), 71(50), 57(100), 43(60).	338(3)
1267	Branched C_{24} alkane?	127(5), 113(10), 99(10), 85(40), 71(60), 57(100), 43(70).	338(-)?
1327	n-C_{26} alkane	127(5), 113(10), 99(15), 85(50), 71(70), 57(100), 43(80).	366(-)
1348	Branched C_{26} alkane?	196(10), 141(10), 127(10), 113(15), 99(20), 85(60), 71(80), 57(100), 43(90).	366(-)

Table 15. (cont'd.)

Scan	Compound	*m/z* (r.a., %)				$M^{\oplus}$(%)
1361	Branched C_{26} alkane?	127(5),	113(5),	99(10),	85(70),	366(2)
		71(50),	57(90),	43(100).		
1378	*n*-C_{30} alkane	127(5),	113(10),	99(20),	85(30),	422(1)
		71(5),	57(100),	43(60).		
1384	Branched C_{30} alkane	168(10),	127(5),	113(10),	99(20),	422(1)
		85(60),	71(60),	57(100),	43(90).	
1404	Branched C_{30} alkane	127(20),	113(10),	99(10),	97(15),	422(-)
		85(50),	71(70),	57(100),	43(60).	

3.4.4 Behavioural Bioassay for the Aggregation Pheromone

The aggregation-activity of an extract was determined using the bioassay developed by Ishii and Kuwahara.[122,123] A filter paper was cut into 5 x 6cm strips and folded to make 'W's as shown in Fig. 14. The strips were treated with either the extract (80μl, 1mg/ml "treated") or dichloromethane (80μl, "control"). Both kinds of strips were left for 15 minutes to allow the solvent to evaporate. Five of these folded strips (one treated and four controls) were placed in a Perspex box (28cm x 16cm x 10cm), 20 first instar nymphs (*ca.* 1:1, male:female) were added, and the box was covered. The box was left overnight (12h) and the relative positions of the cockroaches were noted.

3.4.5 Bioassay Results (Appendix I)

1. "Fresh" faeces from BASF, Limburgerhof, West Germany, stuck on with Sellotape to the filter paper strips. Tested on 15 adult *B. orientalis*.

Replication	I	II
Treated	2	-
Controls	11	13
Elsewhere	2	2

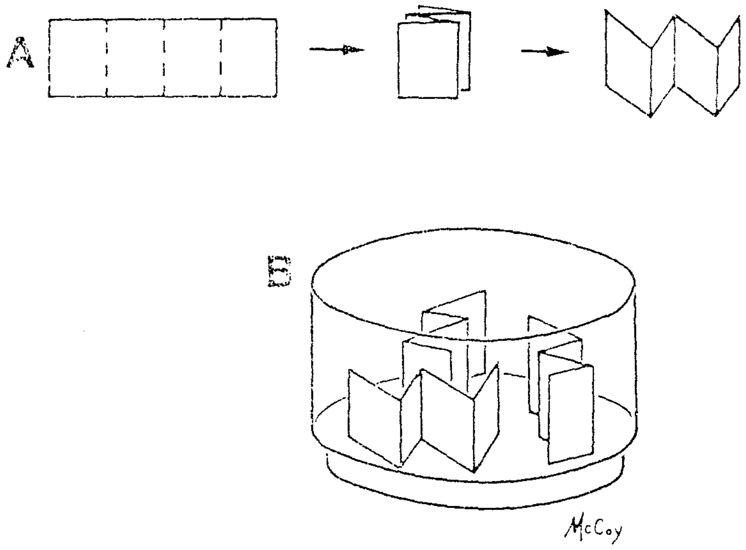


Figure 14. The *B. orientalis* aggregation pheromone bioassay. (A) The 3 x 7 cm filter paper strips were folded into 'W' forms, and (B) the strips were placed upright into a finger bowl and covered.

2. Faeces from BASF stuck on to the filter paper strips with Sellotape. Tested on 25 first instar nymphs.

Replication	I	II
Treated	-	-
Controls	8	15
Elsewhere	17	10

3. Methanol extract of faeces from BASF (25 first instar nymphs).

Replication	I	II
Treated	4	2
Controls	21	23

4. Dichloromethane extract of faeces from BASF (25 young nymphs).

Replication	I	II
Treated	-	3
Controls	25	10

5. Pure major sesquiterpenoid tested on 25 young nymphs.

Replication	I	II	III
Treated	-	-	-
Controls	3	20	20
Elsewhere	22	5	5

6. Freshly soiled filter paper. Tested with 40 first instar *B. orientalis*.

Replication	I	II
Treated	34	43
Controls	-	7
Elsewhere	6	-

7. Dichloromethane extract from freshly soiled filter paper tested on 20 young nymphs.

Replication	I	II	III	IV	V	VI	VII	Total	Mean	s.d.
Treated	18	16	19	9	20	9	20	111	15.86	4.88
Controls	0	4	1	11	0	10	0	26	3.71	4.86
Elsewhere	2	0	0	0	0	1	0			

χ^2 value = 52.74 ($P > 0.01$). Statistically significant aggregation-attraction.

8. Fresh filter paper extract after treatment with excess diazomethane tested on 20 first/second instar nymphs.

Replication	I	II	III	IV	V	VI	VII	VIII	IX	X	Total	Mean
Treated	0	19	15	13	0	19	0	1	19	19	105	10.5
Controls	20	1	5	7	20	1	20	19	1	1	95	9.5

χ^2 value = 0.5 from the total ($P > 0.01$). Aggregation-attraction is not statistically significant.

9. Freshly soiled filter paper after one extraction with dichloromethane. 20 young nymphs tested.

Replication	I	II
Treated	20	18
Controls	0	2

10. Freshly soiled filter paper after one extraction with methanol. 20 young nymphs tested.

Replication	I	II
Treated	1	2
Controls	19	18

11. Analysis of fractions from flash column chromatography (20 young nymphs tested in each case).

Fraction 1	Replication	I	II	III	IV
	Treated	0	0	1	0
	Controls	20	20	19	20
Fraction 2	Treated	1	2	1	1
	Controls	19	18	19	19
Fraction 3	Treated	8	12	0	0
	Controls	12	8	20	20
Fraction 4	Treated	18	13	1	2
	controls	2	7	19	18
Fraction 5	Treated	19	0	10	0
	Controls	1	20	10	20

Mixture of all 5 fractions	Replication	I	II	III	IV
(50μl from each)	Treated	1	0	3	0
	Controls	19	20	17	20

12. Analysis of product from preparative GC (5% OV101, 50-12-300°C). Tested on 20 young nymphs.

Replication	I	II	III	IV
Treated	0	3	1	0
Controls	20	17	19	20

13. Analysis of fractions from preparative HPLC (20 young nymphs tested in each replication).

Fraction 1	Fraction 1	Replication	I	II	III	IV
		Treated	0	4	11	5
		Controls	20	16	9	15
Fraction 2		Treated	0	0	0	1
		Controls	20	20	20	19
Fraction 3		Treated	0	0	1	1
		Controls	20	20	19	19
Fraction 4		Treated	1	0	0	0
		Controls	19	20	20	20
Fraction 5		Treated	1	0	0	0
		Controls	19	20	20	20
Fraction 6		Treated	0	1	2	0
		Controls	20	19	18	20
Mixture of all 5 fractions		Replication	I	II	III	IV
(50μl from each)		Treated	0	15	10	8
		Controls	20	5	10	12

14. Bioassay of carboxylic acids identified by GC/MS as methyl esters (20 young nymphs tested in each replication).

Benzoic acid	Replication	I	II	III	IV	V	VI
	Treated	-	-	-	2	4	10
	Controls	20	20	20	18	16	10
(E)-Cinnamic acid	Treated	-	-				
	Controls	20	20				
(E)-9-Hexadecenoic acid	Treated	3	-	2	-		
(palmitelaidic acid)	Controls	17	20	18	20		

	Replication	I	II	III	IV				
Z-9-Hexadecenoic acid (palmitoleic acid)	Treated	8	1	8	-				
	Controls	12	19	12	20				
Linoleic acid (Z,Z-9,12-octadecadienoic acid)	Treated	1	8	1	-				
	Controls	19	12	19	20				
Oleic acid (Z-9-octadecenoic acid)	Treated	5	-	15	-				
	Controls	15	20	5	20				
Palmitic acid (hexadecanoic acid)	Treated	2	14	5	0				
	Controls	18	6	15	20				
Pentadecanoic acid	Treated	-	-	-	1	1	-		
	Controls	20	20	20	19	19	20		
Phenylacetic acid	Treated	-	4	2	1				
	Controls	20	16	18	19				
Stearic acid (octadecanoic acid)	Treated	5	6	3	5				
	Controls	15	14	17	15				
Mixture of all the carboxylic acids	Treated	-	18	10	-	-	14	10	-
	Controls	20	2	10	20	20	6	10	20

(40µl from each 1µg μl^{-1} solution)

15\. Analysis of the stability of the *B. orientalis* aggregation pheromone after standing for 20 days (20 young nymphs tested per replication).

Replication	I	II	III	IV
Treated	2	1	4	5
Controls	18	19	16	15

CHAPTER FOUR

Attempted Isolation of a Sex Pheromone for the Bruchid Beetle, *Callosobruchus chinensis* (Linnaeus), Coleoptera: Bruchidae

4.1 Introduction

4.1.1 The Role of Pheromones in Control of Stored Product Pests

The Bruchid beetle (azuki bean weevil), Callosobruchus chinensis L., is a major worldwide pest of stored legumes.[126] The increasing consumption of legumes worldwide has led to an intensification of the cultivation and storage of legume grains, particularly in developing countries, where there is a shortage of animal protein.

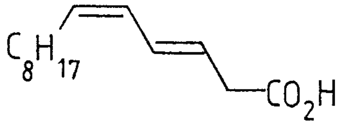
The main methods used to control stored product pests are fumigation and insecticide spraying, but both of these control measures suffer from disadvantages. For example, fumigation with insecticides can cause problems of food contamination and also environmental pollution. Insecticides can also encounter problems of insecticide resistance, and minor pests can be elevated to major pest status as predators and competitors are progressively eradicated. A lack of insecticidal selectivity may mean that many beneficial insects are killed along with the target species.[127,128]

In addition to these problems, most of the farmers in the developing countries are small holders, which renders application of fumigants impractical, while pesticides are likely to remain prohibitively expensive. Thus, a cheap, clean and safe alternative method for controlling such stored product pests would be highly desirable.[129-131]

In this context it has been suggested that pheromones will have a role to play in pest management systems of the future[127,132] owing to their lack of toxicity, negligible environmental pollution and competitive cost; there also appear to be few problems with the development of insect resistance to pheromones and very low dosages are required relative to the insecticides currently available.

Pheromone systems are particularly suited to the monitoring and control of stored product pests, because the closed storehouse environment (constant temperature and humidity combined with a lack of wind and rain) allows uninterrupted flight and minimises the movement of non-target insects.[133,134] With knowledge of an insect's active period it may be possible to trap selectively at a particular time of day.

There has been considerable progress in the use of pheromones for monitoring and control of stored product pests, since the sex pheromone of the black carpet beetle, Attagenus unicolor (=megatoma) was identified in 1967 as (3E,5Z)-tetradecadienoic acid (87).[135]



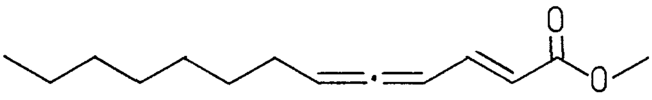
(87)

Pheromones from nearly all the important species have been identified.[136,137] New traps have also been developed and employed in pheromone-based monitoring and management systems.[132]

4.1.2 Sex and Aggregation Pheromones

Two general types of communication and reproductive strategies characterise stored product insects.[132] Adults are either short-lived (less than one month) and require no feeding for reproduction to occur, or they are long-lived (greater than one month) and must feed in order to reproduce. Short-lived insects, such as the moths, dermestids, bruchids and anobiid beetles, rely on sex pheromones for communication. These pheromones are usually produced by the female. Long-lived adults, such as the grain weevils, grain borers, and the flour and grain beetles, generally use male-produced aggregation pheromones for long-distance communication. Both males and females respond to these pheromones in the general case.

The only sex pheromone of a Bruchid species identified to date is (R)-(-)-methyl tetradeca-2E,4,5-trienoate (88), an unusual allenic ester produced by the male Dried Bean Beetle, Acanthoscelides obtectus.[138] Both enantiomers of (88) were synthesised by Pirkle and Boeder in 1978.[139]

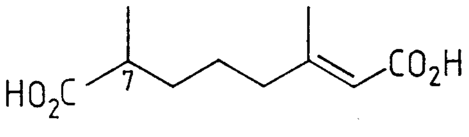


(88)

4.1.3 C. chinensis Pheromones

The existence of an ether-extractable female sex pheromone for C. chinensis was demonstrated by Honda and Yamamoto in 1976,[140] and the sex pheromone was tentatively identified as a straight-chain unsaturated acetate of between 16 and 18 carbon atoms based on GC, TLC and microchemical reaction data. However, the chemical structure of the sex pheromone has never been confirmed.

In 1981 Yamamoto et al isolated and identified the copulation release pheromone of C. chinensis, which induces the male to extrude his genital organ and to attempt copulation.[141,142] This pheromone was named 'erectin' and found to consist of two synergistically acting fractions. One was a mixture of methyl-branched hydrocarbons such as 11,15-dimethyltritriacontane. The other was a new monoterpene dicarboxylic acid, which was named callosobruchusic acid (15 ng per female).[141] Based on the mass spectrum of the corresponding methyl ester (the only available spectral information) and other mass spectral data, Yamamoto et al proposed the structure of callosobruchusic acid to be (E)-3,7-dimethyl-2-octene-1,8-dioic acid (89) without assigning the absolute configuration at C-7.[141] Both enantiomers of (89) have been synthesised by Mori et al[143] and each enantiomer was shown to be biologically active. The absolute stereochemistry of natural callosobruchusic acid is still unknown.



(89)

The initial aim of this project was to identify and synthesise the sex pheromone component(s) of C. chinensis with a view to field application for monitoring and management of this pest.

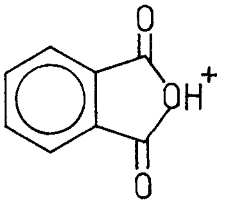
4.2 Results

4.2.1 Pheromone Source

Four different extracts were prepared and investigated for pheromonal activity. These extracts were obtained by solvent extraction of filter paper lining the female chamber, and by air entrainment using Tenax, Porapak Q and charcoal as adsorbents.

4.2.2 Filter Paper Extract

Filter paper (Whatman No. 1), which had been used to line glass chambers containing female *C. chinensis*, was washed with dichloromethane (10ml). The extract was concentrated by distillation at atmospheric pressure and analysed by GC and GC/MS (5% OV101, 100-12-300°C). Compounds identified included two phthalate esters, together with n-C_{20}, n-C_{21} and n-C_{22} alkanes (Table 16, Figure 15); several other compounds were present, but in such small quantities that they could not be identified. Plasticisers such as di-*n*-butyl phthalate are the most common contaminants of insect extracts; they are readily recognised in GC/MS by their characteristic *m/z* 149 base peak (90):

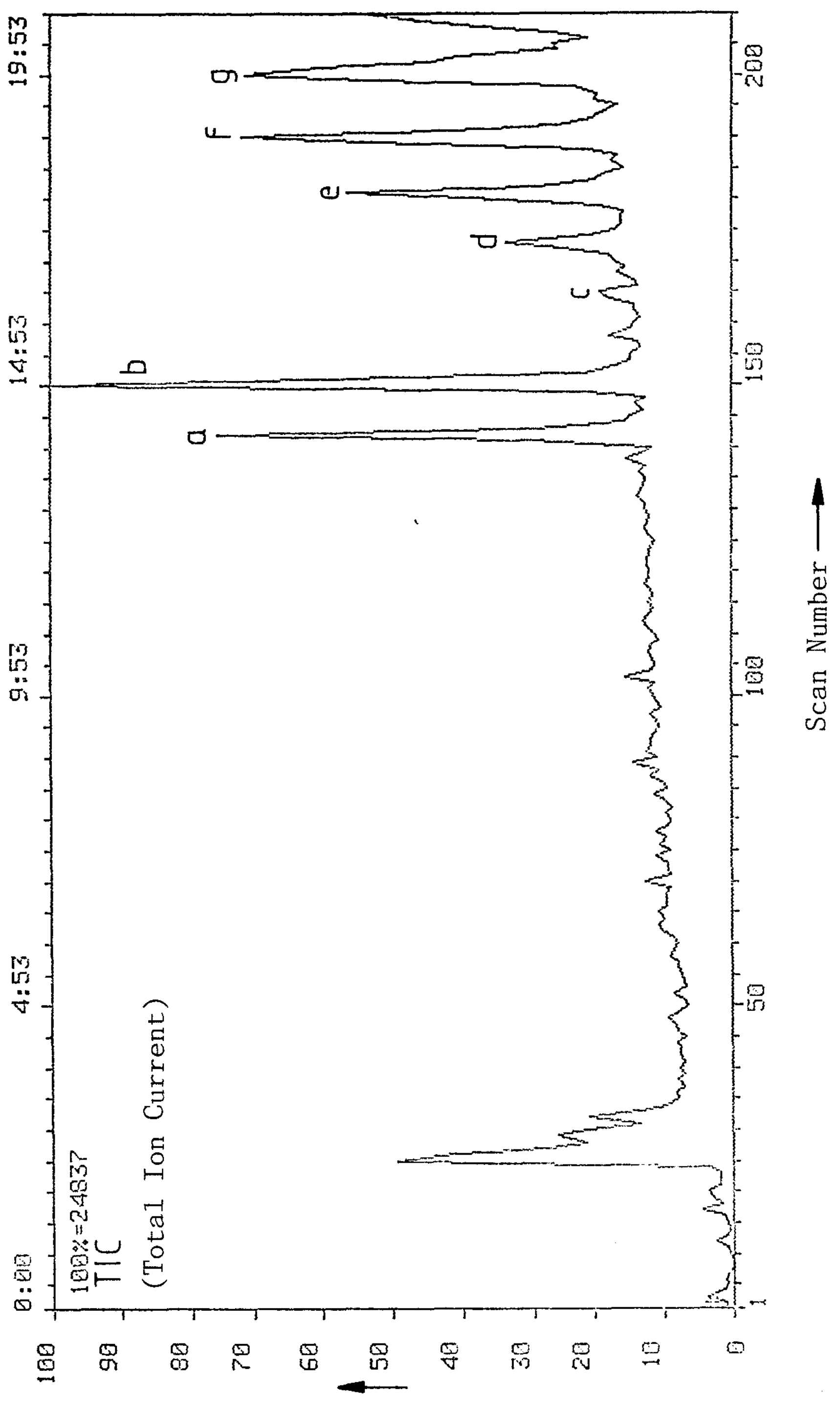


(90)

The concentrated filter paper extract was fractionated by micro-scale Florisil column chromatography using a Pasteur pipette (5cm x 5mm O.D.) and a gradient elution (ether-hexane, 100% hexane to 100% ether with 5% increments). Sex pheromonal activity was found to be localised in the 15% ether-hexane fractions, as reported by Yamamoto *et al.*[141] This biologically-active extract showed no significant peaks on packed-column GC analysis, but capillary GC (BP10, 50, 2-8-230°C) demonstrated that a large number of components were present, albeit in low nanogram quantities.

Following several extractions on a large scale over a one month period and repeated Florisil column chromatography, the combined 15% ether-hexane fractions were purified further by preparative GC (5% OV101, 150-8-300°C, then 5% FFAP, 150-8-240°C) with one minute fractions being taken. The biological activity was located in the fractions eluting between 250-265°C on 5% OV101, and after 4-6 minutes at 240°C on the 5% FFAP column. These results appear to indicate that the *C. chinensis* sex pheromone is a relatively polar compound with a

Figure 15. GC/MS analysis of virgin female *C. chinensis* filter paper extract (5% OV101, 100-12-290°C). See Table 16 for peak identification).



relative molecular mass of at least 200. The combined active fractions from preparative GC were shown to contain eight main components by capillary GC analysis (Figure 16).

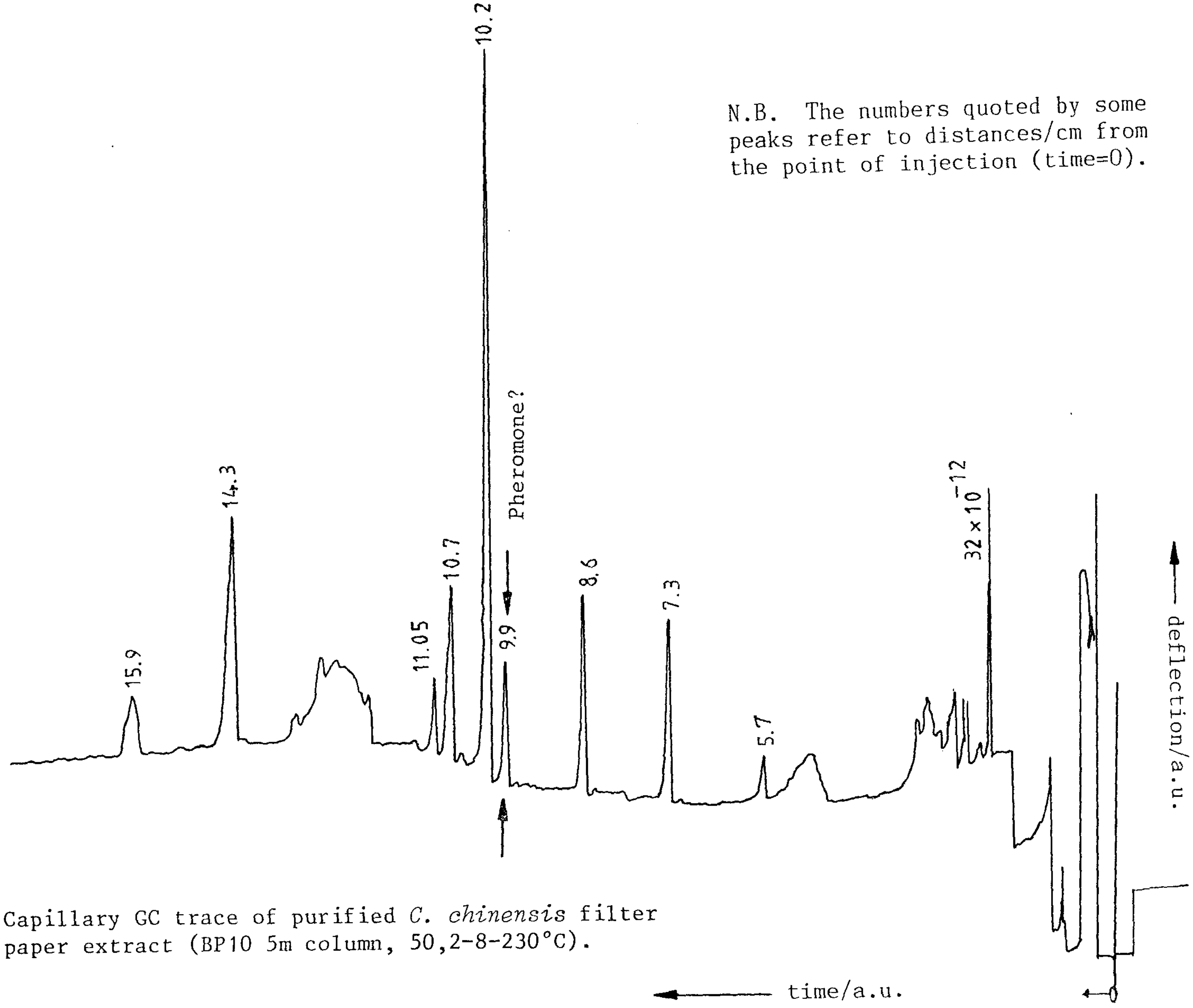
This biologically-active, purified sample was analysed using capillary GC/MS by Dr. Fred Mellon of the Agricultural and Food Research Council at Norwich. A Kratos MS80 instrument was employed using the Alternate Chemical and Electron impact (ACE) technique with ammonia as reagent gas, and a BP10 50m capillary column (50, 2-8-230°C). The GC/MS results are shown in Figure 17 and Table 17 (Section 4.4.2). The active, purified sample was also analysed by Dr. John Pickett of Rothamsted Experimental Station, Harpenden, Herts., using a VGMM 7070F instrument linked to a 2035 Data System and similar ACE conditions with electron impact at 70eV.

4.2.2.1 Microchemical Reactions

Microchemical reactions combined with behavioural bioassay showed that methanolic potassium hydroxide and lithium aluminium hydride in THF destroyed the activity of the sex pheromone (Table 18). The reactions were followed by capillary GC and it was apparent that only the peak at scan number 310 (Figure 17, Table 17) disappeared after treatment with methanolic potassium hydroxide and lithium aluminium hydride in THF. It is also known that sex pheromone production effectively ceases in female *C. chinensis* after mating,[144] and it is significant that the peak of interest is absent from the filter paper extract of mated females.

Table 18. Microchemical Reactions on *C. chinensis* extracts.

Reagent	Activity after reaction?	Conclusions
Lithium aluminium hydride in THF	-	Ester, acid, aldehyde, ketone present
Methanolic potassium hydroxide	-	May be an ester, amide (or epoxide)
Dilute bromine solution	+	Double bonds absent
Sodium borohydride	+	Aldehyde or ketone absent
Aqueous 1 moldm^{-3} hydrochloric acid	+	Epoxide or acetal absent
Aqueous 1 moldm^{-3} sodium hydroxide	+	Acid not present
Bis(trimethylsilyl)acetamide	+	Alcohol absent
Trimethylsilylimidazole	+	" "
Diazomethane solution	+	Not a carboxylic acid



N.B. The numbers quoted by some peaks refer to distances/cm from the point of injection (time=0).

Figure 16. Capillary GC trace of purified *C. chinensis* filter paper extract (BP10 5m column, 50,2-8-230°C).

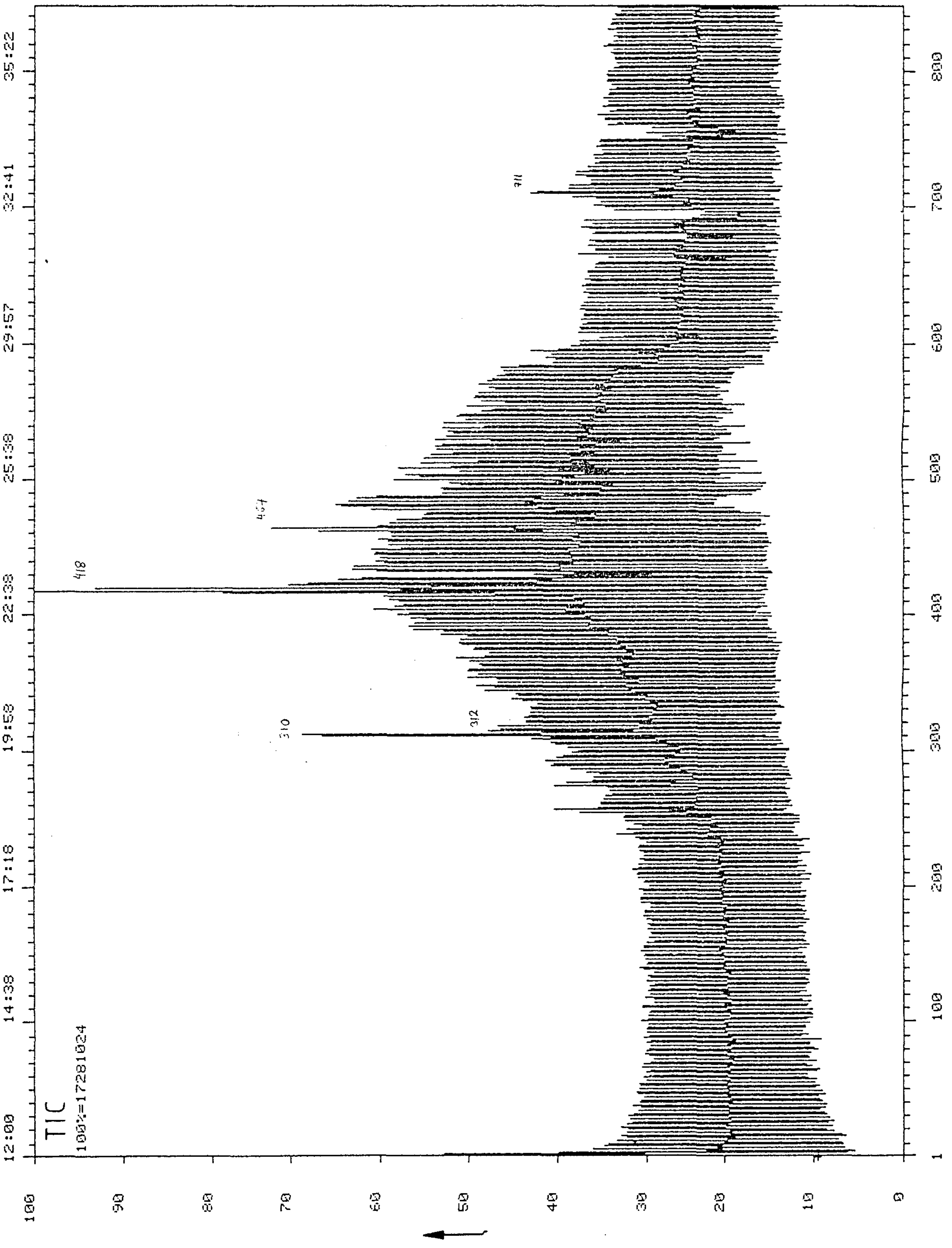


Figure 17. Capillary GC/MS trace of purified filter paper extract from virgin female *C. chinensis* (BP10, 50,2-8-230°C) ACE, ammonia).

4.2.2.2 Mass Spectrum of the *C. chinensis* Sex Pheromone

The observations discussed in Section 4.2.2.1 indicate that the component at scan number 310 in Figure 17 is probably the sex pheromone of *C. chinensis*. The EI and CI mass spectra of this compound are shown in Figures 18 and 19, respectively. Unfortunately, this mass spectral data did not match any of the information stored on the NIH database and is not readily interpretable.

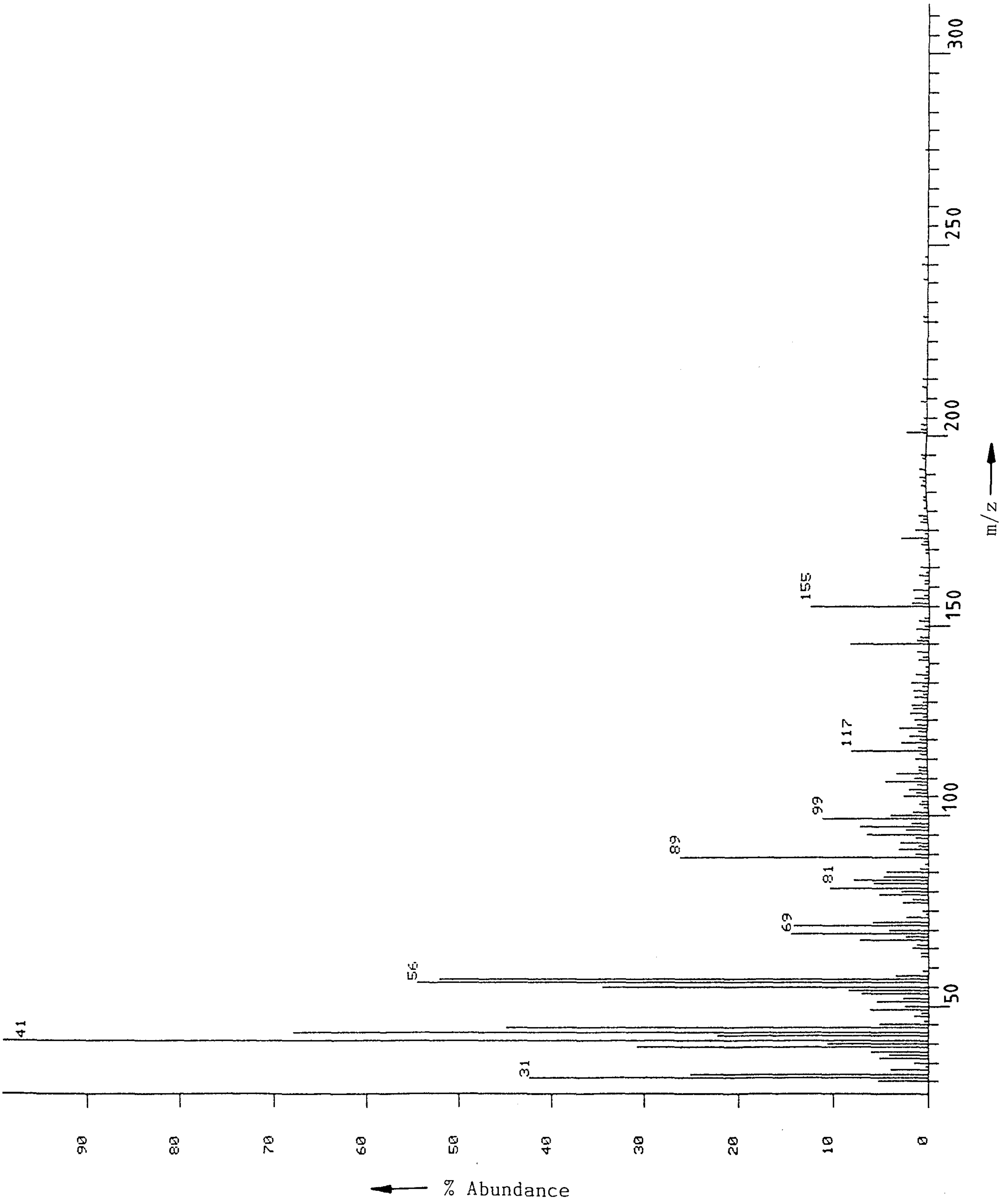
The CI mass spectrum (Figure 19) indicated that the relative molecular mass of the unknown was likely to be 228 owing to the prominence of the [M+1] ion at *m/z* 229. This ion can lose either $[CO^{\bullet}]$ or $[C_2H_4^{\bullet}]$ to form the intense ion at *m/z* 201 (50%), and may gain *m/z* 17 $[NH_4^{2\oplus}]$ to give the ion at *m/z* 246 (M + 18, 5%). The molecular formula of the unknown is probably $C_{14}H_{28}O_2$. The base peak ion at *m/z* 144 may correspond to the molecular formula $C_8H_{16}O_2$; the high intensity of this ion can be rationalised in terms of its highly oxygenated nature. The *m/z* 144 ion may be produced by a loss of a butyl radical $[C_4H_9^{\bullet}]$ from the ion at *m/z* 201 (i.e. M - $C_6H_{12}^{\bullet}$).

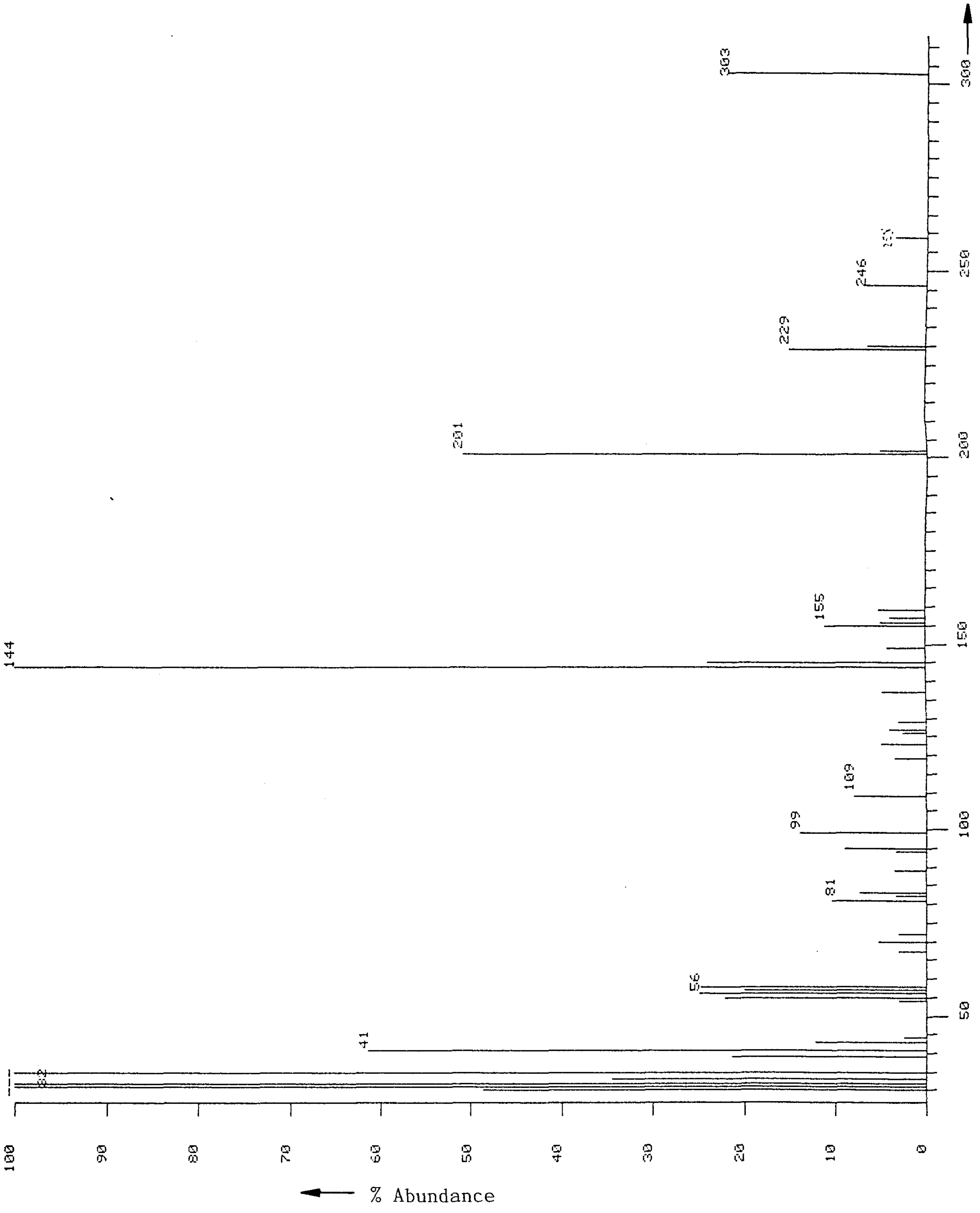
The EI mass spectrum (Figure 18) showed significant ions at *m/z* 201(3%), 173(4), 155(10), 145(8), 117(8), 99(10), 89(25), 81(10), 69(15), 57(52), 56(55), 44(45), 43(68) and 41(100). The molecular ion is notably absent from the EI mass spectrum. The ion at *m/z* 145 may result from a loss of *m/z* 56 ($C_4H_8^{\bullet}$) by the *m/z* 201 ion.

4.2.3 Tenax Aeration Extract

In an effort to obtain larger quantities of the *C. chinensis* sex pheromone for detailed structure elucidation, Tenax GC[145] (a polymer of 2,6-diphenyl *p*-phenylene oxide) was employed to trap volatile compounds from a stream of air passed over virgin female *C. chinensis*. The Tenax trap wash (pentane) was analysed by GC and GC/MS (Figure 20, Table 19). There was only one major component in the biologically-active aeration extract, which could not be identified, and GC analysis using the BP10 capillary column showed no significant peaks in the region of interest.

Figure 18. Electron impact mass spectrum of the proposed *C. chinensis* filter paper extract (BP10 5m column, 50,2-8-230°C).





<u>Figure 19</u>. Chemical ionisation (ammonia) mass spectrum of the putative *C. chinensis* sex pheromone.

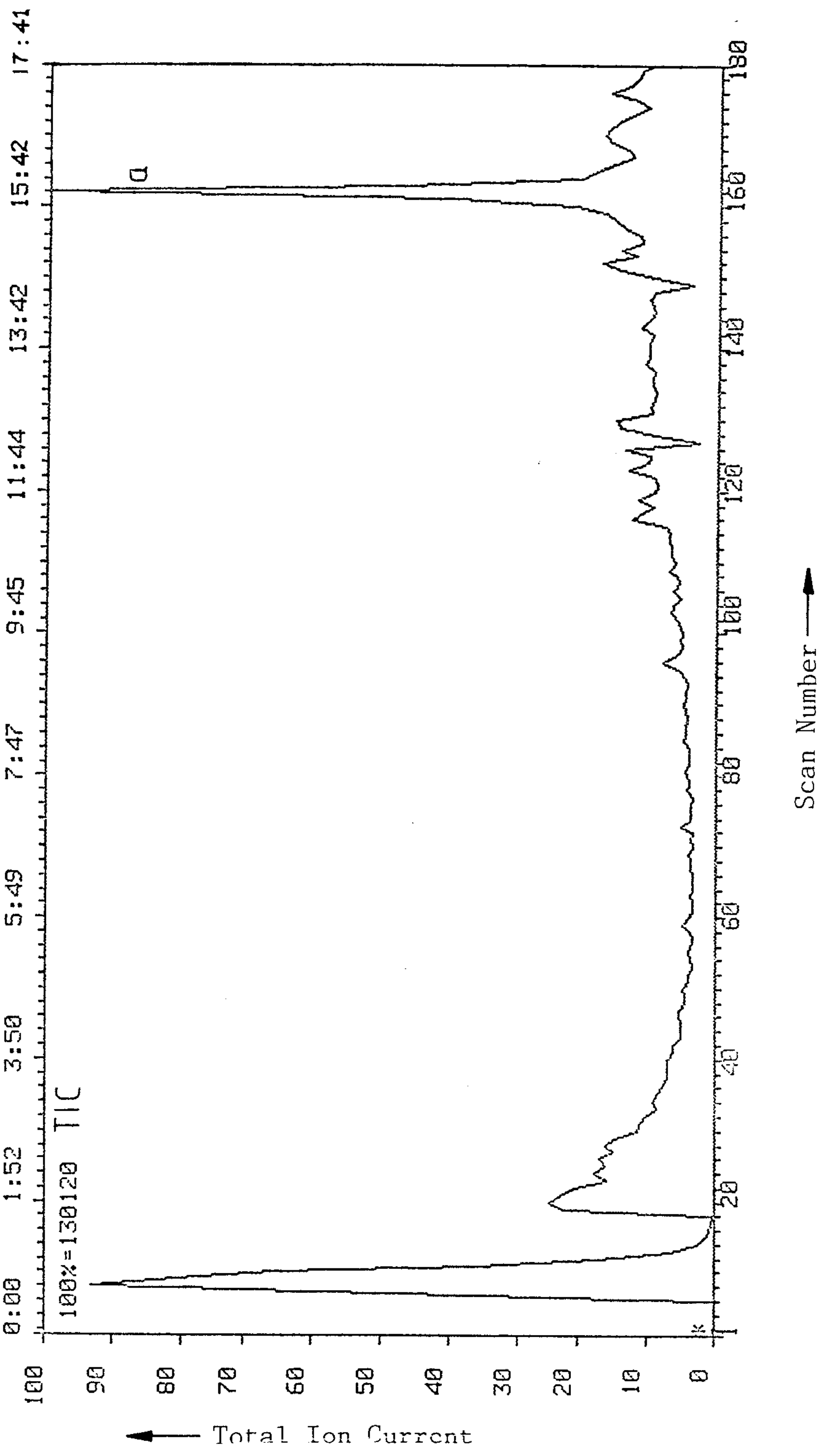


Figure 20. GC/MS trace of Tenax aeration extract from virgin female *C. chinensis* (5% OV101, 100-12-290°C).

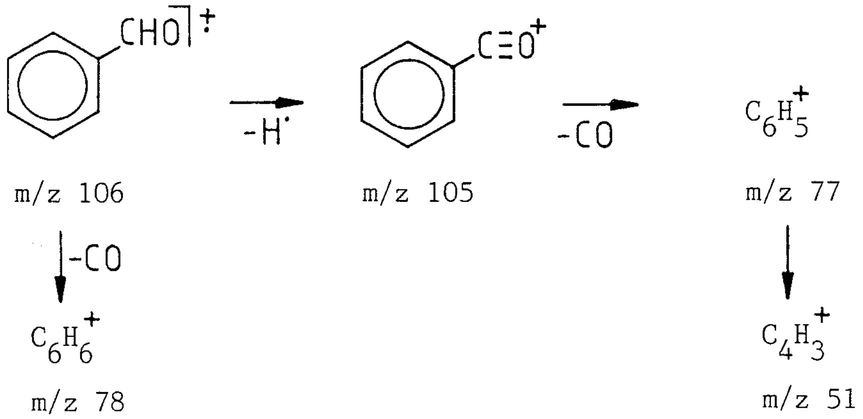
4.2.4 Porapak Q Aeration Extract

The Porapak Q[146] (a polymer of styrene and divinylbenzene) aeration extract was analysed by GC and GC/MS (Figure 21, Table 20), but, once again, there were no large peaks in the pheromone region (despite high sex pheromone activity). GC/MS analysis revealed the presence of two phthalate esters together with an unknown having a probable relative molecular mass of 229.

4.2.5 Charcoal Aeration Extract

Benzaldehyde (C_6H_5CHO) and an unidentified sesquiterpene ($C_{15}H_{24}$) were identified as major components of the charcoal[147] aeration extract by GC and GC/MS analysis (Figure 22, Table 21), but this method also failed to afford viable quantities of the sex pheromone for ^1H NMR study. A monoterpene ($C_{10}H_{16}$) was also present as a major component in the aeration extract, together with several other unidentified minor components. The mass spectral fragmentation of benzaldehyde[47] is shown in Scheme 9.

Scheme 9. Mass spectral fragmentation of benzaldehyde.



Benzaldehyde is a relatively common compound in beetles. For example, the tiger beetle, *Megacephala australis*,[148] was recently shown

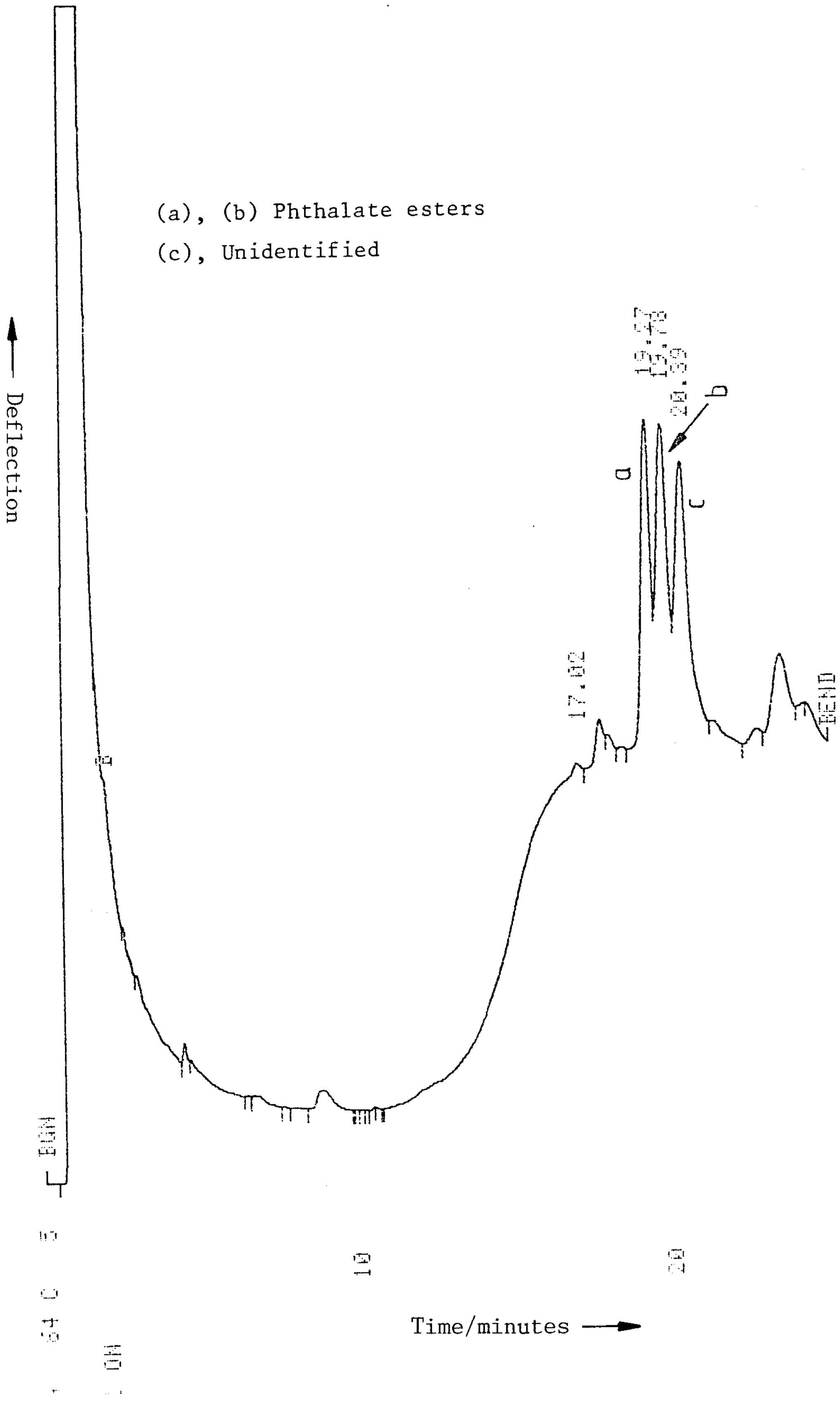


Figure 21. GC trace of Porapak Q aeration extract from virgin female *C. chinensis* (5% OV101, 100-12-290°C).

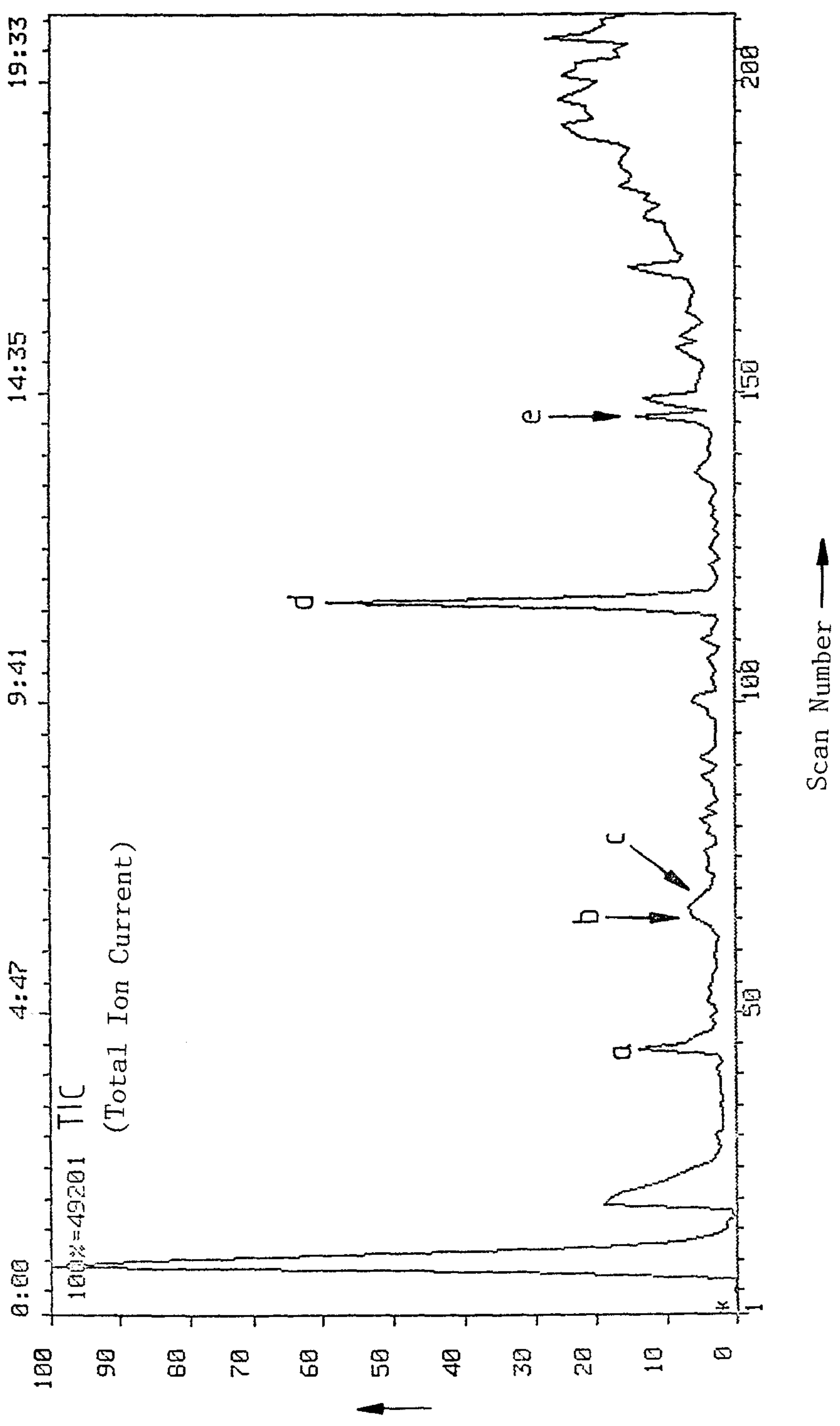


Figure 22. GC/MS trace of virgin female *C. chinensis* charcoal aeration extract (5% OV101, 100-12-290°C). See Table 21 for peak identification.

to produce benzaldehyde in its pygidial glands, while in *M. virginica* and *M. carolina*[149] this aromatic aldehyde is accompanied by hydrogen cyanide.

4.3 Conclusion

The sex pheromone of *C. chinensis* has been investigated and seems likely to be a relatively polar compound with a relative molecular mass of 228 and molecular formula $C_{14}H_{28}O_2$. Microchemical studies suggest that the sex pheromone may be a saturated ester, but is unlikely to be an acetate, carboxylic acid, aldehyde, ketone, alcohol, epoxide or acetal.

Identification of the sex pheromone clearly requires collection on a much larger scale, followed by improved methods of purification (possibly involving preparative HPLC) in order to provide enough pure pheromonal material for ^1H NMR analysis.

4.4 Experimental

4.4.1 Materials and Methods

A culture of *Callosobruchus chinensis* was maintained in the insectary on dried garden peas by Mr. Mohammed Nurul Islam, whose help and advice is gratefully acknowledged. The culture was initiated with beetles from the Agricultural Research Council's Pest Infestation Control Research Laboratory, Slough, and from the Bangladesh Agricultural Research Institute, Dhaka, Bangladesh. The insectary was kept at 27.5 ± 1°C and 70 ± 5% relative humidity, on a 16:8h light-dark cycle at a light intensity of 1000 lx.

A batch of 2000 beetles was aerated for five days (see Section 1.3.4) and the volatiles secreted by the beetles were trapped on activiated charcoal, Porapak Q or Tenax GC (all 1g). Porapak Q (Alltech Associates, Carnforth, Lancashire, 50-80 mesh) and Tenax GC (Phase Separations Limited, Queensferry, Clwyd) were rinsed with pentane, allowed to dry and then conditioned in a stream of dry nitrogen at 190°C (12h) prior to use. The concentrated aeration extract was analysed by packed-column GC (Perkin-Elmer 8310, either 5% OV101 50-16-290°C or 5% FFAP 50-12-240°C), capillary GC (Varian 3700, BP10 SCOT column 0.2mm O.D. x 12m, 50, 2-8-230°C), and GC/MS (see Section 2.4 for General Procedures and Instrumentation).

Capillary GC/MS was carried out by Dr. F.A. Mellon of AFRC, Norwich, using a Kratos MS80 instrument coupled to a DS-55 Data System, and by Dr. J.A. Pickett of Rothamsted Experimental Station, Harpenden, Herts., using a VGMM 7070F instrument interfaced with a 2035 Data System. In both cases the Alternate Chemical Ionisation-Electron Impact (ACE) technique was employed with ammonia as the reagent gas.

Preparative GC was carried out using the all-glass splitter system described by Baker *et al*[124] as adapted by Attygalle and Morgan.[125] Flash column chromatography was performed according to the technique of Still *et al.*[83] Neutral alumina (100-120 mesh) was obtained from Aldrich Limited (Gillingham, Dorset) and Sephadex LH20 (Merck, 100-200 mesh) was kindly supplied by Dr. Graeme Russell, DSIR, Palmerston North, New Zealand, whose advice and assistance is gratefully acknowledged.

Microscale chemical reactions were performed by addition of the reagent (1µl) to the biologically-active sample (2µl) in dichloromethane (30µl) in a microvial. The reagent and the sample were thoroughly mixed by manual shaking and allowed to react (60 minutes). The solution was washed with water (5µl) prior to GC analysis; 1µl of the solution was then taken up in a syringe and injected into the gas chromatograph in the normal way, while the remaining 1µl was kept for bioassay. The following reagents were employed: lithium aluminium hydride (2mg) in THF (100µl), an aqueous methanolic solution of potassium hydroxide, a 1% aqueous bromine solution, an aqueous ethanolic solution of sodium borohydride, an aqueous solution of 1 $moldm^{-3}$ hydrogen chloride, an aqueous 1 $moldm^{-3}$ solution of sodium hydroxide, bis(trimethylsilyl)-acetamide, trimethylsilylimidazole and diazomethane.

Diazomethane was produced by treating 1-methyl-3-nitro-1-nitrosoguanidine with a few drops of 40% w/v aqueous potassium hydroxide, as shown in Figure 23.[150] The resultant gaseous diazomethane was condensed into the dichloromethane insect extract, left to stand at room temperature (1h), and the excess diazomethane removed by a rapid stream of dry nitrogen before GC analysis.

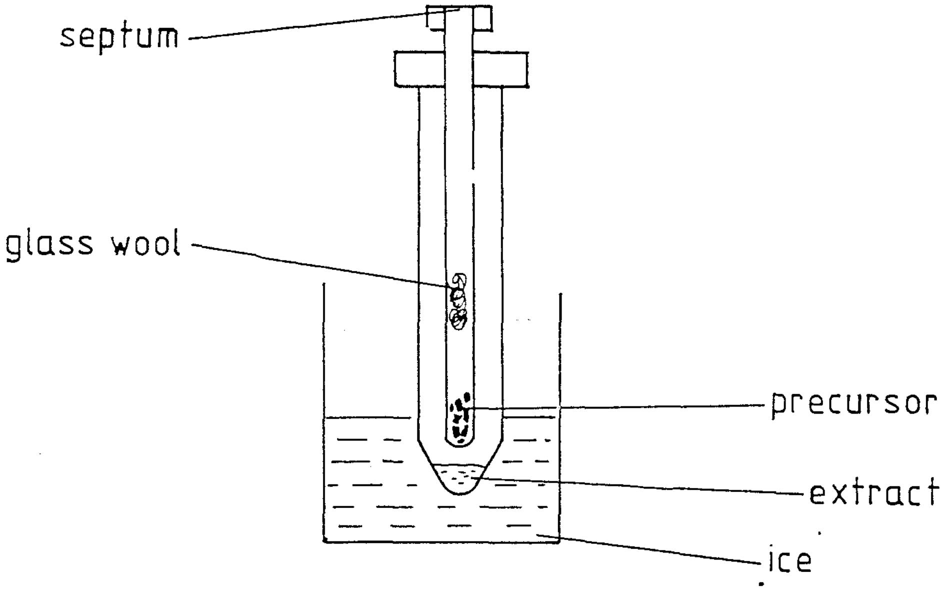


Figure 23. Apparatus for diazomethane preparation.

Biological testing was carried out by Mr. M.N. Islam of the Chemical Entomology Unit, Southampton University, using a specially-designed dual-choice olfactometer for sex pheromone bioassay.[144]

4.4.2 GC/MS Data

Table 16. GC/MS Analysis of Virgin Female *C. chinensis* Filter Paper Extract (5% OV101, 100-12-290°C).

Peak		*m/z* (r.a., %)				$M^{\oplus}$(%)
(a)	Phthalate plasticiser	149(100), 205(5)	41(15), 104(5),	57(10), 93(5),	223(5) 76(5)	278(1)
(b)	Phthalate plasticiser	149(100), 205(5),	41(15), 104(5),	57(10), 93(5),	223(5) 76(5)	278(1)
(c)	Unknown	32(100), 71(20),	40(50), 208(15),	44(30), 281(10),	57(25) 191(10)	281(10)?
(d)	Unknown	44(100), 281(30),	57(80), 119(30),	208(45), 195(30),	75(40) 191(25)	281(30)?
(e)	*n*-$C_{20}H_{42}$	57(100), 41(25),	43(65), 99(10),	71(50), 113(5),	85(40) 127(5)	282(1)
(f)	*n*-$C_{21}H_{44}$	57(100), 41(20),	43(60), 99(10),	71(50), 113(5),	85(30) 127(5)	296(1)
(g)	*n*-$C_{22}H_{46}$	57(100), 41(30),	43(55), 99(10),	71(50), 207(10),	85(30) 141(5)	310(2)

Table 17. Capillary GC/MS Analysis of Filter Paper Extract (purified by preparative GC) from Virgin Female *C. chinensis* (BP10, 50, 2-8-230°C) (ACE, NH_3).

Scan Number (EI or CI)	*m/z* (r.a., %)				$M^{\oplus}$(%)
256(EI)	41(100),	69(80),	43(70),	57(30)	228(3)
	55(27),	53(25),	91(22),	81(20)	
274(EI)	41(100),	69(80),	55(45),	67(30)	?
	95(25),	43(25),	79(20),	91(18)	
288(EI)	69(100),	41(98),	81(52),	43(48)	257(5)?
	55(45),	67(30),	93(22),	79(20)	
292(EI)	41(100),	69(80),	55(50),	95(40)	?
	43(40),	105(38),	81(36),	91(32)	
310(EI)	41(100),	43(68),	56(55),	57(53)	228(1)
	44(45),	31(42),	55(35),	89(28)	
311(CI)	144(100),	41(60),	201(50),	56(25)	229(M+1, 15)
	54(25),	145(25),	55(20),	229(15)	
312(EI)	41(100),	56(65),	43(60),	57(50)	228(1)
	44(45),	89(40),	55(25),	155(20)	
418(EI)	57(100),	43(100),	71(70),	41(60)	296(1)
n-$C_{21}H_{44}$	55(45),	85(40),	69(25),	97(15)	
464(EI)	57(100),	43(95),	41(65),	71(57)	310(1)
n-$C_{22}H_{46}$	55(55),	85(35),	69(32),	97(20)	
711(CI)	41(100),	70(70),	167(65),	55(50)	?
	57(45),	95(35),	104(30),	81(30)	

Table 19. GC/MS Analysis of Virgin Female *C. chinensis* Tenax Aeration Extract (5% OV101, 100-12-290°C).

Peak		m/z (r.a., %)				$M^{\oplus}$(%)
(a)	Unknown	41(100), 57(30),	57(70), 56(20),	112(55), 44(20),	39(35) 139(15)	269(1)

Table 20. GC/MS Analysis of Virgin Female *C. chinensis* Porapak Q Aeration Extract (5% OV101, 100-12-290°C).

Peak		m/z (r.a., %)				$M^{\oplus}$(%)
(a)	Phthalate plasticiser	149(100), 205(5),	41(15), 104(5),	57(10), 93(5),	223(5) 76(5)	279(1)
(b)	Phthalate plasticiser	149(100), 205(5)	41(15), 104(5),	57(10), 93(5),	223(5) 76(5)	279(1)
(c)	Unknown	43(100), 71(30),	102(70), 211(25),	60(60), 229(20),	228(40) 129(20)	229(20)?

Table 21. GC/MS Analysis of Virgin Female *C. chinensis* Charcoal Aeration Extract (5% OV101, 100-12-290°C).

Peak		m/z (r.a., %)				$M^{\oplus}$(%)
(a)	Benzaldehyde, PhCHO	106(100), 50(30),	77(80), 78(20),	105(70), 52(10),	51(50) 74(10)	106(100)
	Standard spectrum	77(100), 32(40),	105(98), 43(35),	106(94), 40(26),	51(44) 50(21)	106(94)
(b)	Unidentified monoterpene, $C_{10}H_{16}$	121(100), 68(45),	93(55), 105(40),	136(50), 91(40),	107(50) 79(35)	136(50)
(c)	Unknown, $C_{10}H_{14}$?	119(100), 67(25),	68(70), 41(20),	134(30), 91(20),	93(25) 39(20)	134(30)
(d)	Unidentified sesquiterpene, $C_{15}H_{24}$	41(100), 81(60),	107(75), 91(60),	93(70), 53(50),	79(65) 105(50)	204(25)
(d)	Unknown, possibly an amide	43(100), 228(30),	60(60), 55(25),	102(50), 129(20),	41(40) 73(20)	229(15)

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