INSECT PHEROMONES

THE SEX PHEROMONE

OF

Nudaurelia cytherea cytherea Fabr.

(Lepidoptera : Saturniidae)

by

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SUMMARY

Studies leading to the isolation, identification and synthesis of the sex pheromone from the female pine tree emperor moth Nudaurelia cytherea cytherea Fabr. (Lepidoptera : Saturniidae) are described.

A small quantity (320 µg) of a pure compound, showing activity in field tests and on the electroantennogram (EAG) was isolated from crude extracts of the terminal abdominal segments from 34,000 virgin female moths. Initial purification was effected by low-temperature precipitation of inactive fats, followed by steam distillation and column chromatography, while preparative gas-liquid chromatography was used as the final purification technique.

From an interpretation of the spectral data obtained on the isolated compound, augmented by the results of reactions on crude female extracts it was deduced that the active component was a decenyl isovalerate ester showing unsaturation in the five position. Confirmation was obtained by synthesis of the ester and comparison with its spectral data. Further confirmation was given by the synthesis of the Δ^4 and Δ^6 positional isomers and the analogue cis-8-methylnon-4-en-1-yl valerate having the isopropyl group on the alcohol moeity. Comparison with their spectral data showed marked discrepancies.

Final verification was given by bioassay in the pine plantations where the synthetic ester was found to attract male moths in large numbers. The sex pheromone is then

cis-dec-5-en-1-yl isovalerate (I)

$$CH_3.\left[CH_2\right]_3.CH{=}CH.\left[CH_2\right]_4.O.CO.CH_2.CH(CH_3)_2 \qquad I$$

The cis and trans forms of Δ^4 and Δ^6, and the trans Δ^5-decenyl isovalerate failed to attract male moths in the plantations. The trans isomer of the pheromone did not inhibit male response when mixed in moderate quantities with the active cis form.

Finally, the electroantennogram was shown to be unsuitable as a critical bioassay in the final confirmation of structure in that antennae responded equally to the synthetic pheromone and the positional isomers.

CONTENTS

CHAPTER			
		Acknowledgements	viii
		Summary	i
I		INSECT PHEROMONES	
	1	Introduction : Definition and function	1
	2	Insect sex pheromones	4
		1. Isolation studies	5
		2. Sex pheromones of the Lepidoptera	8
		(i) Chemical structure	9
II		THE PINE TREE EMPEROR MOTH	
	1	Introduction	16
	2	Life history	18
	3	Collection and storage of pupae	19
	4	Sexing of pupae	22
	5	Collection of "wild" females	23
III		BIOASSAY METHODS	
	1	The electroantennogram (EAG)	25
		1. Materials	29
		2. Experimental	32
		(i) Rest period	32
		(ii) Stimulus-response curve	34

2 Field tests

IV ISOLATION AND STRUCTURAL STUDIES

1 Preliminary studies leading to the final isolation procedures and structural studies 39

1. Pilot study 40

(i) Preparative gas-liquid chromatography 41

(ii) Mass spectrometry 45

2 Final study leading to the identification of the sex pheromone 46

1. Extraction procedures and initial purification 47

2. Preparative gas-liquid chromatography

(i) Stationary phase FFAP 48

(ii) Stationary phase OV 25 49

3. Interpretation of the spectral data 53

4. Comparison with the spectral data from synthetic decenyl isovalerate esters 58

V THE SYNTHESIS OF cis-DEC-5-EN-1-Yl ISOVALERATE AND RELATED COMPOUNDS

1 Introduction 60

2 Syntheses 60

1. cis and trans-Dec-5-en-1-yl isovalerate 61

2. cis and trans-Dec-4-en-1-yl isovalerate 63

3. cis and trans-Dec-6-en-1-yl isovalerate 65

4. cis-8-Methyl-non-4-en-1-yl valerate 66

3 Discussion 66

1. Synthesis of the substituted acetylenes 67

2. Stereospecific reduction of the disubstituted acetylenes

(i) cis Olefin 69

(ii) trans Olefin 70

VI BIOLOGICAL EVALUATIONS

1 Final verification of structure through field tests 72

2 Bioassay of the synthetic pheromone and its positional isomers 73

1. Male pine tree emperor moth response to a mixture of the geometric isomers 73

2. Male pine tree emperor moth response to some positional isomers 79

3. A comparison of male pine tree emperor moth response to the synthetic pheromone and extracts from virgin female moths 81

3 Bioassay using the antennogram

1. Experimental 83

2. Discussion 85

VII EXPERIMENTAL

1 Bioassay techniques

1. Electroantennogram - General procedure 87

2. Field tests - General procedure 91

2 Isolation of the natural pheromone

1. Apparatus and techniques 92

2. Preliminary study on an extract from 12,000 female moths

(i) Purification by column chromatography 94

(ii) Marking of the active area on g.l.c. 95

(iii) Preparative isolation of the pheromone peak 99

(iv) Mass spectrometry 100

3. Final study on an extract from 34,000 female moths

(i) Solvent extraction of abdominal tips 100

(ii) Low-temperature precipitation of lipids 101

(iii) Purification by steam distillation 102

(iv) Purification by column chromatography

(v) Preparative gas-liquid chromatography

(a) Polar phase FFAP 104

(b) Less polar phase OV 25 107

3 N.m.r. spectroscopy 113

4 Mass spectrometry 115

5 Infrared spectrophotometry 116

6 Chemical studies on crude extracts 117

7 The synthesis of cis-dec-5-en-1-yl isovalerate and related compounds

1. cis-Dec-5-en-1-yl isovalerate 119

(i) 4-Chlorobutan-1-yloxytetrahydropyran 119

(ii) Hex-5-yn-1-yloxytetrahydropyran 120

(iii) Dec-5-yn-1-yloxytetrahydropyran 120

(iv) cis-Dec-5-en-1-yloxytetrahydropyran 122

(v) cis-Dec-5-en-1-ol 122

(vi) cis-Dec-5-en-1-yl isovalerate 123

2. trans-Dec-5-en-1-yl isovalerate 124

3. cis-Dec-4-en-1-yl isovalerate 127

4. trans-Dec-4-en-1-yl isovalerate 129

5. cis-Dec-6-en-1-yl isovalerate 130

6. trans-Dec-6-en-1-yl isovalerate 134

7. 8-Methylnon-4-en-1-yl valerate 135

8. Decanyl isovalerate 137

APPENDIX

The spectra of the synthetic pheromone and related compounds

REFERENCES

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CHAPTER I

INSECT PHEROMONES

1. Introduction : Definition and function

Demonstrations of the effective use of chemical signals by organisms as a mode of communication between individuals are well documented in the insect world and have recently extended to the primates. These chemical messengers, recently called"semiochemicals" by Law and Regnier[1], function as intraspecific signals when produced by a member of one species in communicating with other individuals from the same species or as interspecific signals when produced by individuals of one species and interpreted by differing species. These intraspecific chemicals acting through the sensory organs of individuals are known as PHEROMONES and are characterised according to the effect they have upon the receiving individual. If this effect is immediate in releasing a specific behavioural response the chemical is known as a RELEASER PHEROMONE. For example, individuals in an ant colony will take immediate retaliatory action on detecting the alarm pheromone of the colony while male moths on detecting the female sex pheromone will suddenly orientate into the wind in search of a mate. Alternatively the effect may be delayed and similar to that of the hormones in that a slow physiological change results. These chemical messengers are known as PRIMER PHEROMONES of which the

queen substance from the honeybee (Apis mellifera) is an example. This pheromone trans-9-oxodec-2-enoic acid assists in maintaining the various castes in the hive where it is known to supress the development of ovaries in the worker bees.[2]

The pheromones of the insects have been the most extensively studied where they are largely of the releaser type and may be grouped according to the behavioural response they evoke. There are the alarm pheromones which form an integral part of the defence system of the social insects. An example here is the use of isoamyl acetate as an alarm substance by workers of the honey bee (A. mellifera). Traces of this compound left at the sting site arouse other workers and induces them to attack at the same location.[3] The social insects are also known to rely on trail marking pheromones to increase their foraging efficiency. Here, workers of the Texas leaf cutting ant (Atta texana) use methyl 4-methylpyrrole-2-carboxylate as a trail substance to guide other workers to a new food source.[4] Substances having a similar function in the flying insects are known as recruiting pheromones. For example workers of the honey bee (A. mellifera) use monoterpene acids, alcohols and aldehydes for marking food sources.[5]

Some members of the beetle families are known to use aggregation or assembling pheromones in the propagation of species. This may be illustrated by reference to the male bark beetle (Ips confusus)which, when boring in ponderoso

pine, produces a mixture of compounds that are carried to the exterior in the frass. This mixture is attractive to both male and female I. confusus causing a combined attack on the same host plant, thereby assembling a breeding population.[6]

The use of sex pheromones in the communication between male and female is common to most insect species and can be traced to the early observations of the French naturalist Jean Henry Fabr who at the turn of the century, investigated the ability of sexually mature females of the great peacock moth (Saturnia pyri) to attract large numbers of male moths, seemingly over long distances.[7] Further studies have revealed that in addition to the long range attractants, some insects rely on short-range stimulants or aphrodisiacs to orientate or prepare a mate for copulation. Here, males of the danaid butterflies Lycorea ceres ceres and Danaus gillipsis berenice produce sex pheromones which are applied, during flight, to the antennae of a female inducing her to alight and orientate for paring.[8, 9] Also known is the use of a sex pheromone as a repellant. Males of the mealworm beetle (Tenebrio moliter)having located a female, emit a pheromone which inhibits the response of other males to the same female. This pheromone may even be transferred to the female to make her less attractive to other males.[10]

The identification of these chemical messengers is an active field of research today involving the collaborative efforts of chemists and entomologist. These compounds are readily synthesised in the laboratory where their availability and

purity promote a variety of interdisciplinary studies. Further, the synthetic sex pheromones are of value to the agricultural sector where their function as attractants has been exploited in survey programmes to monitor the spread or appearance of a particular insect pest. This is illustrated by reference to the use of "Trimedlure" as an attractant for the Meditteranean fruit fly. This compound was used as a survey tool in Florida in 1956 to determine the localities of infestations. In the following year insecticides were applied to these areas to eradicate the pest. The cost was $11 million against an estimated annual loss to the citrus industry of $20 million.

2. Insect sex pheromones

The use of pheromones in the sexual communication between male and female has been demonstrated in a large number of insect species from various orders. Many of these species have been listed by Jacobson.[11] The majority have been demonstrated in the order Lepidoptera where some of the most spectacular examples of long range attraction can be found. In most cases the pheromone is produced by the female where it may attract a male of the species from a distance or serve to excite a male which is in close proximity, say on the same host plant. Fewer examples of male pheromones dominating the sexual communication system have been demonstrated. In many of these the male pheromone functions as an aphrodisiac in stimulating the female and making her more receptive

for copulation. The pheromone of the male butterfly L. ceres ceres is a case in point.[8] Alternatively, the pheromone may act as an arrestant in preventing the female from escaping thus fulfilling the same function. The pheromone ($C_6H_5.CHO$) of the male southern army worm moth Pseudaletria separata is an example of this type.[12] Some species produce one pheromone only, while others rely on a complex communication system where several pheromones govern the behavioural responses leading to mating. Several species produce the same chemical as a sex pheromone but are reproductively isolated in the field by differing seasonal and diurnal cycles. For example males of Nedra ramosula and Choristoneura fractivitana are both attracted to cis-tetradec-11-en-1-ol but are active at midnight and 8 p.m. respectively.[32] Alternatively the pheromone is associated with a second chemical which may act as an inhibitor or synergist thereby giving the female more specificity than obtained with a single compound. These factors are of particular importance to the Lepidoptera where many species are shown to produce the same sex pheromone.

1. Isolation studies

Although the occurence of sex pheromones has been widely demonstrated in the Insecta relatively few of these compounds have been isolated and structurally identified. Progress in this respect has accelerated during the past two decades since the pioneering work of Butenandt and

Karlson[38] on the ecdysones from insects and Butenandt's group [13] on the sex pheromone from the industrial silk-moth (Bombyx mori). During this period the increased demand for alternatives to the insecticide control of insect pests has paralleled the development and refinement of sophisticated physical and chemical techniques for the isolation and characterisation of microgram quantities of natural compounds. Even though these techniques are readily available isolation attempts are faced with several difficulties. These maybe summarised as follows: i) The quantity of pheromone extractable from one insect is normally at the submicrogram level which requires the collection of large numbers of insects to yield enough pure pheromone for spectral analysis. These large populations must be obtained by either (a) controlled breeding which frequently necessitates the use of artificial media and may therefore influence the formation of the pheromone or by (b) collection in the field which entails extensive organisation. To illustrate the former, reference is made to the studies in these laboratories on the sex pheromone of the false codling moth (Argyroploce leucotreta) where 1,700 000 moths were raised on an artificial media before exact details of the isolation stages were worked out.[14] Further, the reality of these microgram quantities is presented by reference to some of the isolated pheromones from the Lepidoptera (Table 1).

Table 1

The quantities of natural pheromone isolated from some species of the Lepidoptera.

Species	Quantity of isolated pheromone (in mg)	No. of males X 10^3	Ref.
Argyroploce leucotreta Meyr.	$\angle$ 0.1	$\pm$500	14
Argyrotaenia velutinana Walker	0.2	40	15
Bombyx mori L.	12.0	500	13
Pectinophora gossypiella Saunders	1.5	850	39
Prodenia eridania	3.9,0.8	305	16
Porthetria dispar L.	0.01	78	17
Spodoptera frugiperda T. E. Smith	0.9	135	18

ii) Several isolation stages are required to separate the micro quantities of pheromone from the bulk quantities of the initial crude extracts. The stability of the pheromone during these stages must be thoroughly investigated before the techniques

required can be applied.

iii) Difficulty is often encountered in the development of a valid laboratory assay which is an accurate reflection of male response in the field. Such an assay using controlled artificial conditions in the laboratory is of inestimable value in monitoring the isolation steps as the alternative use of field tests is influenced by the seasonal appearance of the insect and prevailing environmental factors.

iv) Due to the extreme biological activity of many of the sex pheromones it is not unlikely that a seemingly active compound showing a symmetrical peak on various g.l.c. columns will show no activity on subsequent identification and synthesis. The active component is therefore masked by the large peak from which it might be difficult to separate.

v) Further, the pheromone can on occasion be more than one substance so that small quantities of individual compounds have to be isolated and tested as mixtures.

2. Sex pheromones of the Lepidoptera

In the order Lepidoptera are found the butterflies and the moths. While the former are largely daylight fliers where the importance of pheromones in the attraction between the sexes is exceeded by that of visual stimuli, most moths are nocturnal in their habits relying largely on chemical attraction between male and female. This

chemical attraction is highly developed in some moth species such as the wattle bagworm (Acanthopsyche junodi) and the lappet moth (Mesocelis) where the adult females do not leave their cocoons but protrude only their abdomens for copulation; males of these species therefore rely entirely on chemical stimuli to find the females. Alternatively, in the feeding species such as the false codling moth (A. leucotreta) pheromone attraction may be augmented by food lures and host plant odours.

The pheromone glands of the female Lepidoptera are generally located in the intersegmental membrane between the terminal abdominal segments where they appear in a variety of evertible forms. [19]

As the intersegmental membrane is withdrawn into the second last segment during the resting period release of the pheromone is achieved by stretching the abdomen and exposing the gland to the air. In the male butterflies the pheromones are typically dispensed from extrudible brushlike structures called "hairpencils" while males of the greater wax moth (Galleria mallonella) produce a pheromone in glands located on the wings. [20]

i) Chemical structure

The moth pheromones which have been isolated from crude extracts of the sex glands and subsequently identified show a marked similarity in structure. With four exceptions they are all long chain unsaturated alcohols (C_{12}-C_{16}) or their acetate derivatives, of the general structure (I).

$$CH_3.\left[CH_2\right]_n CH{=}CH.\left[CH_2\right]_m .O.R \qquad I$$

R , H or $.CO.CH_3$; m, 5 - 11

These pheromones are listed in Table 2a and 2b. Sex attractants are not included.

TABLE 2a

The moth sex pheromones which appear as alcohols and other types.

ALCOHOLS			
Family	Chain length	Unsaturation	Ref
Bombycidae Bombyx mori L.	16	trans-10,cis-12	13
OTHERS			
Hadenidae Leucania impura	$C_6H_5.CHO$		31
Pseudaletia separata	$C_6H_5.CHO$		12
Lymantriidae Porthetria dispar L.	$CH_3.\left[CH_2\right]_9.CH\text{-}CH.\left[CH_2\right]_4 CH(CH_3)_2$ (O bridge)		17
Galleriidae Galleria mellonella L.	$CH_3.\left[CH_2\right]_9 CHO$		20

TABLE 2b

The moth sex pheromones which appear as acetates. Species producing two pheromones are shown *

Family	Chain length of alcohol moeity	Unsaturation	Ref
Gelechiidae			
Pectinophora gossypiella Saunders	13 (propyl branching)	trans-5	39
Tortricidae			
Adoxophyes fasciata *	14	cis-9;cis-11	21
Adoxophyes orana F.U.R. *	14	cis-9;cis-11	22
Argyroploce leucotreta Meyr	12	trans-7	14
Argyrotaenia velutinana Walker	14	cis-11	15
Choristoneura rosaceana	14	cis-11	23
Grapholitha molesta Busk	12	cis-8	24
Noctuidae			
Prodenia eridania *	14	cis-9; cis-9;trans-12	25
Spodoptera frugiperda J. E. Smith	14	cis-9	18
Trichoplusia ni Hubner	12	cis-7	26
Phycitidae			
Ephestia elutella	14	cis-9,trans-12	27
Plodia interpunctella Hubner	14	cis-9,trans-12	28
Olethreutidae			
Paralobesia viteana	12	cis-9	29
Pyralidae			
Cadra cutella	14	cis-9,trans-12	30

The pheromones which appear as esters are all acetate derivatives of C_{12} or C_{14} unsaturated alcohols where the double bond(s) is predominantly located at Δ^7, Δ^9 or Δ^{11} having a cis configuration. The P. dispar pheromone differs in that it is an epoxide but still retains the long aliphatic chain. It is of interest that the pheromones of G.mellonella, L. impura and P. separata which show the greatest deviation in structure are produced by the male moths in each case where they function as short-range stimulants or arrestants in preparing the females for mating. It is evident that some pheromones are not family or species specific as cis-tetradec-9-en-1-yl acetate is the pheromone of S. frugiperda and P. viteana . It also occurs in A.orana and P. eridania where it is associated separately with cis-tetradec-11-en-1-yl acetate and cis, trans-tetradeca-9, 12-dienyl acetate while the same acetate cis, trans-tetradeca-9, 12-dienyl acetate occurs as the pheromone of A. velutinana, C. rosaceana and C. cutella.

The structural similarity of the moth sex pheromones is further strengthened by comparison with the sex attractants discovered for numerous moth species by field or electro-antennogram screening tests. For example, new sex attractants were found for 37 moth species, by Roelofs and Comeau, during field trials using traps baited with 26 monounsaturated alcohols and acetates.[32] These attractants cannot be rightly regarded as the sex pheromones of the responding species as they were not identified by isolation and structural

studies. However, strong indication of their possible function in the pheromone system is given by the consistent response of the male moths to one out of the 26 compounds presented. Further, the majority of species were attracted by the acetate derivatives of C_{12} and C_{14} unsaturated alcohols with unsaturation at Δ^7, Δ^9 or Δ^{11} which is in agreement with the findings from the isolated moth pheromones. Some of the attracted species formed groupings of taxonomic interest. In the family Tortricidae four species of the subfamily Olethreutinae responded to the 12 carbon chain acetates while six species of the subfamily Tortricinae were attracted by C_{14} acetates and alcohols with unsaturation in the 11 position. Also, two pairs of closely related or sibling species showing similar seasonal and diurnal cycles were reproductively isolated in the field by way of their attraction to different geometrical isomers.

Other male sex attractants have been discovered by initially using the electroantennogram as a rapid screening procedure with subsequent bioassay in the field. Examples here are cis-tetradec-11-enyl acetate for males of the larch bud moth Zeiraphera diniana [33] and trans, trans-dodeca-8, 10-dien-1-ol for males of the codling moth Laspeyresia pomonella.[34]

In comparison the sex pheromones of the butterflies show a greater variation in structure. Similar long chain unsaturated acetates have been isolated as sex pheromones from the hair pencil secretions of species from the

sub-family Danaina where cis-hexadecanyl acetate and cis-octadec-11-en-1-yl acetate (11) are produced by the male butterfly Lycorea ceres ceres Cramer.[8]

$$CH_3.\left[CH_2\right]_5.CH{=}CH.\left[CH_2\right]_{10}.O.CO.CH_3 \qquad \text{II}$$

Other sex pheromones from the hair pencil secretions include the terpene alcohols trans, trans-3,7-dimethyl-deca-2,6-dien-1,10-diol (IIIa) and 3,7-dimethyloct-2-en-1,8-diol (IV) from males of L. ceres ceres [35] and the African Monarch butterfly;[36] and the terpene acids trans, trans-10-hydroxy-3,7-dimethyldec-2,6-dienoic acid (IIIb) and trans, trans-3,7-dimethyldec-2,6-dien-1,10-dioate (IIIc) of Danaus plexippus.[37]

$$R^1.\left|CH_2\right|_2.C(CH_3){=}CH.\left[CH_2\right]_2.C(CH_3){=}CH.R \qquad \text{III}$$

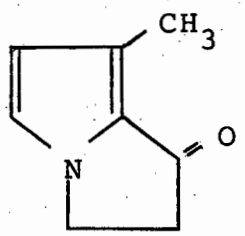
IIIa R,.CH_2OH ; R^1,CH_2OH

IIIb R,.CO_2H ; R^1,CH_2OH

IIIc R,.CO_2H ; R^1,CO_2H

$$HO.CH_2.C(CH_3).\left[CH_2\right]_3.C(CH_3){=}CH.CH_2.OH \qquad \text{IV}$$

Further, the heterocyclic ketone 2,3-dihydro-7-methyl-1H-pyrrolizin-1-one (V) is a sex pheromone common to all these species.



V

With all this information available to him, the author set out to isolate the sex pheromone from the female pine tree emperor moth Nudaurelia cytherea cytherea Fabr. (Lepidoptera:saturniidae) which is a pest in the timber plantations of Southern Africa.

CHAPTER II

THE PINE TREE EMPEROR MOTH

1. Introduction

The pine tree emperor moth (Nudaurelia cytherea Fabr.) (Lepidoptera : Saturniidae) is indigenous to Southern Africa and has long been known as a pest of certain shrubs and timber trees. A typical male and female moth is shown in Fig. II.1.

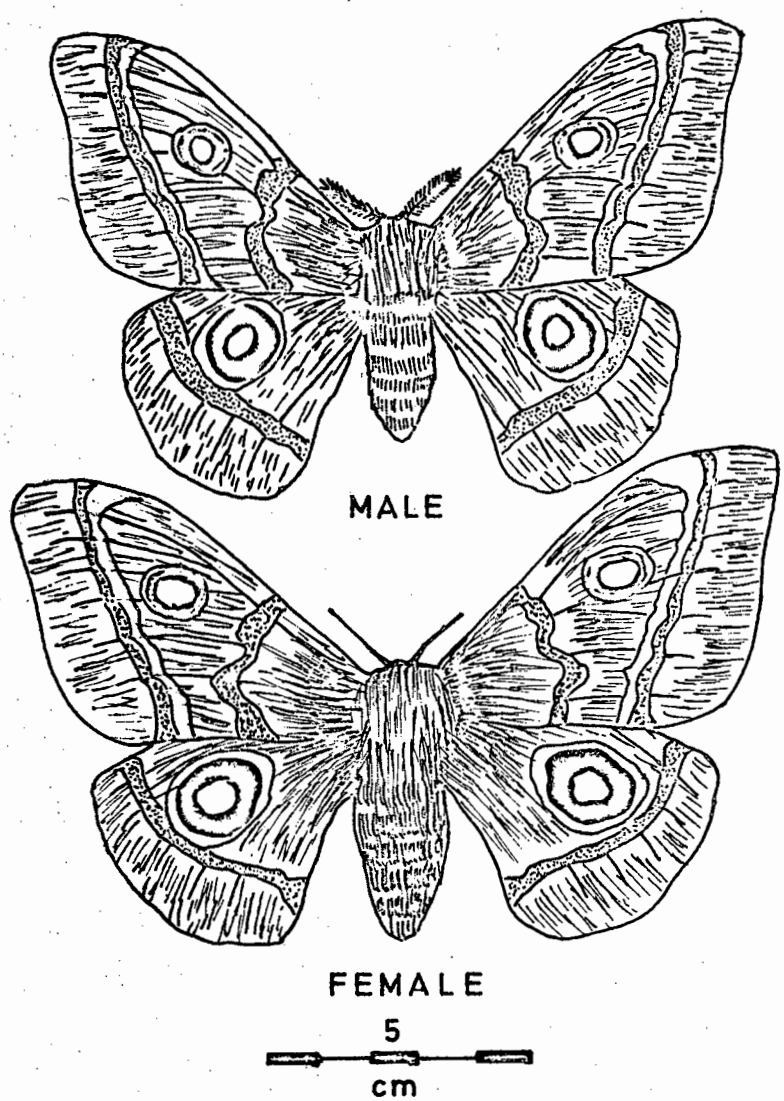


Figure II.1. The pine tree emperor moth Nudaurelia cytherea cytherea (Fabr.).

In a recent study by Geertsema[40] it was shown that two subspecies occur in South Africa. These are N. cytherea clarki and N. cytherea cytherea. The former subspecie is found in the Transvaal, Natal and Swaziland where it occurs sporadically in epidemic numbers on trees mainly of Pinus patula Schl. et Cham. The latter subspecie is found in the coastal belt of the Eastern, Southern and Western Cape where it is mainly a pest of timber trees (Pinus radiata D. Don) and windbreaks. A third subspecie N. cytherea lucida (Rothsch.) is found in Tanganyika but is not known as a forest pest.

The sex pheromone study discussed in this thesis has been confined to the subspecie N. cytherea cytherea, and was prompted by the importance of this insect as a pest of timber trees where it was anticipated that identification of the sex pheromone from the female moth would promote exciting ecological studies and play an important role in survey and control programmes. The larvae cause considerable damage to pine plantations through defoliation of the trees. If this defoliation is continuous, a decrease in the wood increment and a lack of growth results.[41] Scorching of the bark by the sun is also known as is deformation of the crown and tree. Fortunately the proliferation of this insect is partially controlled by numerous species of egg parasites, a virus which attacks the larvae, and numerous predators. Control measures employed to date include: 1) The keeping of wild pigs and guinea fowl in the plantations; 2) the manual collection

of larvae; and 3) the aerial spraying of chemical and biological insecticides.

It would seem that females of the two South African subspecies (N. cytherea cytherea and N. cytherea clarki) utilise the same sex pheromone, as a moth population in which both subspecies interbreed freely, is found in the Transkei. Furthermore, crude sex pheromone extracts from females of each subspecie attract males from both subspecies while the male antennae of either subspecies can be used in electroantennogram (EAG) studies. The reaction of male moths of the subspecie N. cytherea lucida to sex pheromone extracts from females of the other subspecies has not been investigated.

2. Life history

The findings of a detailed study into the life history of both N. cytherea cytherea and N. cytherea clarki have recently been reported by Geertsema[42] and in view of this comprehensive work and a number of earlier publications[43,45] a very brief description of the life history of the former subspecie is given below.

The insect is capable of one generation per year with moths being found in various localities in Southern Africa between the months of January to May. The moth flying season is short, rarely lasting longer than ten weeks. During this period males and females are actively engaged

in finding a mate by way of a sophisticated lure and receptor system in which the female sex pheromone acts as an attractant, luring males from long distances. Females emit the sex pheromone at night only, preferring the early hours of the morning. After mating, each female lays approximately 100 eggs, large numbers of which are destroyed by several species of egg parasites.

The larvae emerge after 5 weeks and initially consume a portion of the empty egg shells. If denied access to the shells the larvae refuse to eat and eventually die of starvation. After four months the larvae are fully grown and are almost 10 cm in length. At this stage the body is brownish-red in colour and covered in bright blue, yellow and green spangles. During the fifth instar the caterpillars leave the host plant and burrow into the soil usually at the base of the host plant, to a depth of 4 - 6 cm where a flimsy cocoon is spun and pupation takes place. The duration of the pupal stage varies from between 5 to 9 months

3. Collection and storage of pupae

During the numerous moth seasons in which attempts were made to isolate the female sex pheromone, supplies of pupae were obtained from state and privately owned pine plantations and to a lesser extent from belts of trees serving as windbreaks on farms in the Klapmuts area in the Western Cape. For this study pupae were collected

from infested areas in the municipal plantation at George (Southern Cape) and from the state forest at Kraaifontein (Western Cape). The labour required was either supplied by the Forestry Department or recruited from private sources and paid on a commission basis. Pupae were invariably found near the base of the pine trees, in soft sandy soil or immediately below the needle mat found in most plantations. They were best exposed by means of an ordinary garden fork or rake. An inherent disadvantage was the loss of a fair number of pupae through impalement. These,if not immediately removed soon rotted and made further handling of the live pupae very unpleasant. A new approach to the collection of pupae of the pine tree emperor moth was attempted by other workers [45] during the previous moth season. Mature larvae were gathered in the pine plantations and housed in two rooms, the floors of which were covered by a two inch layer of soil. Foliage was cut daily and fed to the larvae which, when ready to pupate, left the branches and burrowed into the soil. Unfortunately, a virus propogated in one of the rooms destroying almost twenty thousand larvae and a promising means of obtaining large numbers of pupae as the collection of larvae is uncomplicated and does not involve a large labour force.

Past experience had shown that it was essential to collect pupae as early in the pupal stage as possible if high emergence figures were to be anticipated. The reason being that towards the end of the stage the pupal case

weakens considerably and is easily damaged. Robust handling was also found to have a detrimental effect on the emergence figures. Pupae were therefore transported in cardboard boxes with liberal use of pine needle matting as packing material. Handling was cut to a minimum and once sexed and stored the pupae were left undisturbed until emergence. Highest emergence figures were obtained from pupae stored, uncovered, on sawdust beds in a cool well ventilated room. Various attempts at covering pupae with moist sawdust or needle matting did not seem to enhance emergence patterns.

The pupae collected for this study totalled almost 130,000. Roughly half were obtained from the Western Cape and the other half from the Southern Cape. A disappointing emergence figure of 35% was shown by the latter pupal collection. This low return was probably due to the rough handling the pupae received and the unusually cold weather (these pupae were kept in the open under a needle covering). In comparison, the pupae from the Western Cape which were collected early in the season and handled carefully showed an emergence figure of 91%. From the total pupal collection, almost 34,000 female moths were obtained and the abdominal tips made available for extraction processes in two batches of roughly 17,000 each.

4. Sexing of pupae

Through the observation that male and female pine tree emperor moths copulated soon after emergence, under the crowded conditions of emergence in the laboratory, it became essential to separate them into male and female populations. The reason being that extracts from the sex glands of mated females were found by the present author to be unattractive to male moths. This is likely due to a halt in the production of pheromone or to the presence of a male masking pheromone.

Separation of the sexes was readily achieved in the pupal stage by examination of the ventral surface of the last abdominal segment. The difference between male and female pupae (Fig. II.2) is clearly discernible with the naked eye as the pupae are large and easily handled.

Figure II.2. Male and female pupae of the pine tree emperor moth *N. cytherea cythera.*

5. Collection of "wild" females

The relatively high costs involved in the collection of large numbers of pupae and the difficulties encountered in the hiring of the necessary labour force, prompted an attempt at the collection of female pine tree emperor moths from pine plantations as a means of obtaining substantial numbers for crude extract purposes.

Initially, collection involved the night attraction (before 12 p.m.) of moths to strategically placed mercury vapour lamps. Although many moths were caught (3300 ♂, 145 ♀; 640 ♂, 44 ♀), the method proved to be unsatisfactory due to the small number of females attracted. This method was therefore abandoned and replaced by the daily collection of newly emerged females from the same section of the plantation. The abdominal tips from these females were amputated and macerated in methanol. A portion (10 ♀ ME) of the crude extract obtained was bioassayed on the EAG and in field tests where it was found to be inactive. A similar finding has been reported by Casida, Coppel and Wantanabe[46] for crude sex pheromone extracts from "wild" females of the introduced pine sawfly (D. Similis) and by Read[47] during isolation studies on the sex pheromone of the false codling moth (A. Leucotreta). In both cases pheromone activity was regenerated in the crude extracts by fractionation on column and gas-liquid chromatography. Portions (500 ♀ ME) of the inactive

extract from "wild" females of the pine tree emperor moth were therefore purified by various chromatographic techniques (column chromatography, t.l.c., g.l.c.) but without success. As a result of these findings, gathering of wild females for crude extract purposes was not included in the collection plans for this study. This result was disappointing in view of the ease of collection and moderate labour demands involved in this procedure.

CHAPTER III

BIOASSAY METHODS

In the identification of insect pheromones the development of a suitable bioassay to monitor the pheromone through the isolation stages is a prerequisite for any meaningful study. The author was fortunate, in this work on the sex pheromone of the pine tree emperor moth, in that two reliable bioassay methods were available. These methods, developed by other workers in this laboratory[48] during earlier exploratory studies, involved the attraction of male moths to baited traps deployed in infested parts of the pine forests and the use of excised male antennae in the electroantennogram (EAG). These procedures are discussed below with reference to the modifications introduced by the author.

1. The electroantennogram (EAG)

The EAG was first used by Schneider and Hecker[49] in their pioneer investigations into the olfactory function in males of the silkworm moth (B. mori). Their technique has since been adopted by workers as a bioassay method for the reception of odours by insects and in the study of insect olfactory receptors.[50,51]

The antennal response to odour stimulation is easily recorded by the EAG. This response is composed of the combined simultaneously elicited receptor potentials in the many olfactory sense cells of the antenna and represents a negative change in the d.c. potential between one electrode inserted near the tip of the antenna relative to the other electrode situated in the haemolymph at the base of the antenna. By means of a carefully designed airflow system a current of air is passed firstly over an adsorbent material impregnated with an active pheromone extract and then over a suitably mounted antenna. The resulting response is amplified and studied through use of an oscilloscope. When recording with moth antennae it is normally possible to work almost as efficiently with severed as with intact antennae. This phenomenon is generally not applicable to non-lepidopterous insects as the antennal responses disappear soon after amputation.[50] Most EAG recordings are therefore carried out on immobilised living insects.

The EAG has found little application as a quantitative bioassay method for the evaluation of the efficiency of purification techniques used during isolation studies on crude extracts. An inherent disadvantage in attempting quantitative analyses is the possible antennal response to other components in such extracts. Coupled with the possible complimentatory antennal responses are the

inhibitory properties shown by some extracts. The inhibition of pheromone response is well known and has been attributed to solvents,[52] to male or female "masking" pheromones[46] or to isomers of the pheromone (see Chapter VI, section 2.1). The EAG has, therefore, functioned mainly as a qualitative assay method indicating the presence or absence of the pheromone in a particular extract or fraction thereof. Here, the EAG proved to be the only laboratory bioassay procedure available for a study of the chemical behaviour of the sex pheromone of the female pine tree emperor moth, during the concentration and isolation processes. Indications that this technique was a valid bioassay for the detection of the pine tree emperor moth sex pheromone were obtained by the author during earlier attempts, which extended over two seasons, at isolating the sex pheromone. During these studies the pheromone was located after each purification step by using the EAG with subsequent confirmation by field trapping experiments. At no stage during these isolation studies was an antennal response elicited by more than one fraction or component of the extract. These findings were taken as an indication that male antennae responded only to the sex pheromone and not to any other compound present in the crude extracts. It was accepted then, that the EAG could be used as a bioassay for the pine tree emperor sex pheromone without the necessary

confirmative field tests. This allowed the present author to rely largely on a laboratory assay thereby saving valuable time and effort normally spent on field assays. Periodic confirmatory surveys were carried out and the results of these tests are given in the experimental section, Chapter VII. As a final safeguard against any irregular responses all fractions and residues remaining after each purification step were labelled and stored at 0^{o}.

During this study the EAG was used purely for qualitative assay to indicate the presence or absence of the pheromone in a particular test fraction. The antennal response to the pheromone fraction was positive, as a peak amplitude on the oscilloscope of between 0.8 - 2.0 millivolts (mV) was always obtained while the response to other fractions from the same extract was equal to, or slightly greater, up to 0.2 mV, than the response (0.2 - 0.5 mV) to the solvent blank (test sample containing solvent only). However, when in later studies it was found that antennae responded equally to the synthetic pheromone and its positional isomers, it became necessary to put the EAG on a semi-quantitative basis. This was achieved by the determination of the stimulus-response curve from which an "active-response level" (ARL) of 0.82 mV was calculated. All responses exceeding this value were regarded as active while responses of a lower magnitude were recorded as inactive. Accordingly, early EAG responses

have been recalculated to present a uniform system throughout this work.

1. Materials

The general procedure for recording antennal responses is described in Chapter VII, Section 1.1.

Antennae from 1-4 day old male pine tree emperor moths were used in this biological assay method. As it was observed that the response of excised male antennae compared favourably with that of antennae from mechanically fixed living male moths the use of the former was preferred as their mounting proved to be less complicated and time consuming.

The micro-electrodes used were prepared from glass capillaries which were drawn to a fine point at one end. These capillaries were filled with O.1N saline solution and fitted with platinum wire electrodes.

Recordings were made with a Tektronix Model 564B Storage Oscilloscope fitted with a Type 2B67 Time Base and a Type 2A61 Differential Amplifier. Unless otherwise specified a sweeprate of 1.7 sec./division was used. The oscilloscope lead and mounting platform were enclosed in an earthed aluminium-screened cage (Fig.III.2) to eliminate static and 50 cycles mains interference. The complete shape of the EAG response curve was found to vary with antennae. All, however,

showed a rapid initial negative change in potential at the recording electrode. The amplitude of this initial change was used as a quantitative measure of the strength of the response and was recorded in millivolts. A typical antennal response to a pheromone source (A) and a solvent blank (B) is shown in figure III.1.

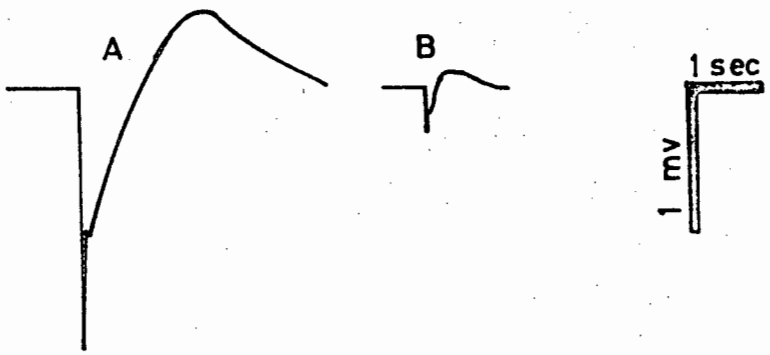


Figure III.1. A typical response, as recorded on the EAG, of a male antenna from a pine tree emperor moth when exposed to an extract A (1 ♀ ME) from a virgin female moth and to a solvent blank B(10 µl, pentane).

The airflow system used is shown in Fig. III.2.

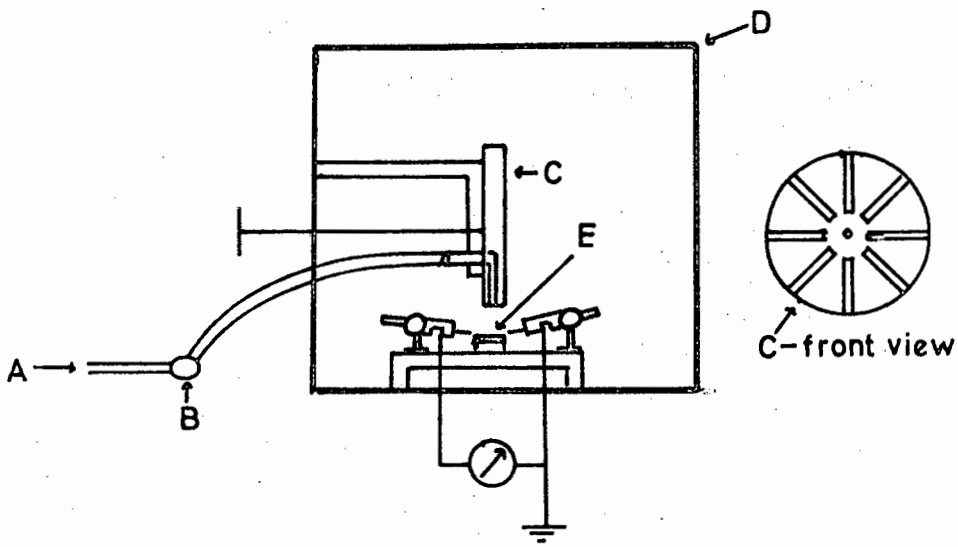


Figure III.2. The airflow system used to deliver odorous puffs of air onto a mounted antenna : A = air supply at 1 Litre/ min.; B = manually operated two-way valve; C = perspex selector disc holding 8 sample cartridges; D = screened - aluminium cage (100 x 45 x 60 cm) ; and E = mounted antenna.

Sex pheromone contamination of the testing room used in earlier studies was minimised by using an ordinary laboratory fan to disperse tested or expelled vapours. Erratic results were obtained on some occasions after prolonged testing due to contamination of the atmosphere. An exhaust fan was therefore fitted to the rear of the aluminium cage for more efficient removal of the pheromone vapours. Further stringent precautions (Chapter VII,

Section 1.1) were taken to prevent misleading results through contamination of the testing apparatus and the atmosphere in the testing room.

2. Experimental

In a continuation of the early EAG work on the N. cytherea sex pheromone by Warren and Bosman[48], the author introduced the use of a two minute rest period between consecutive antennal stimulations. This rest period was required to allow antennae to recover fully from each stimulation in order to obtain comparable responses and to provide sufficient time for the removal of expelled or tested vapours by the exhaust fan. Other variables, which influenced antennal responses, such as 1) the age of male moths, 2) the position of insertion of the glass electrodes into the antennal shaft, 3) the velocity of the air stream through the airflow system, and 4) the distance of the mounted antennae below the exhaust port of the selector disc, were investigated by Warren and Bosman[48] or supplemented by the author. Acquired values of these variables were used in all tests.

(i) Rest Period: Seven antennae were prepared (Chapter VII.1.1) and exposed to a pheromone extract (2 ♀ ME) two minutes after an initial exposure to the same

extract. Similarly five antennae were exposed to a pheromone extract (2 ♀ ME) at a one minute time interval. To standardise the stimulus source a fresh cartridge was used for every antenna. The results of the tests (Fig. III.3) reveal that essentially complete recovery of antennal sensitivity had occurred within both rest periods.

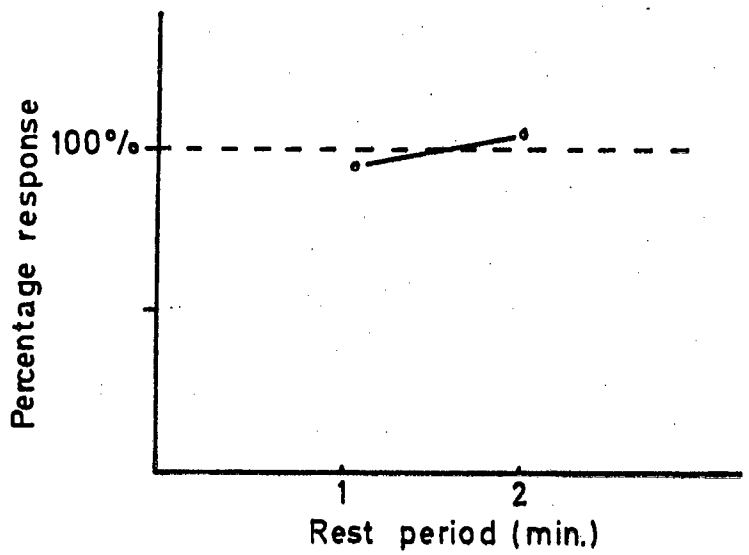


Figure III.3. The mean percentage recovery of male pine tree emperor moth antennae exposed to a natural pheromone extract (2 ♀ ME) at one and two minute intervals after an initial exposure to the same extract.

These results are in keeping with those reported for male antennae of the cabbage looper moth (T. ni),[53] where antennal sensitivity was recovered within 30 to 60 seconds;[50] for males of the silkworm moth (B. mori) and for males of the rice stem borer (Chilo suppressalis).[54]

For practical reasons the two minute rest period was preferred as it allowed the use of a 45 second extraction period to remove expelled vapours and for subsequent air turbulence to subside.

(ii) Stimulus-response curve: A dilution series from a virgin female extract was used as the stimulus source in the preparation of this curve. The responses were accepted as a true measure of the pheromone response as no evidence of masking or synergistic effects by other compounds in the crude extract was encountered in previous studies.

Ten antennae were prepared and exposed to a dilution series of 0.01, 0.1, 0.5, 1, 3, 5 and 10 moth equivalents per treatment. The extract concentrations were presented in ascending order to minimise antennal adaptation to the pheromone. Stock solutions were prepared such that each test sample was presented in a constant volume of solvent (cyclohexane, 30 µl). A response to the solvent blank (pure solvent) was determined 30 seconds before the response to each test sample was recorded. The results of the test are given in Table 1 and are presented as the mean of the responses to each concentration $\pm$ the standard deviation.

TABLE I

EAG responses (mean ± S.D.) of 10 male pine tree emperor moth antennae on exposure to a dilution series from a virgin female extract.

	EAG response (mV)	
	Female extract	Solvent blank
Treatment (♀ ME)	mean ± S.D.	mean ± S.D.
0.01	.70 ± .24	.20 ± .12
0.1	.99 ± .35	.20 ± .13
0.5	1.31 ± .34	.21 ± .12
1.0	1.21 ± .39	.21 ± .12
3.0	1.10 ± .28	.22 ± .15
5.0	1.16 ± .31	.23 ± .15
10.0	1.18 ± .29	.24 ± .16

From a graphical presentation of the results (Fig.III.4) it was evident that the antennal response increased to a plateau between 0.1 and 1.0 ♀ ME thereafter to remain constant.

The active response level was calculated, here, at three times the greatest standard deviation above the highest mean of the solvent blank responses obtained in testing each of the stimuli 0.5, 1, 3, 5 and 10♀ ME/test sample. This gave an active response level (ARL) of -0.72 mV which covers almost 90% of all solvent blank responses. This value was arbitrarily increased by -0.1 mV to give the ARL of -0.82 mV which was used in the re-presentation of all EAG responses.

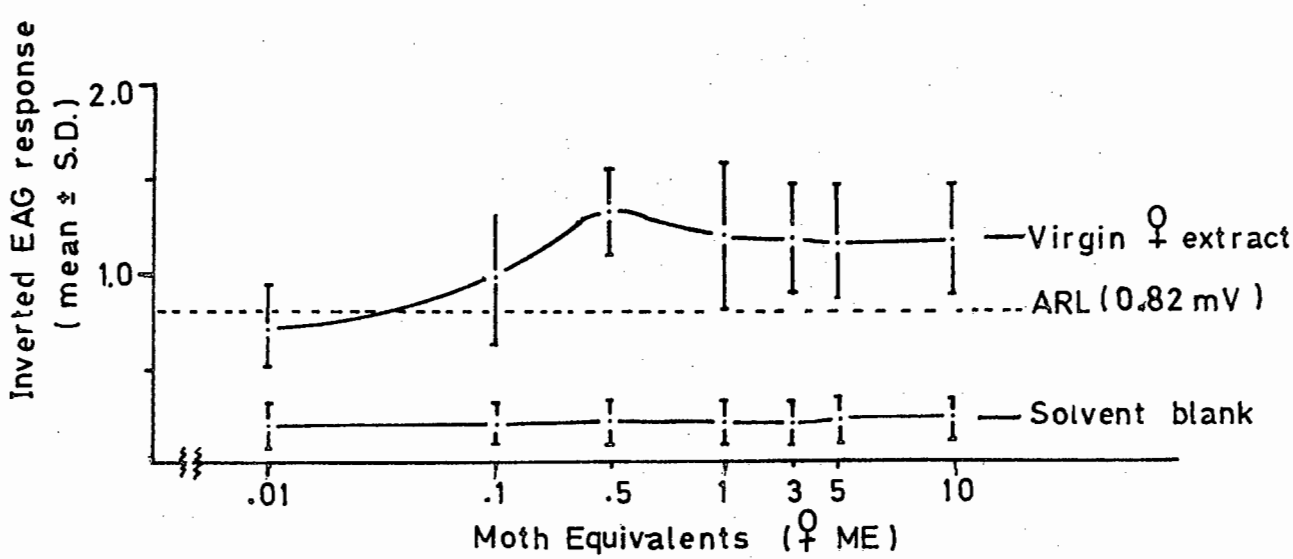


Figure III.4. An inverted plot of the means (-mV ± S.D.) of the antennal responses of 10 male pine tree emperor moth antennae to a dilution series from a virgin female extract (A) and a solvent blank (B) (cyclohexane).

2. Field tests

Field trapping tests were carried out in order to verify those positive responses obtained on the EAG and to provide the final confirmation of the structure of the N. cytherea sex pheromone by the evaluation of male response to the cis and trans isomers of dec-5-en-1-yl isovalerate. A description of the experimental procedures followed in these tests is given in Chapter VII, section 1.2.

1. Materials : Trapping areas were limited to the Western Cape only. The early tests leading towards the isolation of the sex pheromone were carried out in the State plantations at Franschhoek while other tests using the synthetic isovalerate esters were conducted in the State forests at Swellendam and Kraaifontein.

The traps used were of one type only and were identical with those used by other workers in this laboratory in their early field studies on the N. cytherea sex pheromone.[48] The traps consisted of large metal trays (1.2 x 1.2 m) which were filled with water to a depth of 3.5 cm. The attractant source was placed on a gauze covered platform which was positioned in the middle of the tray. A metal box (15 x 15 x 30 cm), open at one end, was inverted over the platform and supported 2 cm above the surface of the water layer.

Males approaching the box were invariably trapped in the water and drowned. Paraffin and detergent were added to the water to increase the effectiveness of the traps while nicotine sulphate was included on some occasions as a killing agent.

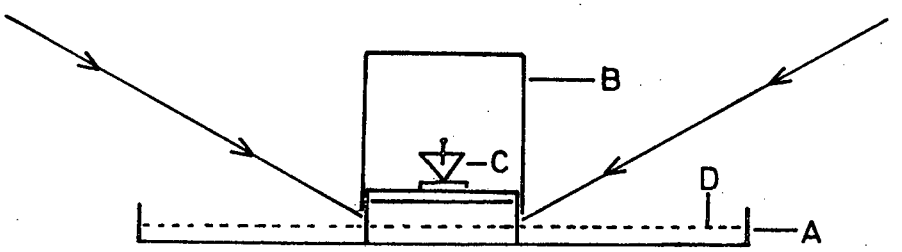


Figure III.5. The traps used in the bioassay of virgin female extracts and the synthetic isovalerate esters : A = metal tray (120 x 120 x 4 cm); B = protective metal box, open at the bottom; C = filter paper wick; D = water : paraffin: detergent mixture.

Wicks consisted of small triangular sections of filter paper pinned to a piece of polyurethane foam.

Weather data was not recorded as the tests conducted were largely qualitative assays seldom requiring more than a one night test period.

CHAPTER IV

ISOLATION AND STRUCTURAL STUDIES

1. Preliminary studies leading to the final isolation procedures and structural studies.

The isolation and identification of the N. cytherea sex pheromone, described in this thesis, is the culmination of many years of careful work by the present author and by other workers in this laboratory. The experience and information gained during the early studies was of inestimable value in the final study where the adbominal tips from 34,000 female moths were made available for extraction purposes. For example it was known that: 1) Extracts of the female sex pheromone could be prepared by two methods which involved the rinsing of female abdomens with pentane and the homogenisation of severed abdominal tips using dichloromethane or methanol as solvents; 2) the EAG could be used as a valid independant bioassay method for monitoring the sex pheromone during the isolation stages while field tests had led to the selection of an efficient trap design and the use of optimum testing conditions; 3) activity in a pheromone extract was not lost on separations by, a) gas-liquid chromatography (g.l.c.), on a variety of stationary phases, b) by thin-layer chromatography using silica gel G or silica gel G impregnated (25%) with silver

nitrate and, c) by column chromatography on neutral alumina, florisil, or silica gel/silver nitrate; and 4) the pheromone was probably a C_{13}-C_{15} unsaturated ester which was not an acetate.

Further, the author was fortunate in having a semi-pure extract from the abdominal tips of 12,000 virgin female pine tree emperor moths made available to him some time before the bulk extracts for the final study became available. This prompted the use of this extract in a pilot study to work out the exact details for the final experimental procedures. The details of this pilot study are discussed below as the column chromatography and preparative g.l.c. procedures adopted were duplicated in the final study. As pheromone losses had to be avoided at all stages special attention was given, here, to procedures such as a) the concentration and storage of the pheromone solutions b) the transfer of pheromone concentrates from one container to another c) the injection of preparative samples for g.l.c. studies and d) the recovery of the pheromone as a pure fraction on preparative g.l.c.

1. Pilot study

The extract used in this preliminary work had been partially purified by steam distillation followed by preparative g.l.c. on a Carbowax 20M column. Before further isolation studies on another g.l.c. phase could be pursued, it was evident that a copious amount of a C_{19} hydrocarbon accidentally introduced into the extract

would have to be removed as it held the same retention time as the pheromone on some g.l.c. stationary phases. The hydrocarbon, introduced during the steam distillation step and originating from a high melting point wax added to the crude extracted oil to prevent excessive foaming, was removed by column chromatography. Florisil (60/110 mesh) was used with development by pentane containing various percentages of acetone. The pheromone was recovered from the pentane-acetone (99:1) eluent while the hydrocarbon was found, by g.l.c. analysis, to have eluted with the pure pentane fraction. The now purified pheromone extract was immediately subjected to isolation studies by preparative g.l.c. in an attempt to obtain a pure active compound for spectral analysis.

i) Preparative gas-liquid chromatography

To minimise the loss of pheromone on g.l.c. studies, special attention was given to conditions of operation, of the gas chromatograph, which allowed analyses at high instrument sensitivity. For example: 1) Care was exercised in the preparation of the g.l.c. columns with preference for lightly loaded packings as they showed little column bleed thereby giving stable base lines on both isothermal and programmed runs and, also caused little contamination of collected samples from preparative runs; 2) interference by septum bleed was minimised by baking each septum before use; and 3) the

flame detector was regularly cleaned.

To assist in the isolation of the pheromone, five methyl esters of long chain saturated fatty acids (C_{11} - C_{15}) were used as reference compounds (markers) to facilitate the accurate determination of the retention time of the pheromone on the g.l.c. stationary phases. These esters were not used as internal standards as they would have interfered in the isolation of the pheromone but were chromatographed as a separate run immediately before the pheromone samples were injected. Both runs were carried out under identical conditions and one chromatogram superimposed upon the other. The methyl esters of fatty acids have been used by Read[47] in these laboratories and by other workers[55] as reference compounds leading to the characterisation of the retention times of unknown substances in terms of their "Equivalent Chain Length" (ECL). This useful index is calculated by logarithmic interpolation between the esters of two fatty acids and is interpreted as the chain length of a hypothetical fatty acid which should appear at the same place as the substance being characterised. The index was used, in this pilot study, in the comparison of the g.l.c. retention times of numerous C_{10} alcohols with those of the pheromone.

The polar phase FFAP (free fatty acid phase) was used as it showed good separation of the extract components and little tailing of ester type compounds. The

retention time of the sex pheromone on this phase was determined by the injection of a portion of the pheromone concentrate (from column chromatography) with the collection of various fractions between the C_{12} and C_{15} ester markers (C_{12} marker is $C_{10}H_{21}.CO_2CH_3$, i.e. $C_{12}H_{24}O_2$). Bioassay of these fractions revealed the active area to be that immediately preceding the C_{14} marker. Before preparative collections were pursued it was necessary to obtain a chromatogram of the pheromone extract and identify the pheromone peak. This was achieved by analysis of a larger portion of the extract with collection and bioassay (EAG) of all peaks appearing between the C_{13} and C_{14} markers. The known active area revealed a single large peak which proved to be active. This chromatogram is shown in Fig.IV.1.

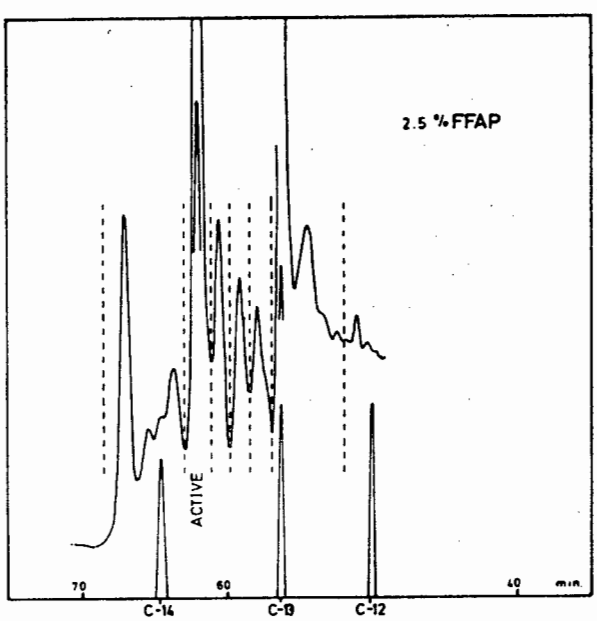


Figure IV.1. A section of the chromatogram of the pheromone extract used in the pilot study showing the pheromone peak in fraction 4.

The compound(s) making up the active peak were isolated in six preparative runs while the collection of the g.l.c. fractions was achieved by passage of the effluent gases through hypodermic needles leading into a cooled solvent. This technique was particularly suited to the gas chromatograph used, where the outlet nozzle of the streamsplitter was hidden by a layer of insulating asbestos, as the long hypodermic needles were readily inserted down the narrow guide tube leading through the asbestos layer to the outlet nozzle. The elegant technique of Witte and Dissinger[56] (note 1) could not be used as modifications to the stream splitter system were not possible under the existing conditions. A quantitative analysis, by g.l.c., of the collected material revealed that an insufficient quantity (ca. 30 µg) had been isolated to allow for further purification using another column. A large portion (80%) was therefore used to obtain a mass spectrum as it was hoped to detect the molecular ion peaks of the compound(s) present and determine their molecular formulae.

Note 1: G.l.c. fractions in this technique are adsorbed on zones of microcrystalline organic material prepared by rapid cooling of a common spectroscopic solvent (chloroform, carbon tetrachloride) in liquid nitrogen. These active filter zones are prepared in glass U-tubes of various dimensions with as little as 2 µl of solvent reported to give an active zone. Collection efficiencies of 99% are reported for a variety of organic compounds.

(ii) Mass spectrometry

A mass spectrum was obtained on a g.l.c. coupled mass spectrometer fitted with a Biemann separator. The gas chromatograph was fitted with the same FFAP column and the sample chromatographed under conditions similar to those employed in the preparative runs. High and low energy spectra showed no peaks higher than a fragment at m/e 138. An accurate mass measurement gave this fragment a molecular formula of $C_{10}H_{18}$. Other significant fragments appeared at m/e 110 and m/e 44 (base peak). It was realised that a $C_{10}H_{18}$ hydrocarbon could not have shown a retention time similar to that of the sex pheromone and was therefore assumed to be an artefact. As artefact production is encountered in the mass spectra of alcohols, chlorides, mercaptans and long chain esters[58] and due to the fact that the phase FFAP is selective in its behaviour in that alcohols are strongly held, it was thought that a C_{10} alcohol may show a retention time somewhat similar to that of the C_{14} marker. Several monoterpene alcohols were readily available and were chromatographed for comparison purposes. It was found that none of these alcohols showed the same g.l.c. behaviour as the sex pheromone as they all held retention times less than that of the C_{13} marker. A detailed interpretation of the mass spectrum obtained was not attempted as the fraction analysed must have consisted of more than one compound to give an overlapping fragmentation pattern.

At this stage, female abdominal tips from the Southern Cape moth collection were made available for extraction and isolation studies.

2. Final study leading to the identification of the sex pheromone

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The experimental details are described in Chapter VII.

The terminal abdominal segments from 34,000 female pine tree emperor moths were made available for extraction purposes in two batches of 17,000 segments each. One batch was obtained from female moths emerging in the early stages of the moth season while the other batch contained the segments from the females which emerged later. It was planned to isolate a sufficient quantity of the sex pheromone from the extract of the first collection of abdominal segments to allow for structural studies and subsequent synthesis before the end of the moth season. This would have enabled the author to carry out immediately the crucial verification of structure by testing the attractancy of the synthetic compound in the field. Although the isolation was completed on schedule the quantity of pheromone obtained proved to be insufficient for n.m.r. analysis. This material was therefore set aside and finally added to the quantity of pheromone isolated from the second extract. Structural studies were then effected on this combined material.

1. Extraction procedures and initial purification

The crude pheromone extracts were prepared by homogenisation of the severed abdominal segments in a Waring Blender using dichloromethane as solvent. The frozen tips were ground to a fine powder in a coffee grinder, prior to homogenisation, as this increased the extraction efficiency.

In past work, the oils obtained from the solvent extraction processes were next steam distilled and the volatile sex pheromone trapped in the steam distillate. In this study it was preferred to follow the procedure of Sekul and Sparks [18] with prior removal of inactive lipids by low temperature precipitation from acetone. It was subsequently found that this procedure gave a tenfold reduction in the weight of the steam volatile components. Whereas previously, large quantities of the methyl esters of myristic, palmitic and oleic acid were found, these acids were now absent.

Steam distillation of the lipid free oils from both batches of the female abdominal segments gave the active distillates S_1 (0.8 g) and S_2 (0.9 g) which were further purified by column chromatography using florisil 60/120 mesh as adsorbent. The columns were developed with pentane and pentane-acetone mixtures with acetone being preferred above diethyl ether as the latter could form peroxides at the column tip and hence destroy activity. This operation was carried out in a cold room

where fractions were collected by hand thereby providing constant supervision; it was considered too great a risk to use a fraction collector as the danger of mechanical failure is ever present. This purification step gave the pheromone concentrates C_1 (first batch) and C_2 (second batch) which were further purified by gas-liquid chromatography.

2. Preparative gas-liquid chromatography

(i) Stationary phase FFAP

The excellent separation of the components of the active residue from the pilot study on the stationary phase FFAP prompted the reuse of this phase in the isolation of the pheromone from the active column chromatographic concentrates C_1 and C_2.

The g.l.c. procedures adopted followed closely those used in the pilot study. The chromatograms obtained from the concentrates were almost identical, with the active fractions in each case showing a single large peak having a retention time slightly less than that of the C_{14} marker. This can be clearly seen in the chromatogram, from the concentrate C_1 , given in Fig. IV.2.

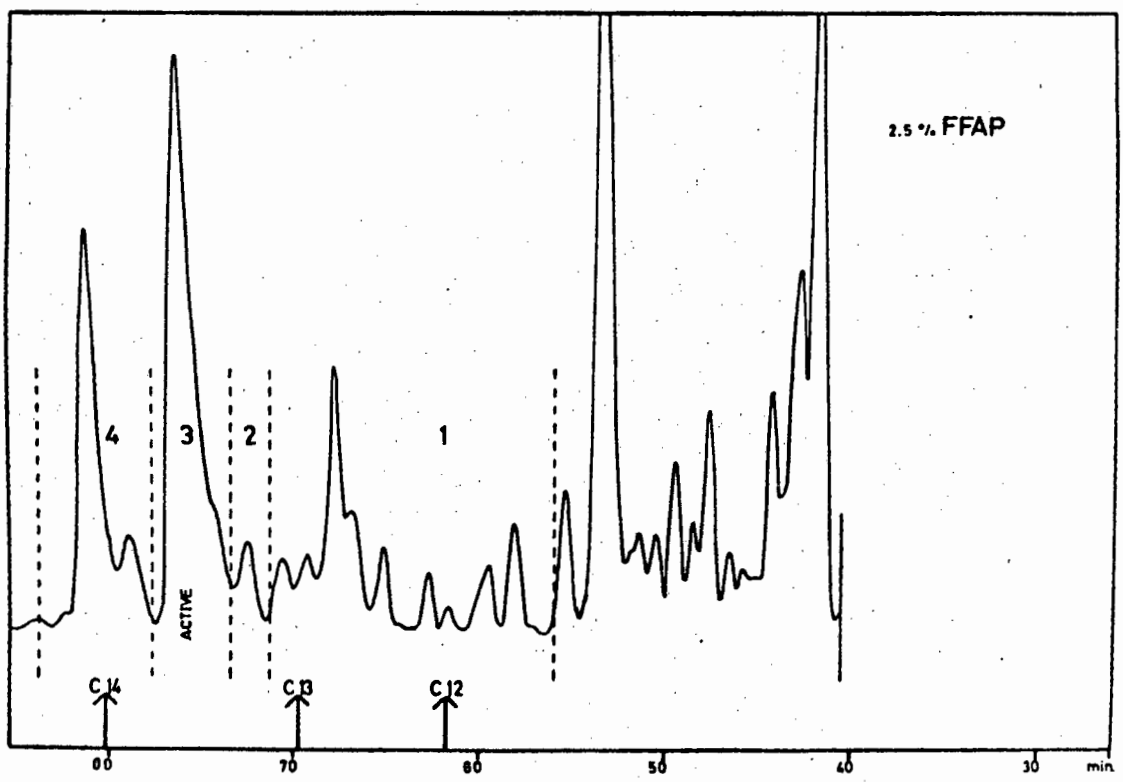


Figure IV.2. A section of the g.l.c. chromatogram from the active column chromatographic concentrate C_1 showing the single large peak in the active fraction 3.

The active compound(s) were then isolated from each concentrate in six preparative runs, using the collection vessels described previously, to give the active semi-pure concentrates G_1 and G_2 which were separately submitted to further preparative collections on another g.l.c. column.

(ii) Stationary phase OV 25

The less polar, phenyl silicone stationary phase OV 25 was selected for the final isolation of the pheromone. A glass column (6.6 m x 0.35 cm i.d.) packed with 4% OV 25 on Chromosorb W was used. The retention time of

the pheromone on this column was determined by superimposing the chromatogram of a portion (50 ♀ ME) of G_1 on the chromatogram of the ester markers with the collection for bioassay (EAG), of 10 fractions between the C_{12} and C_{15} markers. A positive response was elicited by the fraction which gave the pheromone a retention time identical with that of the C_{14} ester marker. A chromatogram of the pheromone concentrate G_1 (Fig. IV.3) was then obtained by injection of a preparative quantity under the same g.l.c. conditions.

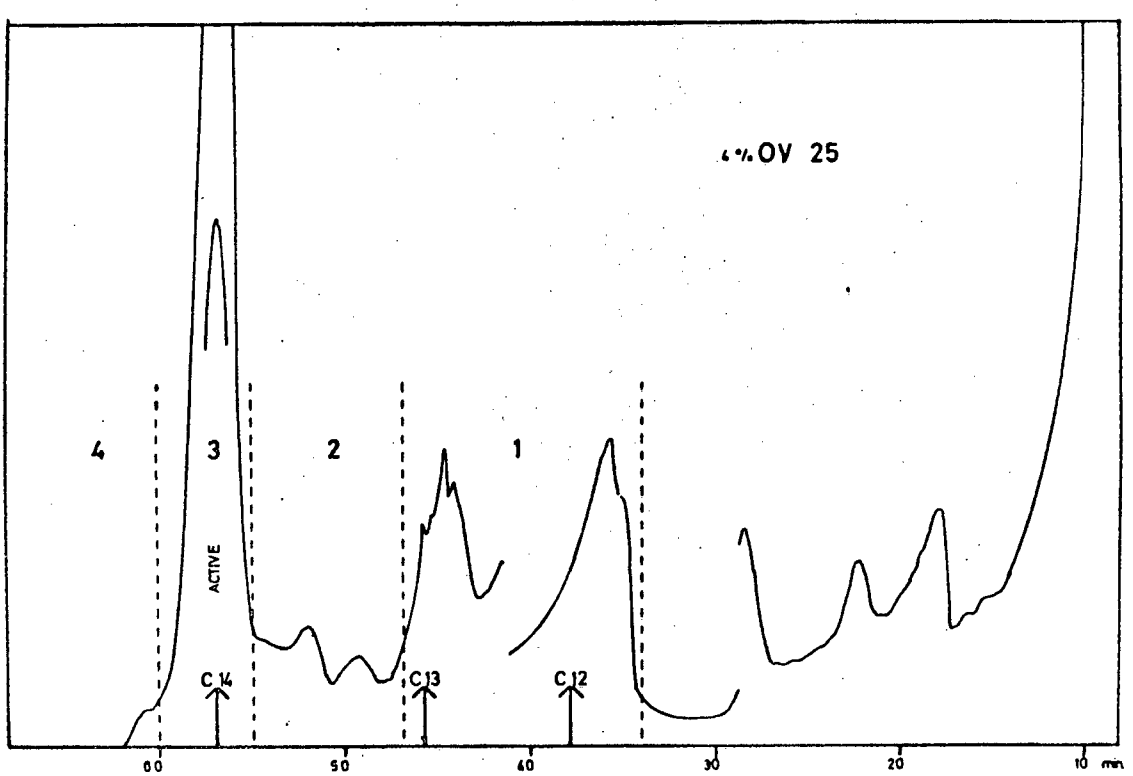


Figure IV.3. A section of the chromatogram from the active concentrate G_1 showing the single large peak in the active fraction 3.

The active area revealed a single large peak with no evident shoulders. This peak was trapped in a preparative collection vessel with spectroscopic carbon tetrachloride as solvent, in anticipation of n.m.r. studies. In all, four fractions were collected and portions of each (10 ♀ ME) bioassayed for activity on the EAG and in a field test in the pine plantations at Franschhoek. Only fraction 3 (single large peak) elicited a response on the EAG while fractions 3 and 4 trapped 150 and 7 moths, respectively, in the field test. The activity in fraction 4 was attributed to tailing of the pheromone on the column. Preparative collection of the active compound from the remainder of G_1 was effected in six runs. The pheromone trapped in fraction 4, was recovered by reinjection of the concentrate from this fraction with collection of the peak which appeared in the active area. The active carbon tetrachloride solution (P_1) was concentrated under a stream of pure nitrogen to a volume of *ca.* 100 µl and quantitatively transferred to a reacti-vial.

G.l.c. analysis of a portion of P_1 under analytical conditions revealed a single large peak from which it was estimated that the total quantity of the active component isolated was *ca.* 170 µg or 0.01 µg/female moth. This compound was considered to be the pheromone as a 10 ♀ ME portion representing 0.1 µg of the compound, had elicited a positive response on the EAG and attracted male moths in a field test. This

quantity (0.1 µg) was one tenth of the minimum quantity of the synthetic pheromone from the cabbage looper moth (T.ni)required to elicit a "significant" EAG response from male antennae;[53] one tenth to one hundredth of the quantity of synthetic (B. mori) pheromone required to elicit strong responses on the EAG[57] and one hundredth of the minimum quantity of the synthetic pheromone from the male queen butterfly (Danaus gilipus berenice) required to obtain a good EAG response from female antennae.[51]

Preparative g.l.c. of the pheromone concentrate G_2 (second batch of moth tips, ex FFAP) revealed a marked difference in the chromatograms from the two concentrates (G_1 and G_2). The chromatogram from G_2 (see Chapter VII, section 2.3.) revealed an additional large peak having a retention time similar to that of the C_{10} marker (estimated). Also, the active peak showed a distinct shoulder at its base which when trapped in a separate collecting vessel and bioassayed (EAG) was found to be inactive. The remainder of G_2 was chromatographed and the active component isolated in carbon tetrachloride. Concentration of this solution gave the pheromone concentrate P2 from which it was estimated that 150 µg of the active component had been isolated.

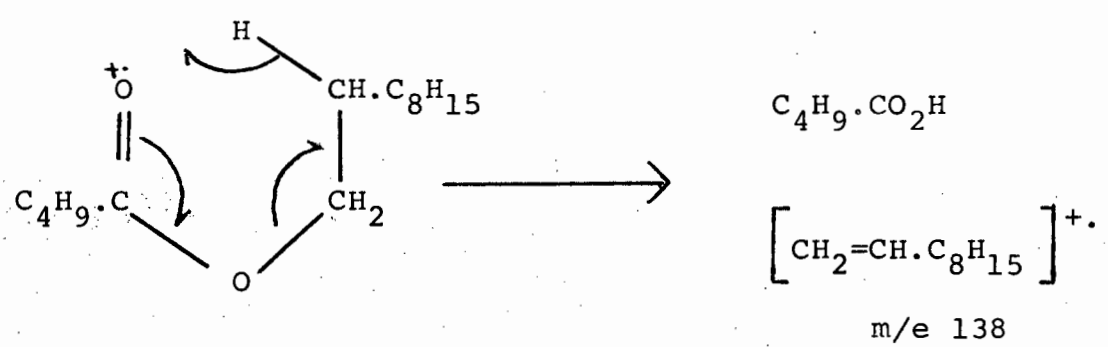
The active concentrates P1 and P2 were combined to give a total of 320 µg (0.01 µg/female moth) of the

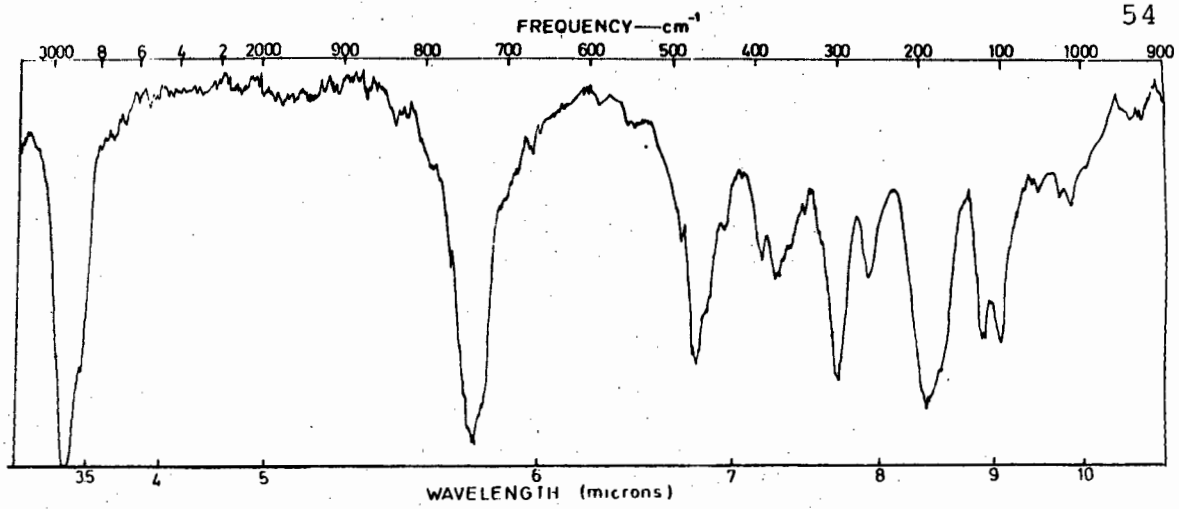
isolate component. This combined material was then used in the determination of the n.m.r., m.s. and i.r. spectra.

3. Interpretation of the spectral data

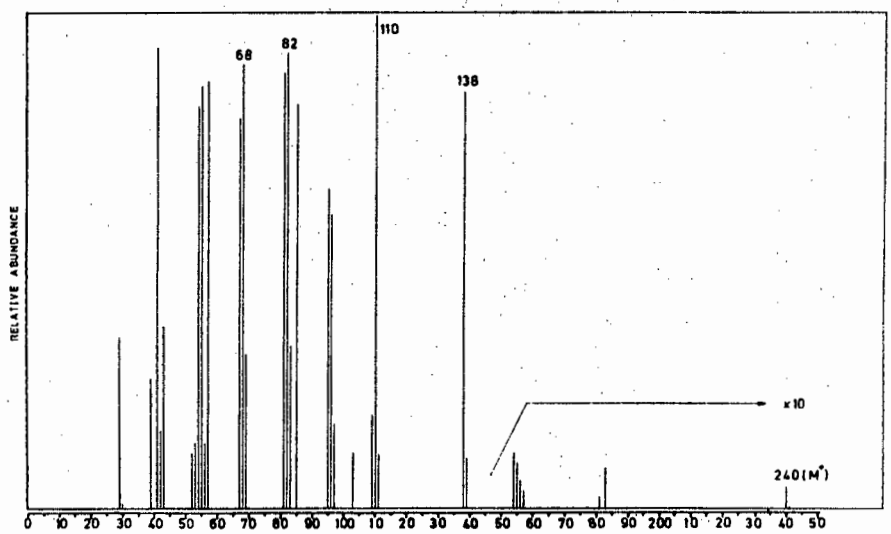
The experimental procedures followed in the determination of the n.m.r., m.s. and i.r. spectra are given in Chapter VII while the spectra obtained are given in Fig. IV.4.

From an accurate mass measurement on the molecular ion peak in the mass spectrum, the molecular formula of the isolated compound was shown to be $C_{15}H_{28}O_2$ (M^+, 0.1%, m/e 240.2089; $C_{15}H_{28}O_2$ requires m/e 240.2097). An aliphatic structure for the compound was revealed by the i.r. spectrum where carbonyl absorption at 1735 cm^{-1} indicated an ester grouping; the position of the carbonyl absorption showed that the acid moiety was not an acetate. This was in agreement with the n.m.r. spectrum which did not show a methyl singlet at τ 8.06 and with the chemical tests on crude extracts where acetylation of the inactive saponification products did not restore activity. The mass spectrum indicated that the acid was valeric acid with the fragment at m/e 138 ($C_{10}H_{18}$) resulting from a 1,2-elimination of the acid by way of a McLafferty rearrangement.[58]

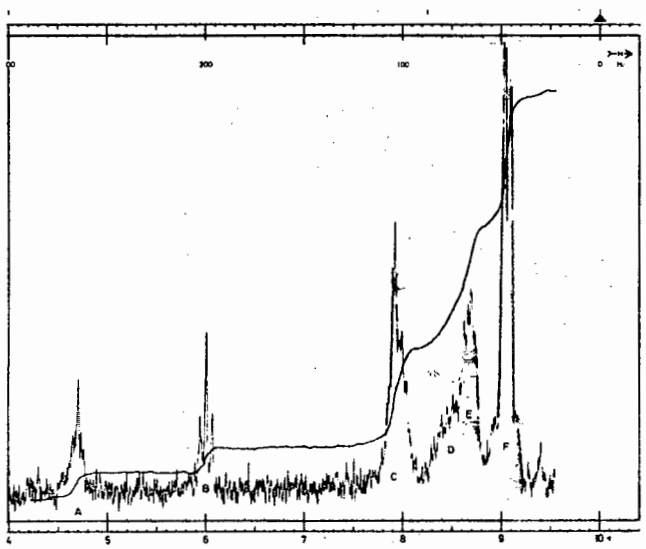




INFRARED SPECTRUM



MASS SPECTRUM



NMR SPECTRUM

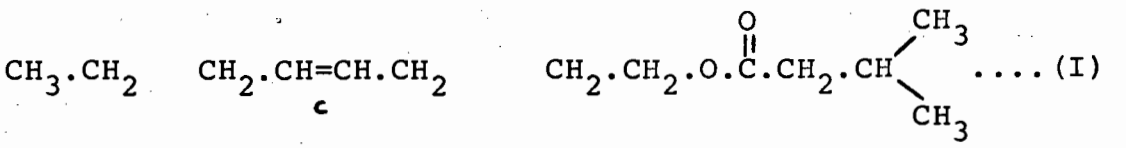
Figure IV.4. The spectra obtained on the isolated compound.

The olefinic signal at τ 4.71 in the n.m.r. spectrum showed a 1:2 proton ratio to indicate monounsaturation. These olefinic proton signals appeared as a multiplet through splitting by adjacent methylene protons. This was shown by decoupling of the methylene protons at τ 7.92 which collapsed the multiplet (τ 4.71) to a singlet. The i.r. spectrum indicated a cis configuration as no absorption band at 960 - 970 cm^{-1} (trans olefin) was evident. The triplet at τ 6.01 in the n.m.r. spectrum revealed that the signals from the methylene protons, adjacent to the oxygen atom, were split by the protons of an adjacent methylene group. The following molecular fragments could be written:

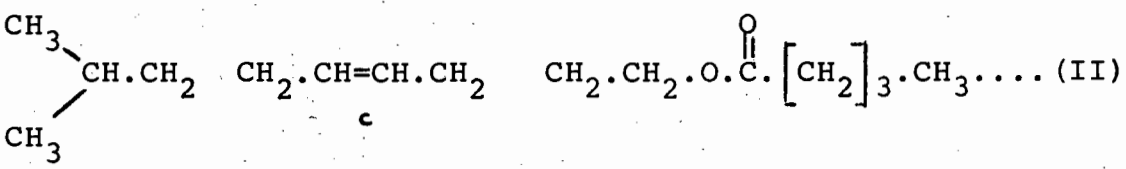
$CH_2.CH=CH.CH_2$ $\qquad\qquad$ $CH_2.CH_2.O.\overset{O}{\overset{\|}{C}}.C_4H_9$

The behaviour of the isolated compound on g.l.c., where it showed a retention time closely associated with that of the straight chain C_{14} and not the homologous C_{15} reference compound (marker) indicated methyl branching. This was confirmed by the characteristic upfield doublet in the n.m.r. spectrum at τ 9.03 which revealed the presence of an isopropyl group; each methyl group is split by the vicinal CH giving rise to two overlapping doublets (J 6.03 H_z). The isopropyl group was also indicated by the double peak in the i.r. spectrum at 1387 and 1371 cm^{-1}. The terminal methyl group signal at τ ca. 9.0, obscured by the isopropyl doublet, was indicated by the proton integration curve which showed a 1:9 ratio. The following

moieties could then be written and arranged in two sequences with the isopropyl group firstly on the acid (I) and then on the alcohol (II) moiety.



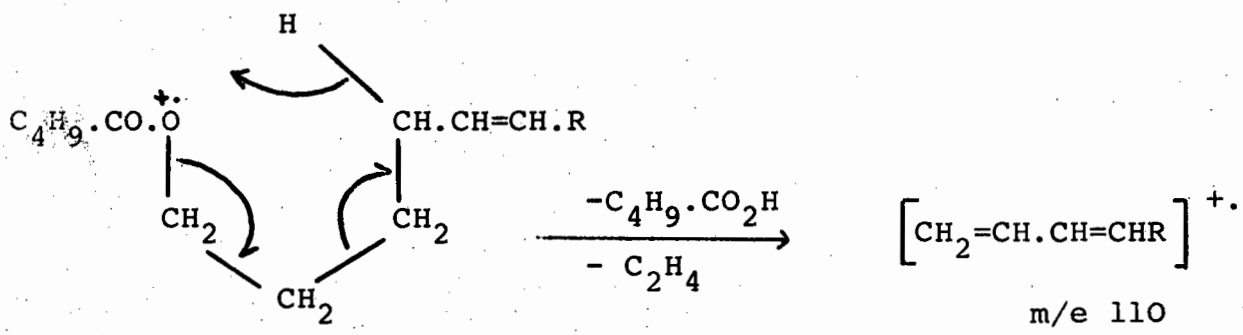
.... (I)



.... (II)

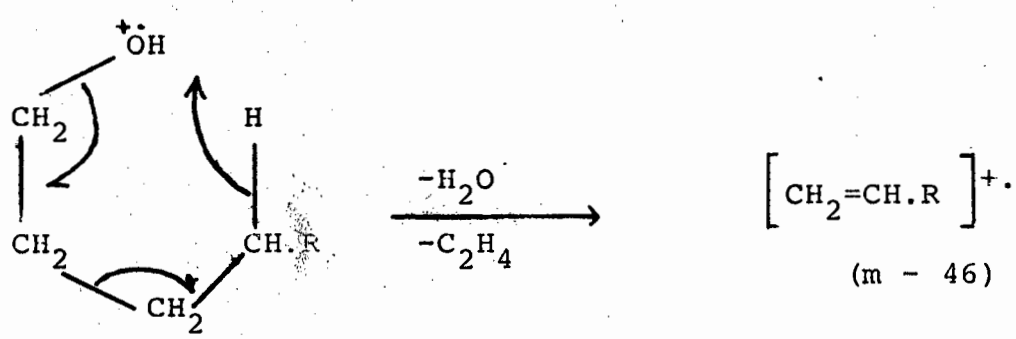
The C_5 acid moiety was envisaged as isovaleric (I) as the shift position of the isopropyl group in the n.m.r. spectrum at τ 9.03 suggested deshielding by the carbonyl group; the upfield doublets of an isopropyl group in a hydrocarbon chain appear at τ 9.13.[59] Further, there was no peak in the mass spectrum at m/e 43 (characteristic isopropyl fragment) as the isopropyl group was lost with the neutral acid fragment ($C_4H_9.CO_2H$).

The structure of the compound was then decen-1-yl isovalerate. The double bond was placed at C_5 to explain the fragment at m/e 110 (C_8H_{14}) in the mass spectrum which could occur by way of a 1,4-elimination of valeric acid promoted by an allylic proton with concomitent loss of ethylene respectively.



$R = C_4H_9$

A similar 1,4-elimination through a six-membered intermediate is encountered in the mass spectra of alkanols possessing more than four carbon atoms where the elimination of water is frequently coupled with the expulsion of ethylene leading to M-46 peaks ($M-H_2O+CH_2=CH_2$).[60]



The position of the double bond and the isopropyl group in the molecule could readily have been established by micro-ozonolysis, with identification of the resulting molecular fragments by g.l.c. This elegant technique[61] which requires a few micrograms of material, has been used in some insect pheromone studies.[17,62,63] Alternatively, oxidative fission using a mixture of potassium

periodate and potassium permanganate (Lemieux oxidation) was possible with subsequent identification of the methylated acid fragments by g.l.c.[64] Neither of these techniques, in which the parent compound is degraded and therefore not recoverable, was applied as any reduction in the quantity of the isolated material was avoided in view of further preparative g.l.c. studies should synthetic cis-dec-5-en-1-yl isovalerate be unattractive to male pine tree emperor moths in field tests.

It was preferred to establish the absolute structure through the synthesis of the proposed ester and comparison of its n.m.r., i.r. and mass spectra with those from the isolated compound. Other positional isomers and analogues were synthesised to provide additional structural confirmation and to allow an investigation into the structure-activity relationship in male sexual response and to promote a preliminary study of the female sex pheromones from other South African species of emperor moths through field screening tests.

4. Comparison with the spectral data from synthetic decenyl isovalerate esters.

The m.s. and n.m.r. spectra obtained on the synthetic isovalerate esters, i.e. cis and trans-dec-5-en-1-yl isovalerate and the positional isomers showing

unsaturation in the 4 and 6 positions, are included in the appendix section.

The spectral data obtained on the synthetic cis-dec-5-en-1-yl isovalerate was found to be identical with that obtained on the isolated active component while the spectra of the positional isomers showed marked discrepancies. The n.m.r. spectra from the latter esters differed most noticeably in the methylene proton signals at τ 8.4 - 8.6 while their mass spectra revealed a variation in the abundance of fragment ions in the mass range m/e 50 - 110. Also the n.m.r. spectrum of the analogous cis-8-methyl-non-4-en-1-yl valerate showed a difference in the methylene proton signals and an upfield shift in the signal from the isopropyl group.

It was evident then that the structure of the active compound isolated from extracts of the female moth was cis-dec-5-en-1-yl isovalerate. This compound was tested for activity in the field during the following moth season where it was found to attract male pine tree emperor moths in large numbers.

CHAPTER V

THE SYNTHESIS OF cis-DEC-5-EN-1-YL ISOVALERATE AND RELATED COMPOUNDS

1. Introduction

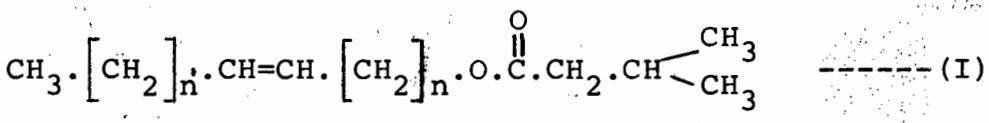
After a review of the syntheses of the known moth sex pheromones and considering the experience gained in this laboratory in the synthesis of the sex pheromone of the female false codling moth (A. Leucotreta) [65] the author elected to synthesise cis-dec-5-en-1-yl isovalerate and its positional isomers through the preparation of monosubstituted acetylenes which were condensed with alkyl halides to give the desired long aliphatic chains. Selective reduction of these acetylenic intermediates gave the pure cis and trans olefins. This method was particularly attractive in that it gave positional and geometric specificity for unsaturation in each of the esters and high yields of the final products. In addition, the method is economically adaptable to bulk syntheses.

2. Syntheses

In view of the extreme biological activity shown by most sex pheromones and the possible inhibitory effects by low concentrations of positional and geometric isomers, great care was exercised to prevent cross-contamination of the synthetic esters. The compounds were synthesised

sequentially to avoid possible mix up of the various reaction products while all apparatus used was thoroughly rinsed with ethanol and acetone and then placed in aqua regia or chromic acid.

The unsaturated valerate esters synthesised for spectral analysis and subsequent biological evaluation are given below.



------(I)

Ia. n' = 3 ; n = 4 (cis - 5)

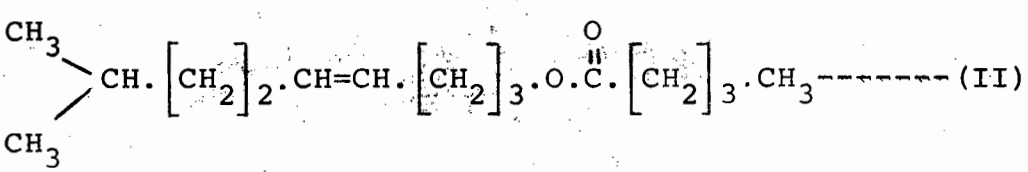
Ib. n' = 3 ; n = 4 (trans - 5)

Ic. n' = 4 ; n = 3 (cis - 4)

Id. n' = 4 ; n = 3 (trans - 4)

Ie. n' = 2 ; n = 5 (cis - 6)

If. n' = 2 ; n = 5 (trans - 6)



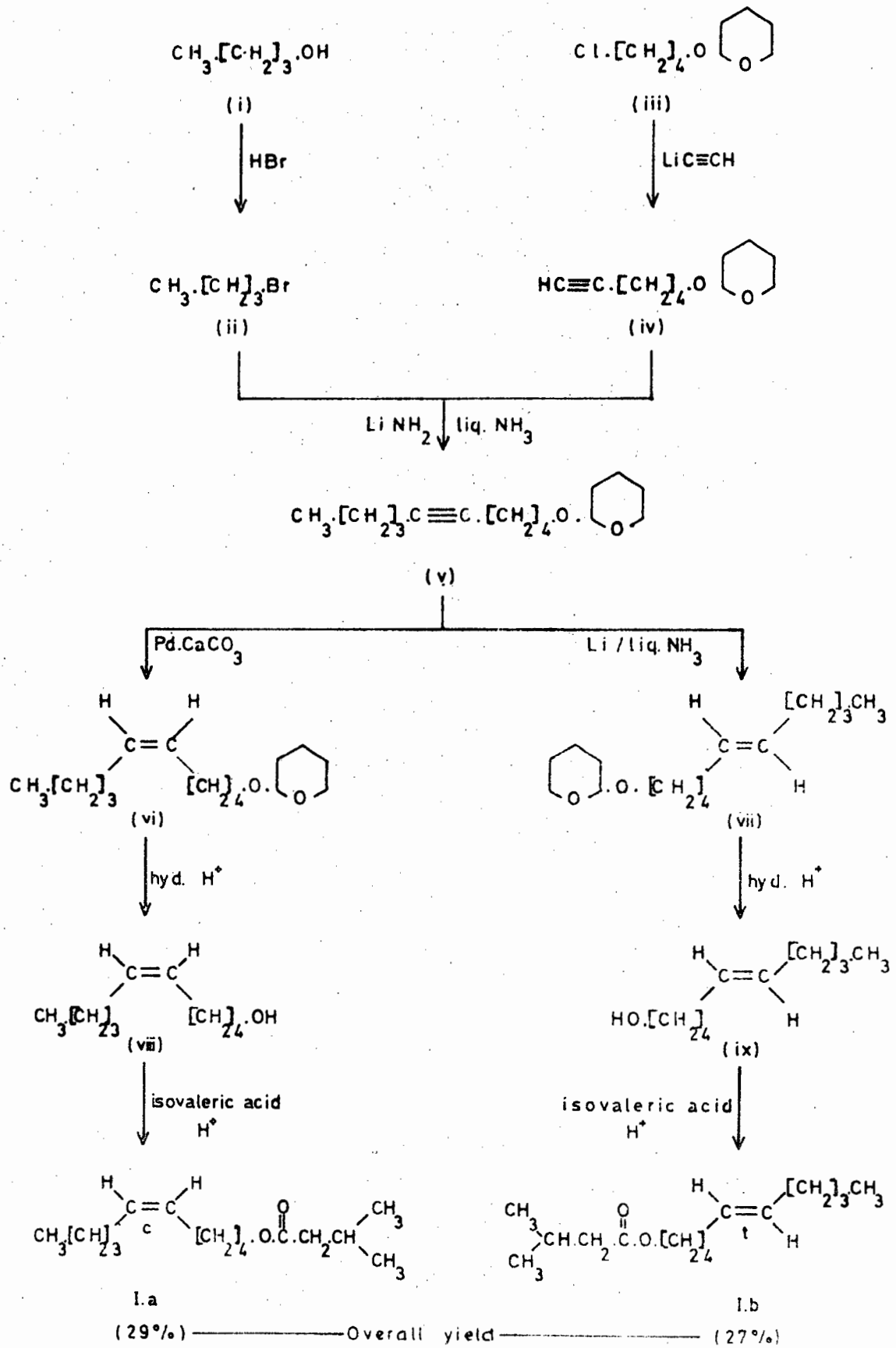
-------(II)

1. cis and trans-Dec-5-en-1-yl isovalerates (Ia and Ib).

The reaction sequence followed is given in Chart V.1. 4-Chlorobutan-1-ol was converted into its tetrahydropyranyl ether (iii) in 90.5% yield by reaction with dihydropyran, and then into hex-5-yn-1-yloxytetrahydropyran (iv) in 73.1% yield by reaction with lithium acetylide-ethylene-diamine complex in dry DMSO. Reaction of this acetylenic intermediate (iv) with sodamide in liquid ammonia and

CHART V.1

Synthesis of cis and trans-Dec-5-en-1-yl isovalerate



subsequent condensation with butyl bromide in the same media, gave dec-5-yn-1-yloxytetrahydropyran (v) in 69% yield. Catalytic reduction over Lindlar's catalyst (Pd-$CaCO_3$, poisoned by quinoline) gave the cis olefin (vi) while chemical reduction by lithium in liquid ammonia gave the trans olefin (vii). Acid hydrolysis of the respective unsaturated tetrahydropyranyl ethers gave separately cis and trans-dec-5-en-1-ol in 90 and 81% yields. Esterification with isovaleric acid by refluxing in benzene and catalytic amounts of conc. sulphuric acid gave the required esters cis-dec-5-en-1-yl isovalerate (Ia, 73% yield) and trans-dec-5-en-1-yl isovalerate (Ib, 74% yield). The overall yields were (Ia), 29% and (Ib), 27%.

2. cis and trans-Dec-4-en-1-yl isovalerates (Ic and Id).

Only the initial reaction sequence is given (Fig. V.1) as the later stages were identical with those given in Chart V.I.

Pent-4-yn-1-ol (ii) prepared in 61.5% yield, through ring scission of tetrahydrofurfuryl chloride (i) by sodamide in liquid ammonia, was converted into its tetrahydropyranyl ether (iii) in 85.5% yield. Formation of the sodio derivative of (iii) by sodamide in liquid ammonia followed by condensation with pentyl bromide gave dec-4-yn-1-yloxytetrahydropyran (iv) in 63.7% yield. Selective reduction gave the pure cis and trans olefins in quantitative yields. Acid hydrolysis gave the cis-decenol in 90% yield and the trans-decenol in

83% yield. Esterification with isovaleric acid gave the desired esters, cis-dec-4-en-1-yl isovalerate (Ic) in 71% yield and trans-dec-4-en-1-yl isovalerate (Id) in 77% yield. The overall yields were (Ic), 25% and (Id), 23%.

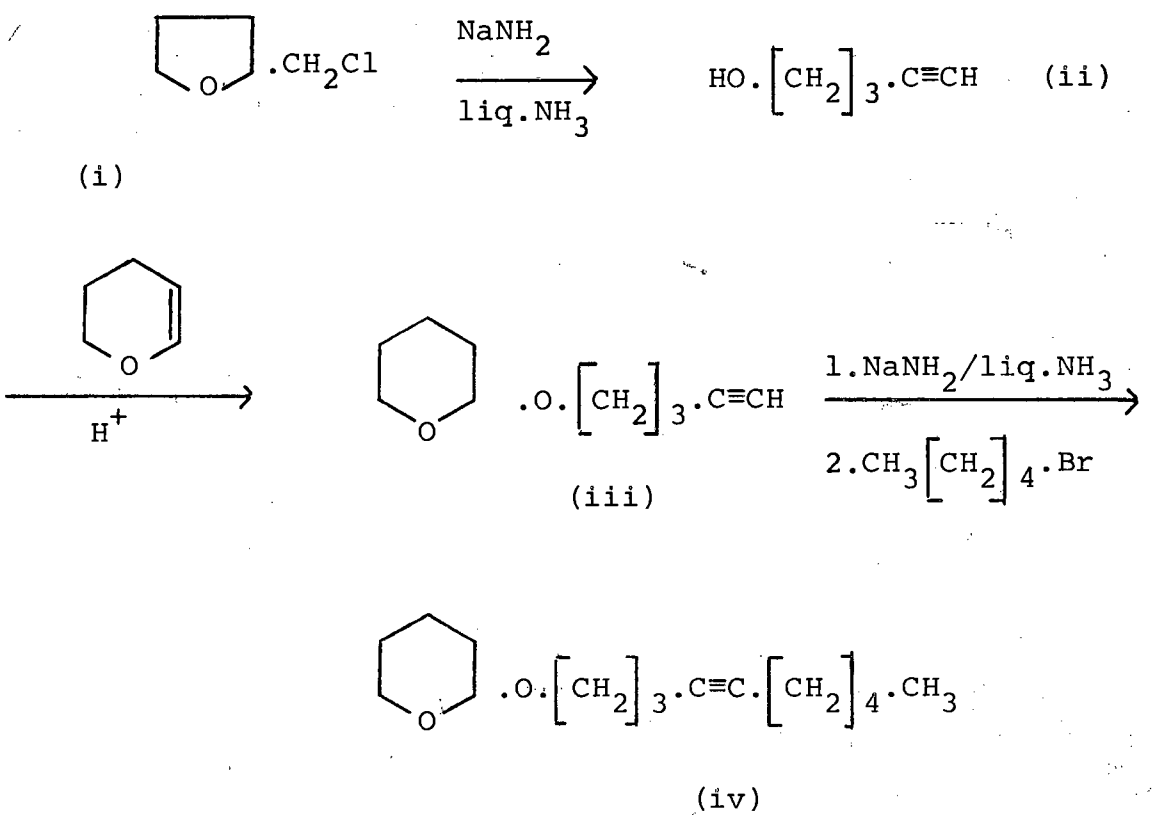


Figure V.1. The initial reaction sequences in the synthesis of cis and trans-dec-4-en-1-yl isovalerate.

3. cis and trans-Dec-6-en-1-yl isovalerate (Ie and If).

Similarly, only the initial reaction sequence is given (Fig. V.2.).

5-Chloropentan-1-ol (i), prepared through ring opening of tetrahydropyran by acetyl chloride followed by hydrolysis of the resulting chloroacetate, was converted into its tetrahydropyranyl derivative (ii) in an overall yield of 65%. Refluxing with sodium iodide in acetone gave the corresponding iodo compound (ii) which was condensed with lithium acetylide in liquid ammonia to give hep-6-yn-1-yloxytetrahydropyran (iii) in 57% yield. Formation of the lithio derivative of (iii) and condensation with propyl bromide gave dec-6-yn-1-yloxytetrahydropyran (iv) in 55% yield. Selective reduction followed by acid hydrolysis and esterification of the resulting decenols gave cis-dec-6-en-1-yl isovalerate (Ie) in an overall yield of 11% and trans-dec-6-en-1-yl isovalerate (If) in an overall yield of 13%.

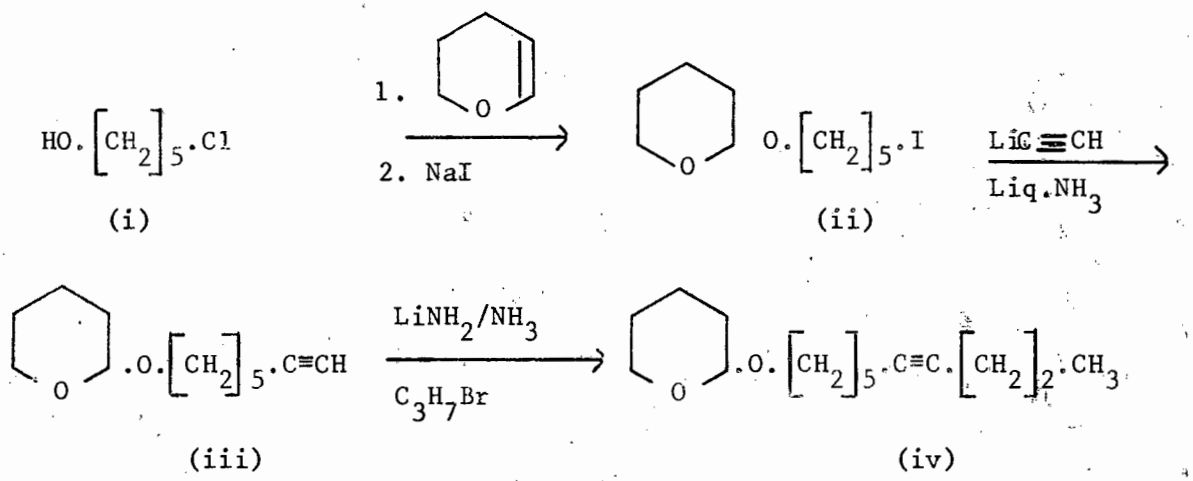


Figure V.2. The initial reaction sequence followed in the syntheses of cis and trans-dec-6-en-1-yl isovalerate.

4. cis-8-Methyl-non-4-en-1-yl valerate (II)

The reaction sequence is not given as it differed only slightly from that in Chart V.I.

Pent-4-yn-1-yloxytetrahydropyran [Fig.V.I.(iii)] was condensed, by way of its sodio derivative with 3-methyl-1-bromobutane to give 8-methyl-non-5-yn-1-yloxytetrahydropyran. Hydrogenation over Lindlar's catalyst, followed by acid hydrolysis and esterification with valeric acid gave cis-8-methyl-non-4-en-1-yl valerate in an overall yield of 20%.

3. Discussion

The key step in the syntheses of the known moth sex pheromones has been the introduction of unsaturation into an alkyl chain containing, in most cases, a hydroxyl group which may be esterified. As the pure cis and trans isomers are required for meaningful biological evaluations most syntheses have proceeded by way of various acetylenic intermediates which provide control of the olefin geometry through selective reduction of the parent alkyne. This method has found wide application in the synthesis of unsaturated fatty acids. Other stereospecific methods for the synthesis of disubstituted alkenes have since been developed. These include:1) Ring scission of 2-alkyl-3-chlorotetrahydropyrans by sodium metal to give the trans form of alk-4-en-1-ols.[66] This method was used in the synthesis of trans-dodec-7-en-1-yl acetate,[67] the stereo-

isomer of the T.ni pheromone; 2) the β-hydroxyphosphonamide method of Corey and Kwiatkowsky[68] which has been used in a number of syntheses,[69,70] and 3) the Wittig syntheses, where a choice of suitable reaction conditions and structural factors (Bergelson and Shemyakin) allows control of the olefin geometry.[71] Applications of the stereospecific Wittig reaction include the syntheses of (a) some prostaglandins,[69] (b) the insecticidle N-isobutylamide of dodeca-2,6,8,10-tetraenoic acid[72] and (c) the hydrocarbon dictyopterene,[73] isolated from an algae (Dictyopteris).

As the syntheses of the known moth sex pheromones have been reviewed on numerous occasions[47,74], further repetition by discussion in this thesis was not envisaged. However, some aspects of the syntheses, in this study, are discussed below.

1. Synthesis of the substituted acetylenes

Monoalkylacetylenes are prepared generally from sodium or lithium acetylide and alkyl halides in liquid ammonia or organic solvents.[75] Although good yields (50 - 80%) are obtained with the lower alkyl halides (C_1-C_{10}) insignificant yields are shown by the higher homologues (C_{13} - C_{16}) due to their decreased solubility in liquid ammonia. Better yields may be obtained by using an autoclave and higher reaction temperatures.[76] Increased use has been made of lithium acetylide stabilised as its ethylenediamine complex, in the syntheses of alkyl acetylenes, as it is a dry powder and can be used in solvents such as diglyme,

dioxane, DMF and DMSO thus avoiding the use of liquid ammonia. Also, it provides a means of obtaining high yields of alkyl acetylenes from alkyl chlorides. This reagent was used, by the author, in the preparation of hex-5-yn-1-yloxytetrahydropyran (iv, Chart V.I.) from 4-chlorobutyloxytetrahydropyran. It was not possible to use the corresponding 4-bromo ether as attempts to prepare this compound from 4-bromobutan-1-ol and dihydropyran consistently gave a mixture of products. Conversion of the 4-chlorobutyl ether into the iodo compound was not attempted due to the general instability of iodoalkyloxytetrahydropyrans.[77, 78, 79] Other methods of synthesising monosubstituted acetylenes, include: 1) the bromination of terminal olefins followed by dehydrobromination by sodamide in liquid ammonia[80] or sodamide and sodium hydride in DMSO;[81] and 2) the reaction of lithio-1-trimethylsilylpropyne with alkyl halides.[82] This reagent is useful in the synthesis of the 1,5-diene system, characteristic of acyclic isoprenoids. Applications include the syntheses of trans, trans farnesol[82] and dl-C_{18} Cecropia juvenile hormone.

In the preparation of trans-dec-4-en-1-yl isovalerate (Ic), the intermediate 4-pentyn-1-ol (ii) was prepared directly by ether cleavage of tetrahydrofurfuryl chloride by sodamide in liquid ammonia.[83] This compound is also obtained through ring scission of dihydropyran by n-amylsodium;[84] if, however, n-butyllithium is used, addition occurs and the reaction product is trans-non-4-en-1-ol.[85]

In the syntheses described in this thesis and the syntheses of the sex pheromones of the cabbage looper moth (T.ni),[67] the false codling moth (A. leucotreta)[65] and the red-banded leaf roller (A. Velutinana),[15] the prepared terminal acetylenes were condensed by way of their sodium or lithium derivatives with aliphatic halides to give the desired long aliphatic chains.

2. Stereospecific reduction of the disubstituted acetylenes

(i) cis olefin: The selective catalytic hydrogenation of acetylenic compounds to cis olefins has been widely exploited. This hydrogenation is readily effected over deactivated palladium catalysts under mild conditions. Palladium on calcium carbonate, deactivated by lead acetate and quinoline (Lindlar's catalyst)[86] was used in this study. It has been reported that palladium on barium sulphate, deactivated by an equal weight of synthetic quinoline is superior to Lindlar's catalyst in both reproducibility and preparation.[87] It is important to note that the hydrogenation is not absolutely stereospecific as a small amount of the trans olefin is formed, which may lead to misleading results on biological evaluations. Berger[77] synthesised cis-dodec-7-en-1-yl acetate, the sex pheromone of the cabbage looper moth (T.ni), using Lindlar's catalyst, and noted that the infrared spectrum showed a weak band at 965cm^{-1} indicating the presence of some trans isomer. A similar weak band was evident in the infrared spectrum of the synthetic cis acetate from an independant synthesis (Green et al.).[67]

Read,[47] in his work in this laboratory on the sex pheromone of the false codling moth (A. Leucotreta) examined a sample of the synthetic T.ni pheromone, supplied by Jacobson's group (Green et al), and noted that it contained at least 10% of the trans isomer. Recent analysis[88] of synthetic (origin not given) T.ni sex pheromone by g.l.c. has indicated the presence of 24% of the trans isomer. In this analysis, the synthetic compound was epoxidised and chromatographed on a Carbowax 20M column which gave efficient separation of the resulting cis and trans epoxides.

(ii) trans olefin: Disubstituted acetylenes are readily reduced, to trans olefins, by solutions of sodium or lithium in liquid ammonia. The yields obtained for these alkali metal reductions, when carried out at the boiling point of liquid ammonia (-32^{o}) and at atmospheric pressure, vary considerably. Jacobson's group using the sodium/liquid ammonia reduction of cis-tridec-9-en-5-ynyloxy-tetrahydropyran and its geometric isomer, the cis-5,9-yloxytetrahydropyranyl ether, report an 80 and 72% yield of the respective trans olefins.[67] They also report an 85% yield of a trans olefin as an intermediate in the synthesis of the pink bollworm (P. gossypiella) sex pheromone. Berger and Canerday,[77] reduced dodec-7-ynyloxytetrahydropyran and obtained only 25% conversion to the trans olefin. Three further reductions gave a product which showed, on g.l.c. analysis, a 90% reduction. Elsner and Paul[76] on treatment of oct-3-yne with sodium/liquid ammonia found no reduction to the trans olefin. However, when the reduction was carried out at room

temperature in an autoclave, almost quantitative conversion was obtained. Similarly, the use of an autoclave by Dear and Pattison [85] gave elaidic acid in 95% yield from heptadec-8-yn-1-oate. It seemed, therefore, that reductions at room temperature through use of an autoclave were inducive to high yields of trans olefins. As an autoclave was not available in this laboratory the reductions were carried out in a sealed metal container fitted with a safety valve which was adjusted to open at a pressure of 14 kg/cm^2; during the reductions the internal pressure rose to 10.6 kg/cm^2. Two successive reductions were necessary before analysis of the products by t.l.c. on silica gel G impregnated (25%) with silver nitrate, revealed almost quantitative conversion to the trans olefins.

CHAPTER VI

BIOLOGICAL EVALUATION

A description of the materials and methods used in the field tests discussed in this Chapter is given in Chapters III and VII.

1. Final verification of structure through field tests

As the seasonal appearance of the pine tree emperor moth is limited to the months April - May in the Western Cape the crucial field assay of the synthetic cis and trans dec-5-en-1-yl isovalerates was delayed for six months and was eventually carried out in an infested area in the State pine plantations at Swellendam. Nine traps were used in the form of a 3X3 Latin square in which treatments were spaced at 40 m intervals. A control treatment of live female moths was not included due to the unavailability of virgin females.

The treatments tested were small quantities of the cis-5 and trans-5 isomers (A and B respectively) and a large concentration of the latter isomer (B'). From the results (Table I) it was evident that the cis isomer only, was attractive to male moths. On the strength of these results and the excellent agreement of the m.s., i.r. and n.m.r. spectra of the synthetic cis-5 isomer with those from the isolated active compound, it was deduced that the sex pheromone of the female pine tree emperor moth is the cis isomer of dec-5-en-1-yl isovalerate.

TABLE I

Numbers of male pine tree emperor moths attracted to traps baited with cis-dec-5-en-1-yl isovalerate (A = 300 µg/wick) and trans-dec-5-en-1-yl isovalerate (B = 300 µg and B' = 1000 µg/wick).

Treatment	Males trapped			Total
A = cis-5 (300 µg)	16	18	14	48
B = trans-5 (300 µg)	0	0	0	0
B'= trans-5 (1000 µg)	0	0	0	0

2. Bioassay of the synthetic pheromone and its positional isomers

The successful identification of the sex pheromone from the female pine tree emperor moth and the availability of 1 - 2 gram quantities of the synthetic pheromone and various positional isomers prompted further field trapping experiments to investigate some factors of importance to a bulk synthesis of the pheromone for use in planned future extensive survey and control work.

One of these factors was the possible masking of the attractancy of the synthetic pheromone by its geometric

isomer as this masking is known to occur in several Lepidopterous species. For example, the synthetic pheromone of the cabbage looper (T.ni), cis-dodec-7-enyl acetate is unattractive to male moths when mixed with a 90% concentration of the trans isomer.[89] Also in two gelechiid species where the female sex pheromones are separately the cis and trans isomers of tetradec-9-en-1-yl acetate, a 1:1 (50% - 50%) mixture of these isomers is unattractive to males of both species.[90] Further, as little as 5% of the trans isomer of the female red-banded leaf roller (A. velutinana) sex pheromone, cis-tetradec-11-en-1-yl acetate is reported to inhibit male response in the field[91] while 15% of the trans isomer of the pink bollworm (P. gossypiella) pheromone is reported to inhibit male response in laboratory assays.[92] It can be appreciated that this phenomenon may well demand a stereospecific synthesis of one geometric isomer only.

Another factor was the possible attraction of male pine tree emperor moths to traps baited with isomers of the pheromone leading to the use of other compounds as attractants. This study was also important in view of the positive EAG responses elicited by the trans isomer of dec-4-en-1-yl isovalerate (this Chapter, section 3.) and the need to understand the structure-activity relationship in male response.

A comparison of the male response to the synthetic pheromone with that shown to extracts from virgin female

sex glands was another factor. Natural female extracts may contain a synergist or second pheromone (which escaped detection) and hence show greater attraction of males in the field. A precedent here is the function of dodecanyl acetate as a synergist for the pheromone of the red-banded leaf roller moth (A. velutinana)[93] and the use of two pheromones by females of the southern armyworm (Prodenia eridania)[16] and summer fruit tortrix moth (Adoxophyes orana).[22]

Before these tests, to investigate these factors, were carried out, various concentrations of the synthetic pheromone were assayed to facilitate the choice of a quantity of pheromone for use as a control treatment. Through the observation of the male response to the synthetic pheromone in the initial test it was realised that the quantity used (300 µg) was excessive as anemotactic behaviour in males stopped 30 - 40 metres from the trap itself and was substituted by a short-range "searching" behaviour leading to a slow approach across the forest floor towards the trap. This was in contrast to male moth behaviour as observed in previous studies when they approached traps baited with 10 - 40 ♀ ME aliquots of virgin female extracts. On these occasions males would fly around the traps and suddenly drop into the collecting tray. It was realised that too large a quantity may well result in lower trap catches as many males might respond to the high release rate by limiting their behaviour to short-range searching some distance away from the traps. There is also the possibility of

male adaptation to the stimulus. Many investigators have noticed that male response to a sex pheromone does not persist for long. Therefore, if males spend too long a period in approaching the traps they may adapt to the stimulus and disperse from the area without having reached the trap.

In the choice of a quantity of pheromone for the control treatments, four quantities of the synthetic pheromone ranging from 5 - 100 µg were tested in the field. In the test 12 traps were used in an arrangement consisting of three rows of traps. Longitudinal rows were spaced 100 metres apart and the traps in these rows 50 m apart. Each quantity appeared once in every row being replicated twice. Treatments were allocated by the use of a table of random numbers. The results of this test (Table II) show that within the range of quantities tested the number of males caught increased with an increase in the quantity of the synthetic attractant. Also at these quantities no repulsion of male response was observed. It was decided to use 50 µg of the synthetic pheromone as a control treatment in the following experiments as this quantity had attracted a sufficient number of males and also would have less influence on the male response to other traps by emitting a weaker signal. During this test it was also observed that males rapidly approached to within ten metres of the traps containing the 50 µg treatments before commencing the short-range searching behaviour.

TABLE II

Numbers of male pine tree emperor moths attracted to traps baited with 5, 10, 50 and 100 µg/wick of the synthetic pheromone.

	Males trapped
Synthetic pheromone	Total from 3 traps
5 µg	5
10 µg	11
50 µg	76
100 µg	100

1. Male pine tree emperor moth response to a mixture of the geometric isomers

The effect of trans-dec-5-en-1-yl isovalerate on male pine tree emperor moth response to the synthetic female sex pheromone was examined on two occasions by assaying a fixed amount of the synthetic pheromone (50 µg) mixed with varying amounts of the trans isomer. A similar trap arrangement, as described previously was utilised except that an extra row was added while treatments were prepared as outlined in the previous test. The numbers

of male moths trapped on both occasions is given in Table III.

TABLE III

Numbers of male pine tree emperor moths attracted to traps baited with the synthetic pheromone S (50 µg/wick) mixed with varying concentrations of its geometric isomer trans-dec-5-en-1-yl isovalerate.

			Male moths trapped	
S= cis-5	trans-5	% trans of total	Test I Total from 3 traps	Test 2 Total from 3 traps
50 µg	+ 25 µg	33%	110	39
50 µg	+ 50 µg	50%	129	37
50 µg	+ 100 µg	66%	124	44
50 µg	+ 1000 µg	95%	28	15
50 µg	____	0%	153	44

From the results of both tests it was evident that admixture of the synthetic pheromone with moderate quantities, up to 100 µg/50 µg, of its geometric isomer showed little or no effect on male response whereas admixture with a larger quantity, 1000 µg/50 µg,

caused a marked decline in the number of males trapped.

The relative insensitivity of male pine tree emperor moths to moderate quantities of the geometric isomer of the female sex pheromone makes it possible to use a mixture of the cis and trans isomers in survey and control experiments. This finding may be of economic significance in the bulk synthesis of the pheromone where a reaction sequence leading to a mixture of isomers may be preferred.

2. Male pine tree emperor moth response to some positional isomers.

Male response to the cis and trans isomers of dec-4-en-1-yl isovalerate and dec-6-en-1-yl isovalerate was examined on two occasions through the bioassay of a low concentration (50 µg/wick) and a high concentration (1000 µg/wick) of each ester. Fifteen traps were employed in the arrangements as described. The results, given in Table IV, showed that no males were attracted to any of the traps baited with the pheromone isomers.

It was expected that the trans isomer of dec-4-en-1-yl isovalerate would show some activity in field tests as it had elicited a positive response on the EAG (this Chapter, section 3.1). The selectivity of the male pine tree emperor moth in responding to one compound only (synthetic pheromone) from a series of positional isomers is in keeping with the high specificity of the sex pheromones from other species of Lepidoptera. Males of the

cabbage looper (T.ni) have been exposed to fifteen isomers and congeners of the female sex pheromone in a study of the structure-activity relationship in male response.[89]

TABLE IV

Numbers of male pine tree emperor moths attracted to traps baited with two quantities (50 µg and 1000 µg/wick) of the synthetic positional isomers of the sex pheromone.

	Male moths trapped	
Isovalerate esters of	50 µg/wick	1000 µg/wick
cis-dec-4-en-1-ol	0	0
trans-dec-4-en-1-ol	0	0
cis-dec-6-en-1-ol	0	0
trans-dec-6-en-1-ol	0	0
Synthetic pheromone (50 µg)	44	56

Although four of these compounds produced copulatory behaviour in males, they were less than 0.2% as attractive as the synthetic pheromone. In a similar study males of the gypsy moth (P. dispar) were exposed to a series of compounds showing slight structural variations and although some elicited slight activity none approached

the activity of the synthetic pheromone cis-7, 8-epoxy-2-methyloctadecane.[94, 95] The present author, in studies on the false codling moth (A. leucotreta) exposed male moths to various isomers and analogues of the female sex pheromone, trans-dodec-7-en-1-yl acetate. These latter compounds were tested at low concentrations only (10^{-6}-10^{-7} g/wick) but none produced copulatory attempts in males while some stimulated flight response only.[65]

3. Comparison of male pine tree emperor moth response to the synthetic pheromone and extracts from virgin female moths.

A comparison of the male response to the synthetic pheromone with that shown to extracts of virgin females was limited to a qualitative assessment as a quantitative study would have demanded considerable field work which was impractical at this stage.

Two surveys were carried out in the pine forests at Franschhoek in the Western Cape. In the first, six traps were used in an arrangement of three rows of two traps each. The rows were set 30 m apart with 30 m between traps. Three traps were primed with an aliquot (3 ♀ ME) from an active virgin extract and with quantities (0.3 µg) of the synthetic pheromone. Trap catches were recorded after two days (Table V).

TABLE V

Numbers of male pine tree emperor moths attracted to traps baited with virgin female extracts (3 ♀ ME) and the synthetic sex pheromone (0.3 µg/wick).

Treatment	Males caught Total from 3 traps
Virgin extract (3 ♀ ME)	4
Syn. pheromone (0.3 µg)	27

In the second test, in which a Latin square (3 X 3) was used, two treatments of the synthetic pheromone and one from a natural extract were assayed for activity. Catches were again recorded over a two-day period (Table VI).

TABLE VI

Numbers of male pine tree emperor moths attracted to traps primed with virgin female extracts (2 ♀ ME) and two concentrations of the synthetic sex pheromone (0.1 µg and 0.03 µg/wick).

Treatment	Male moths caught Total from 3 traps
Virgin extract (2♀ ME)	6
Syn. pheromone (0.1 µg)	6
Syn. pheromone (0.03 µg)	6

This test was unfortunately hampered by adverse climatic conditions and a rapidly declining moth population, but from these preliminary results it was evident that male pine tree emperor moths did not show a marked preference for those traps baited with the natural extracts.

3. Bioassay using the Electroantennogram

A preliminary EAG bioassay using 1 µg quantities of the synthetic isovalerate esters was carried out some time before male response to the same compounds was investigated by bioassay in the field. It was found, here, that antennae showed little selectivity in that positive responses (≥ ARL) were elicited by each ester. Further investigation of this phenomenon was delayed until the completion of the bioassay of the esters in the field where it was found that male moths responded to cis-dec-5-en-1-yl isovalerate only.

1. Experimental

Varying quantities i.e. 1.0, 0.1 and 0.05 µg of the synthetic pheromone and its positional isomers were bioassayed and the antennal responses noted.

The results of the first test (Fig. VI.1) in which 1 µg quantities were used were similar to those obtained in the earlier assay, as positive responses (≥ ARL), comparable to the standard pheromone response, were elicited by each ester. Five antennae were used in this test and prepared as described in Chapter VII, 1.1. The

mean response to the synthetic esters is indicated in Fig.VI.1 by the vertical lines while the mean response to the solvent blank is shown by the dotted section.

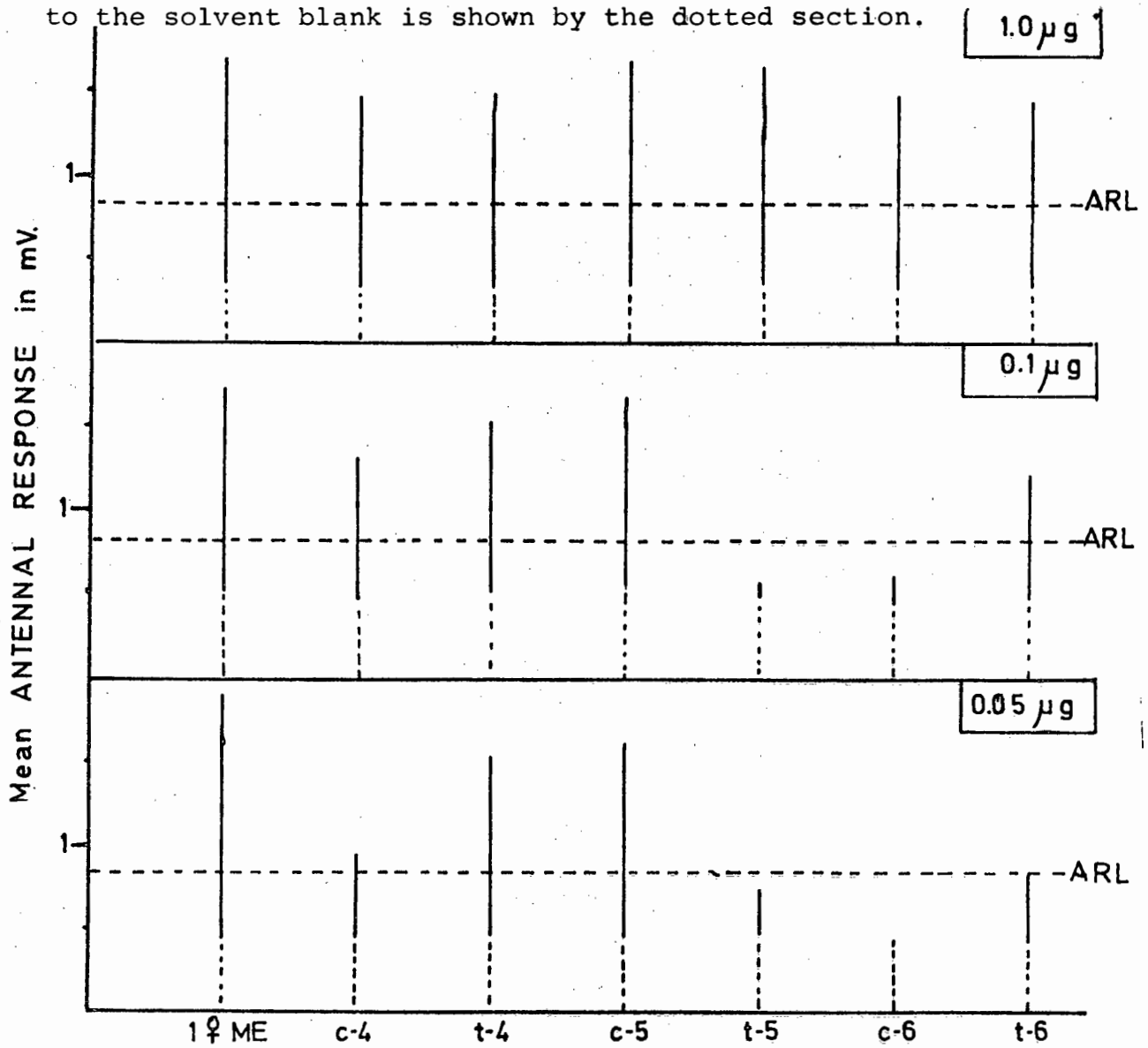


Figure VI.1. Mean EAG responses of male N. cytherea cytherea antennae to varying quantities of the synthetic pheromone and isomers. The response to the esters are indicated by the vertical lines, with the dotted section showing the amplitude of the antennal response to the solvent blank. The esters are listed in relation to the position and geometry of the double bond. The response to a pheromone standard (1 ♀ ME) is included.

A varying response was noted in the second test where a smaller quantity (0.1 µg) of each ester was assayed. Five antennae were again used and the results (Fig.V.I.1) plotted as described. Antennae were found in this test to differentiate between the synthetic pheromone and its geometric isomer in that a positive response, comparable to that from the pheromone standard, was elicited by the former ester while the latter compound gave a negative response ($\angle$ ARL). A negative response was also given by cis-dec-6-en-1-yl isovalerate while its trans-6 isomer gave a relatively weak positive response. A difference in the mean response to cis and trans-dec-4-en-1-yl isovalerates was also evident; whereas the former gave a weak positive response the latter almost equalled the response to the synthetic pheromone.

This pattern was further emphasised in the third test where smaller quantities (0.05 µg) were assayed. Seven antennae were used and the results presented as described. The response to trans-dec-4-en-1-yl isovalerate again almost equalled that given by the synthetic pheromone while the other isomers showed negative or very weak positive responses.

It was not possible to further decrease the quantities assayed as the response to the synthetic pheromone became too weak for comparative studies.

2. Discussion

In this context male antennae of the silkmoth (B. mori) and the gypsy moth (P. dispar) have been shown to yield

meaningful EAG responses to some synthetic isomers and analogues of the female sex pheromones. For the former the synthetic sex pheromone, 10-trans, 12-cis-decadien-1-ol and its geometric isomers were assayed, while for latter moth the synthetic pheromone, 7, 8-epoxy-2-methyloctadecane and 55 related compounds were tested. In the silkmoth study[50] the stimulating ability of the pheromone isomers, on the EAG, compared favourably with their level of activity in a laboratory bioassay while in the gypsy moth study[96] the intensity of the EAG response to each compound, generally, correlated well with its attraction in the field. In view of these results it was expected that trans-dec-4-en-1-yl isovalerate, one of the positional isomers of the N. cytherea cytherea sex pheromone would attract male moths in the field as it had consistently given a response on the EAG comparable with that from the synthetic pheromone. It was shown later that this compound was inactive in the field (Chapter VI,2.2). This phenomenon was also observed in the gypsy moth study where some isomers gave strong responses on the EAG but showed very weak attraction of male moths in field trials. The significance of these findings remains to be clarified but,may indicate synergism or inhibition by a particular isomer. It is apparent though, from these and other studies[22, 97], that antennae may respond to compounds similar to the sex pheromone which therefore calls for a cautious approach to the use of the EAG as an independant bioassay of pheromone activity in any isolation studies.

CHAPTER VII

EXPERIMENTAL

1. Bioassay technique

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1. Electroantennogram - General procedure: Antennae from 1 - 4 day old male moths were used in this technique. Each antenna was amputated at its base by use of a surgical micro-scissors and transferred to a small, cork covered, mounting base (Fig. VII.I) where a strip of filter paper was pinned across the antennal shaft to secure the antenna for mounting. The antennal tip (ca. last 8 segments) was removed and finely drawn glass capillaries, containing O.IN saline, inserted into either end of the shaft to a depth of ca. three segments. Electrodes were originally inserted into the basal segment and into one of the branches located near the tip of the antennae. Insertion of the latter electrode was, however, found to be an exhausting process and was soon replaced by insertion into the shaft as there appeared to be no difference in the EAG responses. Optical control of the capillary insertion was achieved with a binocular compound microscope. The glass capillaries were inserted into perspex clamps prior to insertion and fitted with platinum wire electrodes. After mounting, the securing strip of paper was removed and the cork base slid out from beneath

the antennae. Removal of the base proved to be essential as the cork soon became contaminated giving rise to erratic results. The suspended antennae were positioned (about 3 cm) below the outlet port of the selector disc in the airflow system (Chapter III, section 1.1) and the platinum electrodes connected to an oscilloscope.

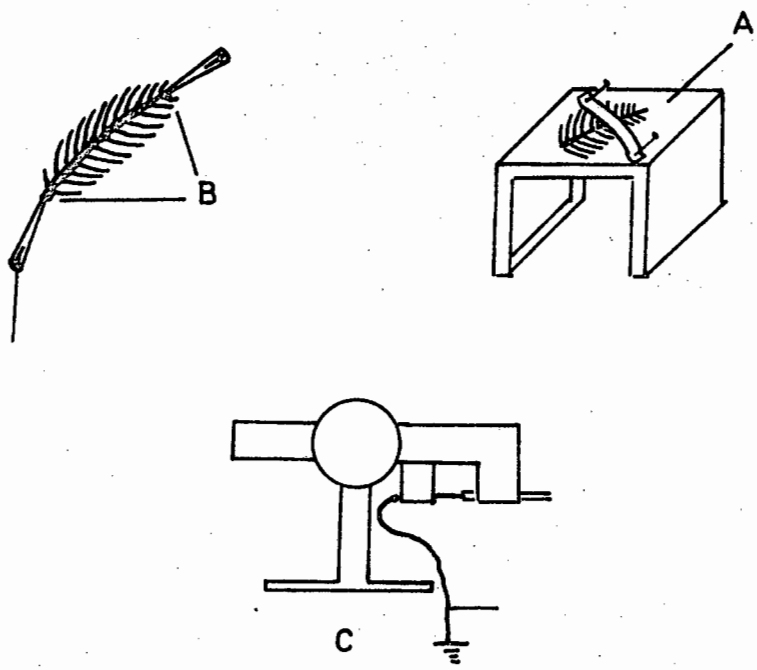

Figure VII.I. A schematic representation of the procedure involved in the mounting of an antenna for testing purposes: A, the cork covered mounting base supporting a secured antenna; B, the position of insertion of the finely drawn glass capillaries in the antennal shaft; C, the perspex clamps for holding the glass capillaries and facilitating their insertion.

Antennae were not exposed to a continuous stream of air into which the test vapours were suddenly inserted, but to puffs of ca. 0.5 sec. duration directed through the selector disc and over filter paper discs containing the test sample. These puffs were created by momentarily placing a thumb or finger over the two-way valve exhaust port of the airflow system.

Test samples were impregnated on to filter paper discs (Whatman No.1,1.8 cm) and air dried for five minutes before testing. These discs were placed into clean glass cartridges (5 x .4 cm. i. d.) which were numbered and inserted into the perspex selector disc. The cartridges were held in position by small sections of rubber tubing. Antennae were allowed a two minute rest period between consecutive stimulations. During this period all expelled or tested vapours were removed from the surrounds by switching on an exhaust fan, attached to the rear of the aluminium cage, for 45 seconds. The antennal response was recorded by measuring the magnitude of the rapid initial negative change in the potential at the recording electrode. All responses were recorded in millivolts.

The antennal response to a known pheromone extract (2 ♀ ME) was recorded in each test for comparison purposes. These pheromone standards were prepared from virgin female abdominal tips macerated in methanol and extracted in cyclohexane. In a preliminary experiment four male antennae were exposed to a dilution series from a virgin female extract of .01, .1, .5 1, 3, 5 and 10 ♀ ME / test sample. The stimulus -

response curve showed a plateau at .1-1.0 ♀ ME thereafter to remain constant. In all EAG tests a portion (10 ♀ ME) of the pheromone extracts, in excess of the amount (1 - 3 ♀ ME) required to elicit the maximum response from each antenna, was assayed to compensate for the loss of pheromone during each purification step. To obtain a true measure of the antennal response to a test sample it was essential to determine the response to the solvents used in the preparation of the sample cartridges. This was achieved by including a cartridge primed with a filter paper disc containing solvent only. Stock solutions of the synthetic pheromone and other isomers were prepared in cyclohexane such that the test sample was delivered in a constant volume of solvent (10 µl).

Before and after the preparation of each batch of sample cartridges, all relevant apparatus was thoroughly washed with ethanol and dried in a stream of air. The author was obliged to wash his hands before each preparation as traces of the synthetic pheromone or extracts were readily transferred to the testing apparatus. Preparation and disposal of the sample cartridges was confined to an adjacent room to avoid contamination of the atmosphere in the testing room. For the same reason, the testing room was not located in the laboratory in which the pheromone and its isomers were synthesised and in addition was positioned some distance from the insectary housing male and female moth populations.

2. Field tests - General procedure: Traps were positioned on the ground in known invested areas. Preliminary studies by Warren and Bosman showed that males responded to the pheromone at night only, reaching a peak in the early hours of the morning (4 am.). Traps were therefore primed as late in the afternoon as possible to avoid unnecessary loss of the test substance. Captured moths were removed from the traps each morning and identified, sexed and counted. Only male moths were caught. Male and female moths of several species of the family Geometridae were found in all traps presumably attracted by the paraffin or water as they were found in primed and unprimed traps. Numerous flies were also attracted to the traps, as were eight butterflies of the species (Dira clytus). Extracts of virgin females were assayed in quantities representing a certain number of female moth equivalents (♀ ME). The synthetic pheromone and isomers were assayed in quantities representing a number of micrograms/wick (µg/wick). A 50 µg quantity of the synthetic pheromone was used as a control treatment in most tests. Stock solutions (1µl = 1µg of ester) of the synthetic isovalerate esters were prepared by diluting each compound (11.5µl = 10,000µg; density = ca. 0.86^{20}_{4}) with cyclohexane to a volume of 10 ml. The traps were primed by pipetting aliquots from each stock solution or female extract on to the filter paper wicks. Cross-contamination was avoided

by rinsing the used pipette with alcohol into a beaker containing sawdust. Contamination of the trapping equipment was minimised by retrieving the metal con - tainers after each test and rinsing them thoroughly with ethanol and acetone.

2. Isolation of the natural pheromone.

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1. Apparatus and techniques: Solvents used were of analar quality and, where desirable, were further purified. Acetone was refluxed for one week over portions of potassium permanganate, until the pink colour persisted and then distilled. Dichloromethane and carbon tetra- chloride used in the preparative g.l.c. collections were initially passed through a column of activated alumina. Where possible, apparatus was constructed entirely from glass which was thoroughly cleaned before use by washing with detergent and solvents such as ethanol and acetone. The utilisation of teflon stoppers and tubing was preferred above that of their rubber counterparts as the latter contain freely extractable plasticisers and waxes.

Preparative and analytical g.l.c. studies, unless other- wise stated, have been confined solely to a Perkin - Elmar Model 900 Gas Chromatograph fitted with a flame ionisation detector. All columns employed were freshly packed at the start of the moth season and conditioned

at 220° for two days. The solid support used, Chromosorb W (60/80 mesh), was sieved to remove fines and coated with the relevant stationary phase by the filtration (solution coating) technique.[98] The coated support was dried by the fluidised drying technique,[99] which is reported to give greater column efficiency and better reproducibility. Septums were regularly changed and, to avoid interference by septum bleed, baked in the vacant injector port at 200° for one day, before use. Where possible Hamilton syringes of the gas tight type were used, to prevent the loss of some material through blow back between the plunger and barrel.

Infra red spectra of the natural pheromone were run on a Beckman I. R. -7 instrument using a micro-cavity cell and carbon tetrachloride as solvent. Spectra of the synthetic pheromone and analogues were run as liquid films on a Perkin-Elmar 137 instrument.

Mass spectra were run on an A.E.I. MS - 902, a high resolution instrument and refer to 70 - 75 ev spectra. They are plotted in terms of relative abundance with the most intense peak (base peak) taken as 100%.

N.m.r. spectra were determined on Varian 60 and 100 MH_z instruments. Deuterochloroform and carbon tetra - chloride were used as solvents with tetramethylsilane as internal standard. Bands are given on the τ scale and coupling constants (J) in c/sec.

2. Preliminary study on an extract from 12,000 female moths

(i) Purification by column chromatography

A column (20 x 1.5 cm) of Florisil (25 g, 60 - 80 mesh), packed in the normal way, was used to remove the large amount of a C_{19} hydrocarbon accidentally introduced into the extract. This was effected by charging the extract to the column and developing with pure pentane (100 ml) followed by portions of pentane (50 ml) containing varying percentages of acetone, increasing from 0.1 - 5%. Fractions (10 ml) from the solvent mixtures were collected and portions (10 ♀ ME) bioassayed (EAG) for activity. The pheromone was detected in fraction 21 after having eluted with the pentane - acetone mixture (99:1) (Fig VII.2). G.l.c. analysis of the initial fractions showed that the contaminating hydrocarbon had eluted with the pure pentane fraction.

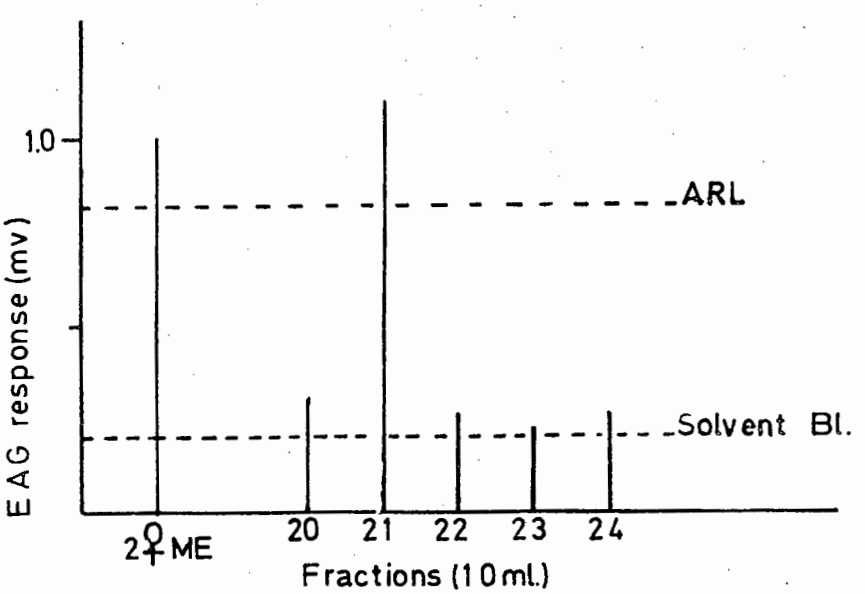

Figure VII.2 EAG responses of one male antenna exposed to aliquots (10 ♀ ME) from each of the pentane-acetone (99:1) fractions.

The content of tube 21 was concentrated by careful removal of the pentane by distillation from a pear-shaped flask (25 ml) fitted with a glass column (10 x 1 cm), packed to a height of 3 cm with glass beads. The distillation, controlled by playing a stream of warm air from a laboratory hair dryer over the flask was discontinued when the volume of pentane in the flask reached approximately 0.1 ml. Pheromone adhering to the glass beads and sides of the column was washed into the flask by running 0.1 ml of pentane down the sides of the column and over the glass beads. An aliquot (0.5 ml)of the distillate (9 ml) was removed and bioassayed (EAG) for activity. An antennal response slightly greater than that of the solvent blank was obtained so it was assumed that very little pheromone had been lost during this concentration procedure. The flask was stoppered and kept at 0^{o} to await g.l.c. analysis.

(ii) Marking of the active area on gas-liquid chromatography

(a) A polar column

The stationary phase FFAP (Free Fatty Acid Phase ; Supelco, Inc. Bellefonte Pa.) was used as it showed minimal tailing of the fatty acid esters and little column bleed. A glass column (6.6 m x .35 cm, i.d.) was packed, as described previously (this Chapter, section 2.1) with 2.5% FFAP on Chromosorb W and conditioned before use. A mixture of methyl esters of long chain fatty acids (C_{11} - C_{15}; Henkel Int.)

was used to establish optimum g.l.c. conditions and as markers to determine accurately the retention time of the pheromone on this column.

The optimum g.l.c. conditions were found to be :

1) an injector and detector - manifold temperature of 190° and 200° respectively;
2) helium as carrier gas at a flow rate of 35 ml/min; and
3) an oven temperature of 60° for an initial period of 12 minutes followed by a programmed run to 135° at 1.5°/min.

A portion (10 µg) of the methyl ester mixture in 10µl of pentane was injected under these conditions and a chromatogram obtained. The C_{16} marker (C_{15} acid + methyl) was found to emerge after a period of 116 minutes. A portion of the active residue (1.0 µl, 60 ♀ ME) was similarly chromatographed under these conditions and the chromatogram superimposed upon that from the methyl esters. No peaks were seen. Various fractions were collected between the C_{12} and C_{15} markers (Fig. Vll.3a) by passage of the effluent gases through hypodermic needles (15 cm) leading into the bottom of narrow glass tubes containing cold (0°) pentane (1-2 ml). Bioassay (EAG) of these fractions (Fig. Vll.3b) showed fraction 6 to be active. The pheromone therefore held a retention time slightly less than that of the C_{14} marker.

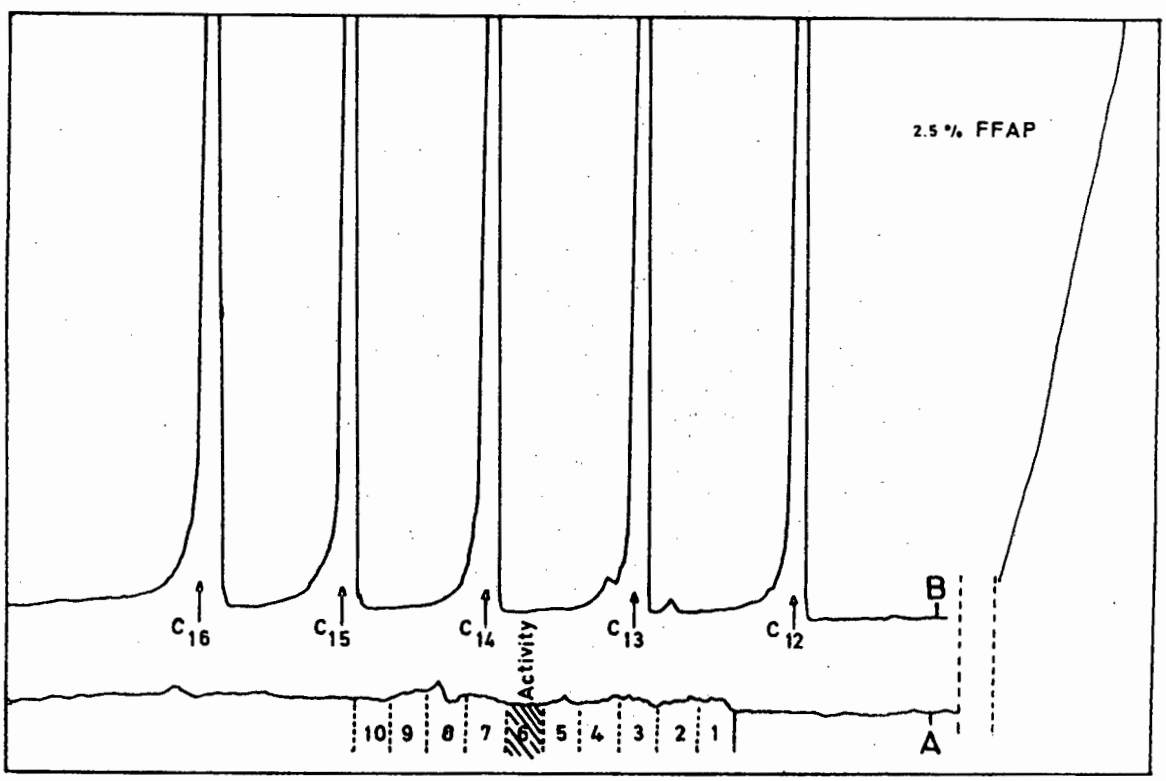

Figure VII.3a. A section of the chromatogram (A) of a portion of the active residue (60 ♀ ME, 1 µl.) superimposed on the chromatogram (B) of the methyl ester markers. The fractions collected are shown by vertical dotted lines.

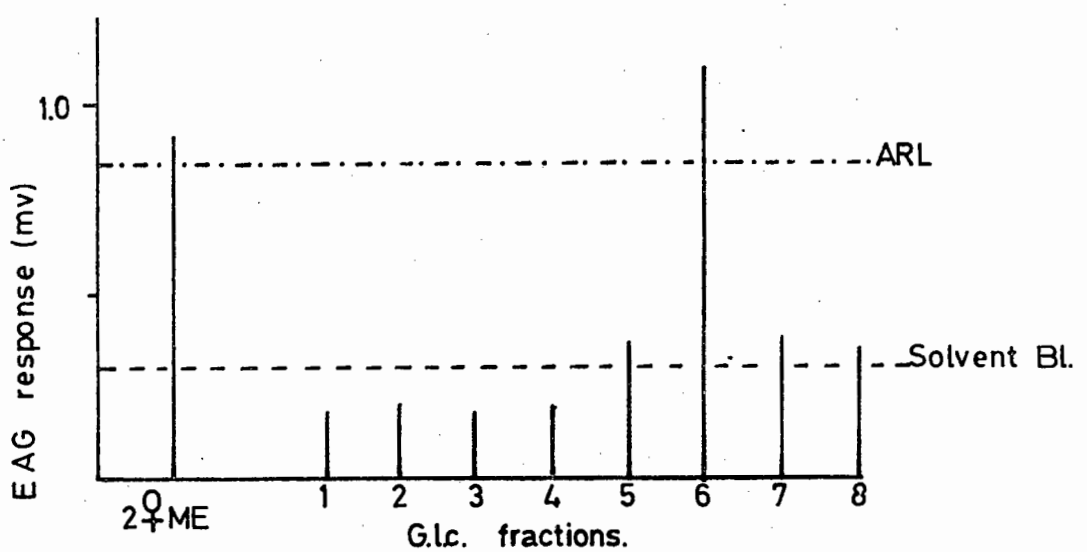

Figure VII.3b. Mean EAG responses of two male antennae exposed to the fractions collected from the FFAP column.

A chromatogram of the active residue (Fig. Vll.4) was obtained by injection of a larger portion (5 µl, 300 ♀ ME) under the same conditions. The stream splitter was altered to conduct the major portion (90%) of the carrier gas to the flame detector. All compounds emerging between the C_{13} and C_{14} methyl esters were collected and bioassayed (EAG) for activity. The known active area revealed a single large peak which proved to be highly active.

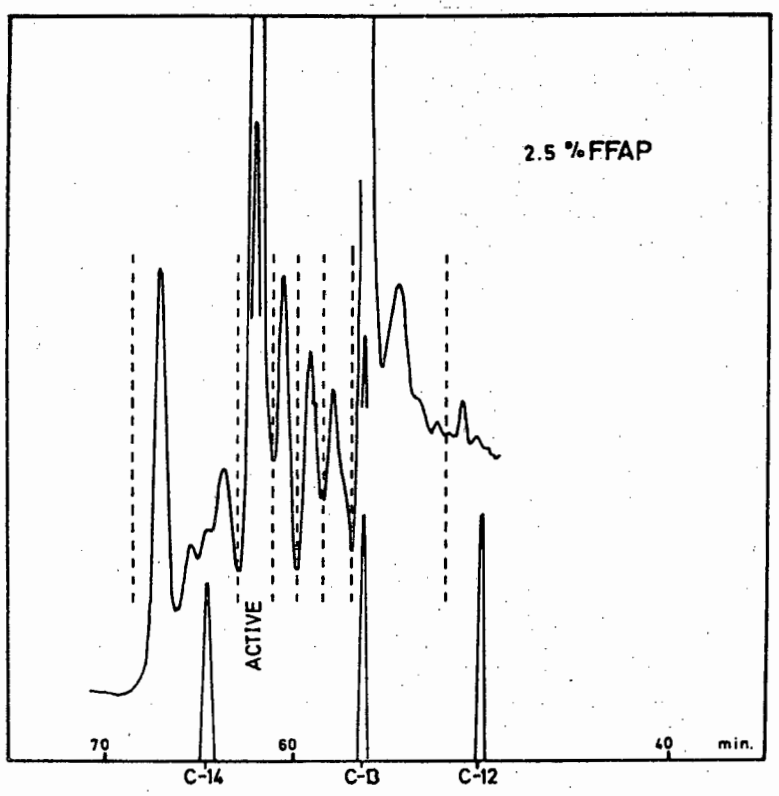

Figure VII.4 A section of the chromatogram from the active residue (5 µl , 300 ♀ ME) superimposed on the chromatogram from the methyl ester markers. The preparative fractions collected, are shown by vertical dotted lines.

(iii) Preparative isolation of the pheromone peak

Before preparative collection of the active fraction from the remaining residue was attempted, the unavoidable loss of material through the flame ionisation detector was minimised by modification of the stream splitter to allow ca. 1 - 3% of the carrier gas to flow to the detector. The same g.l.c. conditions were used except for the injector and detector-manifold temperatures which were lowered to 170° and 190° respectively.

The remaining portion was chromatographed in eight runs (8 x 25 µl) and the compound(s) in the active area collected by passage of the effluent gas through pentane (2 ml) cooled (-80°) by an ethyl acetate/liquid nitrogen mixture. The pentane was held in a pearshaped flask (5 ml) fitted with a teflon stopper containing a hypodermic needle (20 cm long, guage 22) through which the gases were introduced into the bottom of the flask and a short exhaust needle. At the end of a collection run the inlet needle was disconnected from the hot outlet nozzle of the stream splitter and fitted with a teflon stopper to prevent the loss of material, condensed in the needle, through evaporation. When cool, 3 - 4 drops of pentane were passed through the inlet needle to flush all condensed material into the flask.

An analysis of the condensate under analytical g.l.c. conditions showed that an insufficient quantity

(ca. 30 µg) of material had been isolated to facilitate further purification by preparative gas chromatography on a less polar column and still retain enough of the active compound for analysis by mass spectrometry. A large portion (80%) of the isolated material was therefore used to obtain a mass spectrum which would possibly show the molecular ions from the major compounds present and hence afford relative molecular formulae.

(iv) Mass spectrometry

A gas chromatograph was fitted with the same FFAP column and coupled to the mass spectrometer ion source by way of a Biemann separator. The g.l.c. conditions used were similar to those employed in the preparative collections. The chromatogram obtained revealed a single large peak in the known active area. High and low energy spectra of this peak showed no fragment higher that m/e 138. The molecular formula of this fragment was $C_{10}H_{18}$, m/e 138.1410 ($C_{10}H_{18}$ requires 138.1395). Other fragment ions appeared at m/e 110 (25%) and m/e 44 (base peak).

3. Final study on an extract from 34,000 female moths

1. Solvent extraction of abdominal tips

Frozen (-10^{o}) abdominal tips from 17,000 female moths were immersed in liquid nitrogen and then ground to a fine powder by passage through a cold coffee grinder. Lumps that were too large for the grinder were shattered by pounding in a mortar and pestle. The powder obtained

was steeped in three times its volume of cold (5°) dichloromethane (ca. 5 litres) for 12 hours and then homogenised twice (2 x 2 min) in a laboratory blender. Filtration of the homogenate on a Buchner funnel through dichloromethane washed cottonwool, gave a yellow filtrate and a dark purple pulp. The filtrate on concentration by rotary evaporation (25°; 30 cm Hg) gave an active brown oil (E_1; 60 ml) with a characteristic odour. A similar volume of oil (E_2) was obtained from the second batch of 17,000 female tips.

2. Low-temperature precipitation of lipids.

The oil S_1 was taken up in ten times its volume of acetone (600 ml) and kept at -75° (ethyl acetate/liquid nitrogen) for 30 minutes. The precipitated fats were removed by filtration on a cold Buchner funnel and taken up in acetone (50 ml). The solution was cooled (-75°) and filtered. The residue was washed with acetone (2 x 100 ml) to give a hard white solid (18 g) and a light yellow filtrate. To check on the efficiency of purification (0.1 g, ca. 100 ♀ ME) of the precipitated fats and an aliquot (0.4 ml 10 ♀ ME) of the combined filtrates and washings were bioassayed for activity. Only the filtrate gave a response on the EAG.

The acetone solution was concentrated by rotary evaporation (30°; 35 cm Hg) to one third (250 ml) of its original volume and again cooled (-75°) for 30 minutes. Further

fats were precipitated and removed by filtration. These fats were taken up in acetone (20 ml) and precipitated by cooling (-75°) for 30 minutes. Filtration and subsequent washing with acetone (-75°; 2 x 10 ml) gave a flaky, pale yellow residue (6.0 g) which on bioassay (EAG; 35 mg, 100 ♀ ME) was found to be inactive.

The final active solution (filtrate + washings) was concentrated by rotary evaporation (30°; 30 cm Hg) to give a brown oil A_1 (43 g) which proved to be highly active on bioassay (EAG).

Lipids were removed from the crude extract of the second batch of tips in an identical manner to give an active brown oil A_2 (48 g).

3. Purification by steam distillation

The active oil A_1 (43 g) was immediately transferred to a continuous extraction apparatus and the steam volatile compounds extracted in dichloromethane for 54 hours. The dichloromethane used was removed at six hourly intervals for the first 18 hours and then once every 12 hours and replaced by fresh solvent. Bioassay(EAG) of a portion (10 ♀ ME) of each batch of solvent revealed almost complete extraction within 32 hours. The solvent used in this period, was combined and concentrated by slow distillation, initially through a Vigreux column (50 cm) and then through a column

(10 cm) packed with glass beads (see this Chapter, section 2.2.(i)) to give a highly active, (EAG) strong smelling, colourless oil S_1 (0.6 g).

Steam distillation of a small portion of the pulp, remaining after digestion of the milled abdominal segments with dichloromethane and bioassay (EAG) of the concentrated distillate showed that not all the pheromone had been removed by solvent extraction. The entire pulp content was therefore steam distilled for 12 hours and the distillate (0.2 g) added to the active oil S_1.

The oil A_2 from the second batch of female tips was similarly steam distilled to give 0.9 grams of active, steam volatile material S_2.

4. Purification by column chromatography

A column (35 x 1.5 cm) was prepared by slurring Florisil (60/120 mesh; 50 g) in pentane for packing. The active steam distillate S_1 (0.8 g) in pentane (1 ml) was adsorbed on to the top of the column and development achieved with portions (50 ml each) of the following solvents: pentane 100%, pentane-acetone (99.8: 0.2), (99.5:0.5), (99:1), (97:3) and (100 ml each) of pentane-acetone (95:5) and acetone 100 %. An elution rate of 0.5 ml/min was employed. Fractions (5 ml) of each solvent were collected and aliquots (3.5 µl , 10 ♀ ME) bioassayed for activity (EAG). The pheromone was found to elute with pentane-acetone (95:5) over a series of fractions totalling 50 ml of solvent. The active fractions

(66 - 76; EAG) were combined and concentrated initially by careful distillation and then under a steam of high purity nitrogen, to a volume of 0.1 ml. The concentrate C_1 was quantitatively transferred to a conical glass vial of 120 µl capacity (Reactivial, Pierce Chemical, Co.) by means of a 50 µl syringe. The vial was stoppered and kept at 0°.

Chromatography of S_2 (steam distillate from second batch of moth tips) on a similar column showed analogous behaviour and gave a pheromone concentrate C_2 which was transferred to a reactivial.

The preparation and development of the columns was done in a cold room at 12°.

5. Preparative gas liquid chromatography.

(i) Polar phase, FFAP

The excellent separation of the active residue components achieved on this stationary phase in the preliminary study prompted its reuse in the final isolation. The same glass column (6.6 m x .35 cm i.d.) packed with 2.5% FFAP on Chromosorb W was utilised under the following g.l.c. conditions: 1) injector and detector-manifold temperatures of 170° and 180° respectively; 2) helium as carrier gas at a flow rate of 25 ml/min with an inlet pressure of 26 p.s.i. ; 3) an oven temperature set at 60° for 12 minutes followed by a programmed run to 145° at 1.5°/min; and 4) a stream splitter ratio of 98.5:1.5 (effluent-detector).

It was known that the pheromone held a retention time slightly less than that of the C_{14} methyl ester on the phase FFAP (this Chapter, section 2.2.(ii)). A preparative quantity (10µl =10%) of C_1 was immediately chromatographed and four fractions collected between the C_{13} and C_{15} ester markers (Fig. VII.5a). The fractions were condensed in pentane using a series of collecting vessels described in section 2.2.(iii). Aliquots (1.2µl , 10 ♀ ME) from these fractions were bioassayed (EAG) for activity. Antennae responded to fractions 3 (Fig. Vll.5b) which showed a single large peak on the g.l.c. chromatogram.

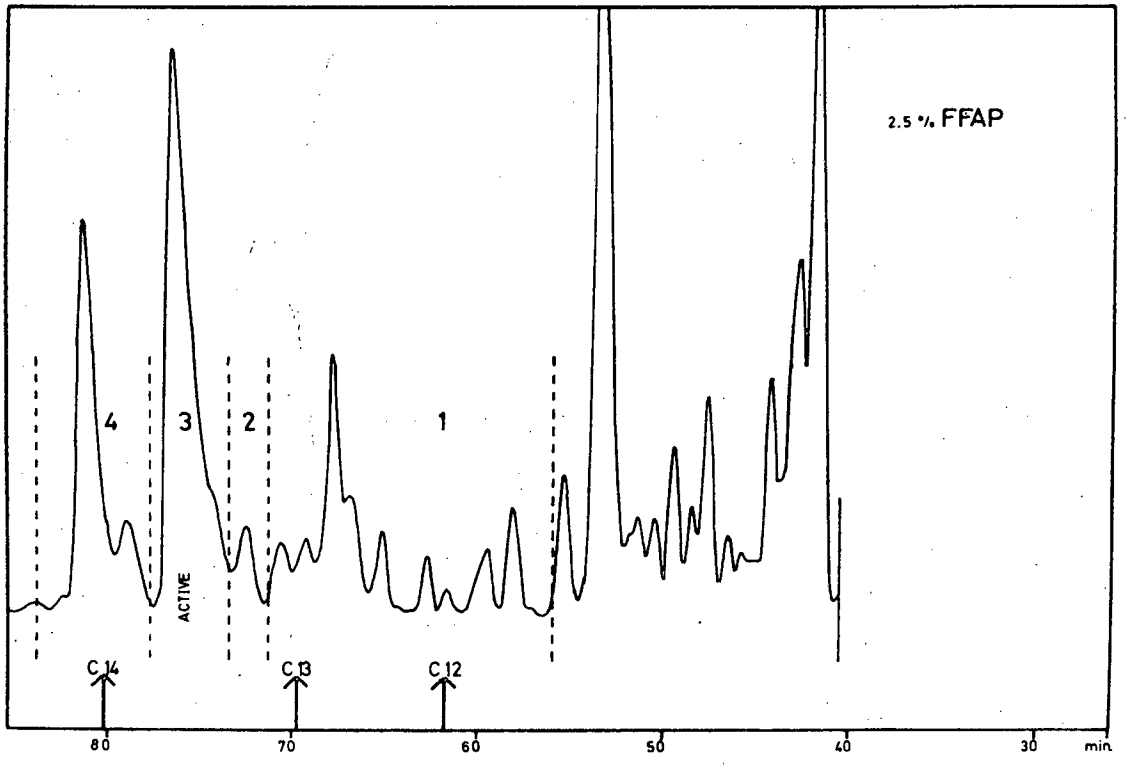

Figure VII.5a. A section of the chromatogram from the pheromone concentrate C_1 showing the four fractions collected (vertical dotted lines) and the large peak in the active (EAG) fraction 3.

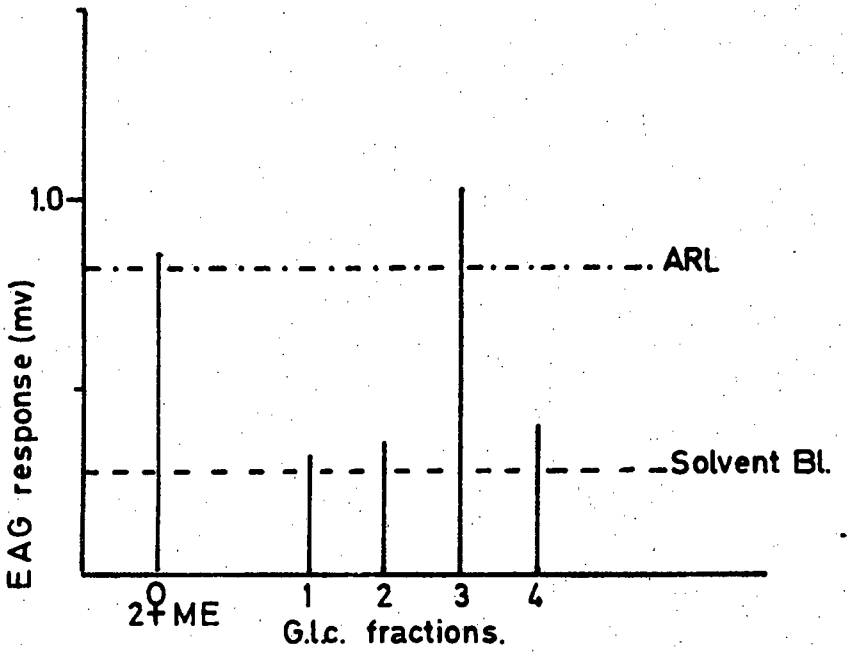

Figure V11.5b. EAG response of one male antenna exposed to aliquots (1.2 µl , 10 ♀ ME) of each of four fractions collected from the stationary phase FFAP in the chromatography of the pheromone concentrate C_1.

The remainder of the pheromone concentrate C_1 was chromatographed in six runs (6 x 15 µl) and the same peaks or fractions collected. To recover much of the active peak trapped in fractions two and four through tailing on the column, the condensates from these fractions were combined and rechromatographed in three runs. The small peak appearing in the active area was collected in the flask containing the condensate from fraction 3 which was subsequently concentrated under a slow stream of pure nitrogen to a volume of 50 µl The nitrogen was purified

by passage through tubes containing activated charcoal and molecular sieve beads. This concentrate G_1 was quantitatively transferred to a small conical glass vial (120 µl capacity, Reactivial), diluted to 100 µl and kept at 0°.

The concentrate C_2 from the second batch of moth tips was similarly chromatographed and the active fraction isolated in pentane. The chromatogram from this extract C_2 is not included as it differed only slightly from that of C_1 (Fig. V11.5a.). This gave a pheromone concentrate G_2 (100 µl).

(ii) A less polar phase, OV 25

A phenyl silicone stationary phase OV 25 (Supelco, Inc., Bellefonte, Pa.) was selected for the second isolation of the pheromone as it was readily available and showed less tailing of ester type compounds than the popular phase SE 30. A glass column (6.6 m x .35 cm i.d.) was packed with 4% OV 25 on Chromosorb W and was utilized under the following g.l.c. conditions: 1) Injector and detector-manifold temperatures of 180° and 190° respectively; 2) helium as carrier gas at a flow rate of 13.5 ml/min, with an inlet pressure of 25 p.s.i.; and 3) an oven temperature of 150°.

The retention time of the pheromone on this stationary phase was determined by superimposing the chromatogram of a portion (0.35 µl, 50 ♀ ME) of G_1 on the chromatogram of the methyl ester markers with the collection of 10 fractions between the C_{12} and C_{15} markers.

The fractions were collected by passage of the effluent gases through hypodermic needles (16 cm in length) leading into the bottom of narrow glass tubes containing pentane (1 - 2 ml). The needles were flushed with pentane (3 drops) and the solutions concentrated for bioassay. Only fraction eight elicited a response on the EAG. This gave the pheromone a retention time identical with that of the C_{14} ester marker.

A chromatogram of the pheromone concentrate G_1 was obtained by injection of a preparative quantity (10 µl) under the same g.l.c. conditions using a constricted stream splitter, conducting 1.5% of the carrier gas to the detector. All peaks appearing in the active area were collected in preparative collection flasks (section 2.2. (iii)) containing spectroscopic carbon tetrachloride (1.5 ml ; -15^{o}) in anticipation of n.m.r. studies. In all, four fractions were collected (Fig. Vll. 6a) and portions (12µl , 10 ♀ ME) bioassayed for activity. Only fraction 3 elicited a response on the EAG (Fig. Vll. 6b.).

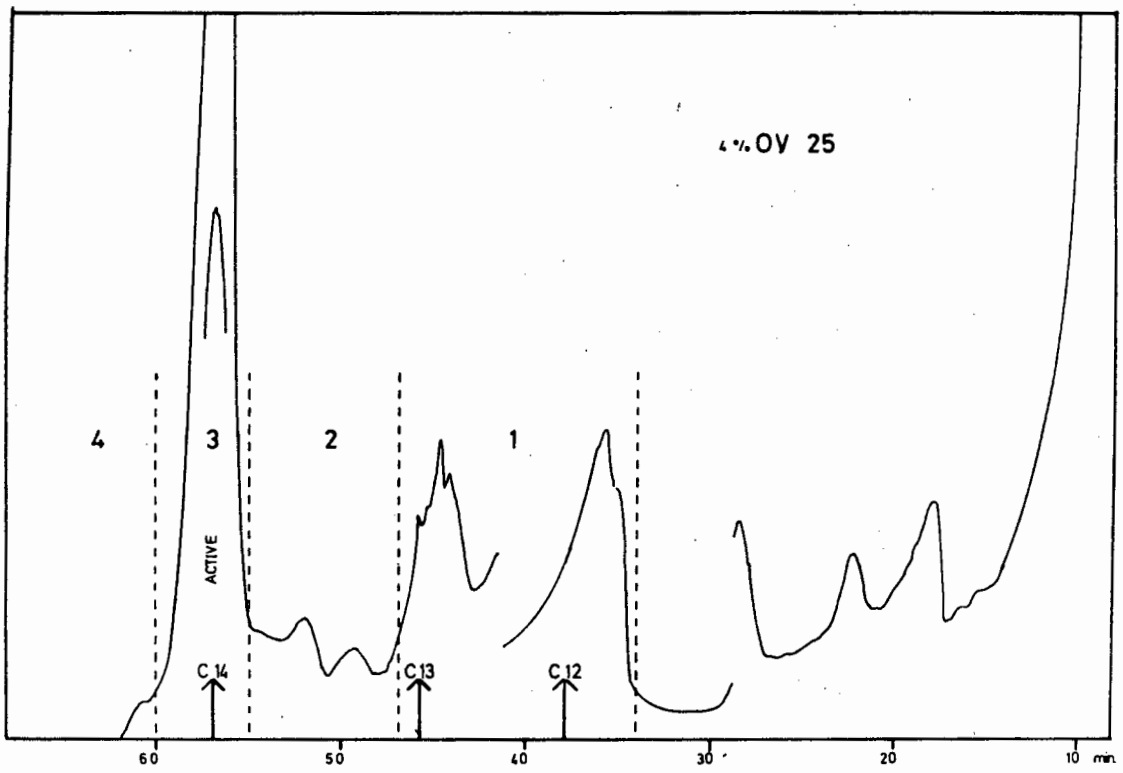

Figure VII.6a. A section of the chromatogram from the pheromone concentrate G_1 showing the preparative fractions collected (vertical dotted lines) portions of which were bioassayed on the EAG and in field tests.

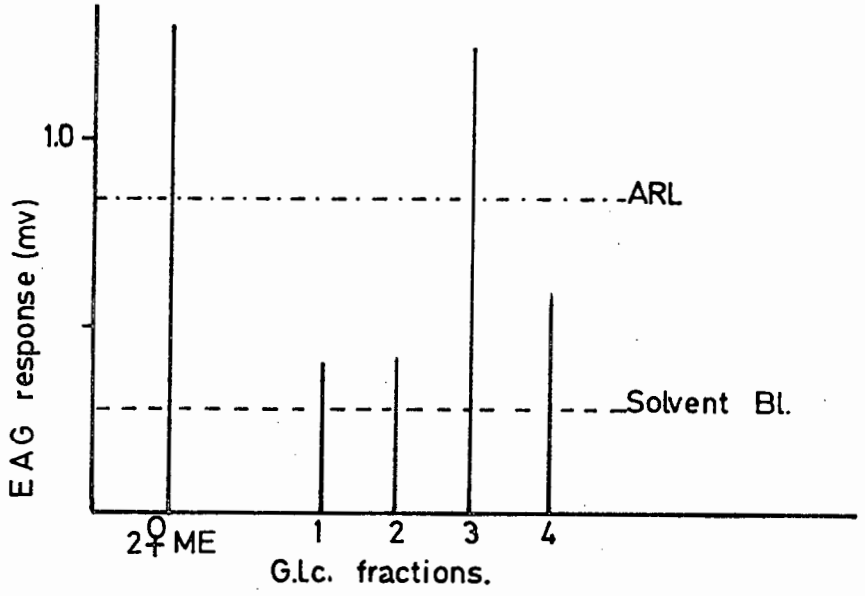

Figure VII.6b. EAG responses of a male antenna to four fractions collected from the g.l.c. of the pheromone concentrate G_1.

Portions (ca. 12 µl , 10 ♀ ME) of each fraction were placed in traps in infested areas in the pine plantation at Franschoek. These traps were positioned in a single row at 30 yd. intervals. Fractions 3 and 4 attracted male moths, trapping 150 and 7 respectively.

The active area (fraction 3) again revealed a single large peak with no evident shoulders. Preparative collection of this peak from the remainder of G_1 was effected in six runs (6 x 15µl). Much of the active peak trapped in fractions 2 and 4 through tailing on the column was recovered by injection of the concentrates from these fractions and recollection. The carbon tetrachloride from the active fraction (F3) was concentrated to a volume of ca. 100µl under a stream of high purity nitrogen, to give a pheromone concentrate P_1 and quantitatively transferred to a reacti-vial by means of a 50µl syringe. Analysis of the concentrate P_1 under analytical g.l.c. conditions using the same column revealed the presence of a single peak from which the total quantity of material isolated was estimated to be ca. 170µg = 0.01 µg/female moth.

The pheromone concentrate G_2 (second batch of 17,000 moth tips) was similarly chromatographed and a single active peak isolated from the effluent gases by con - densation in carbon tetrachloride. The chromatogram obtained from this material G_2 is included (Fig. VII.7) as it differed considerably from that of G_1 (Fig. VII.6a).

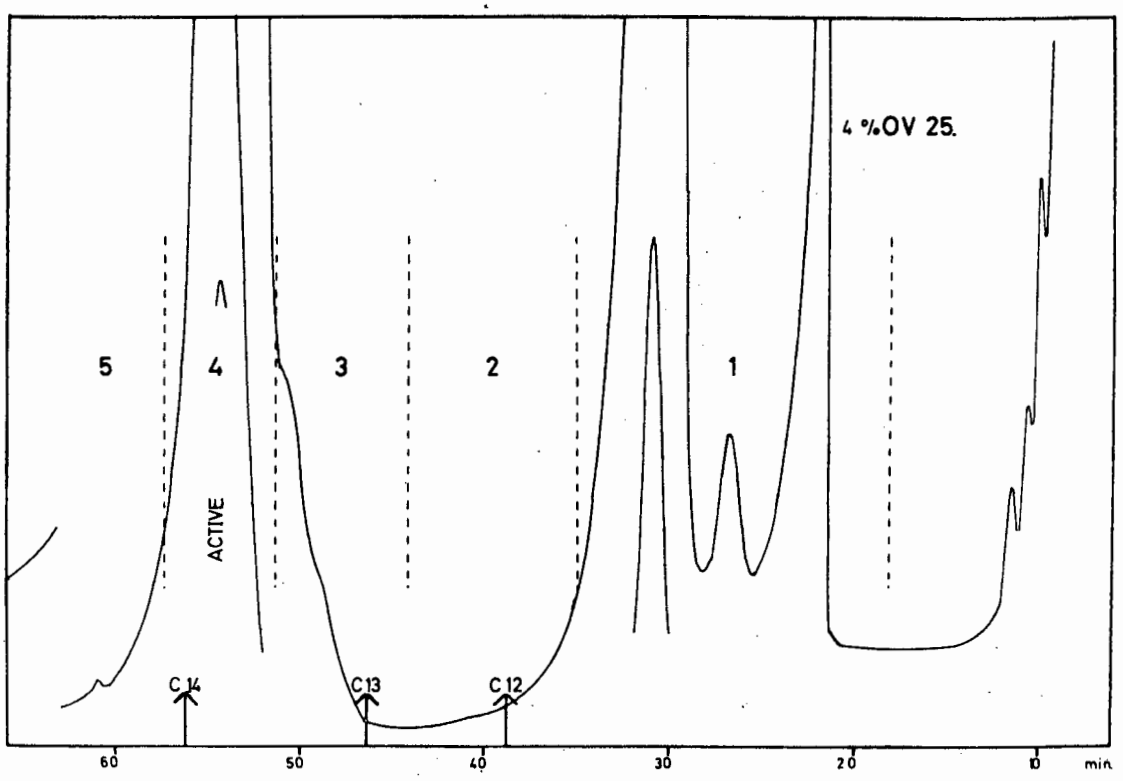

Figure VII.7. A section of the chromatogram from the pheromone concentrate G_2 depicting the fractions collected (vertical dotted lines) in isolation of the active compound from the stationary phase OV 25.

In addition to the presence of a large peak in the vicinity of the C_{10} ester marker (estimated) the active peak (fraction 4) revealed a distinct shoulder which on bio-assay (EAG) (fraction 3; 1.2 µl, 10 ♀ ME) was found to

be inactive. Only fraction 4 (1.2 µl , 10 ♀ ME) gave a response (EAG). Active material trapped in fractions 3 and 5 was recovered by injection of the concentrates from these fractions and collection of the active peak (Fig. VII.8).

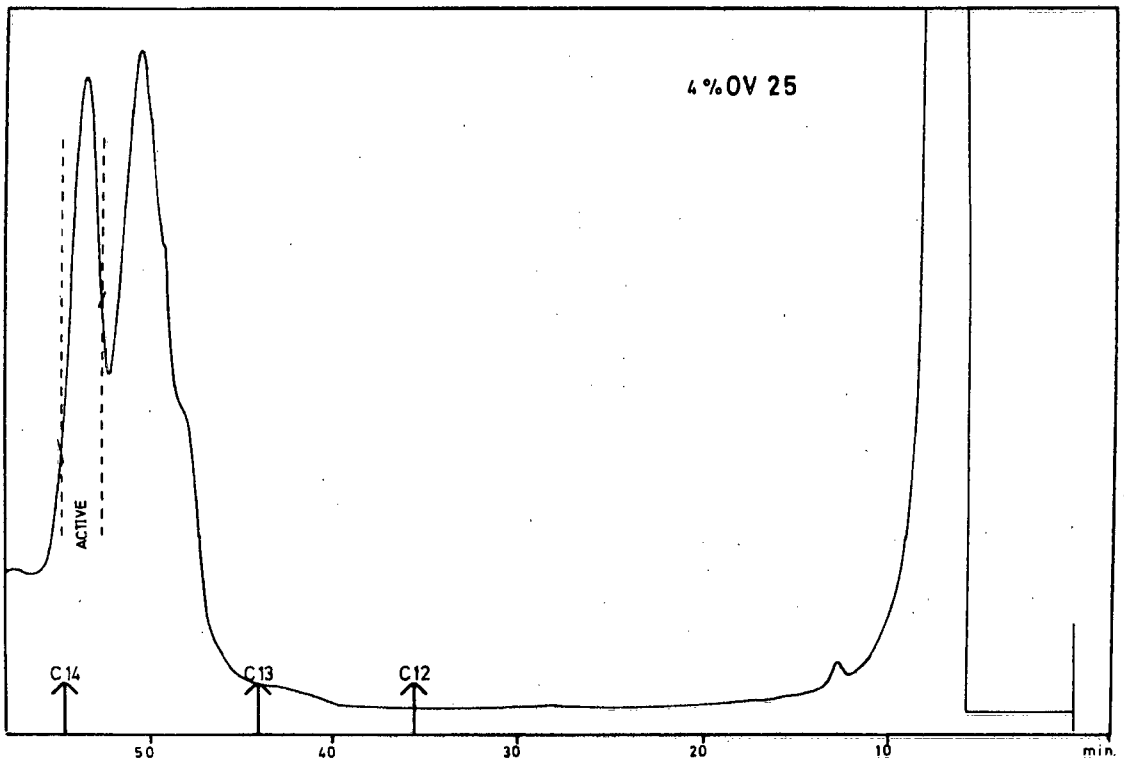

Figure VII.8. The chromatogram obtained on re-injection of the condensates from fractions 3 and 5 depicting the additional quantity of active material collected.

Removal of the stream splitter from the g.l.c. and analysis of the concentrate P_2 from fraction 4, revealed a single peak from which it was estimated that ca. 150 µg (=.009 µg/female moth) of an active substance had been isolated.

3. N.m.r. spectroscopy

The combined pheromone concentrates P_1 (170 µg) and P_2 (150 µg) were quantitatively transferred by means of a hypodermic syringe (50 µl) to a n.m.r. micro-cell (capacity 120 µl , cylindrical bore) and tetramethylsilane (TMS) added as internal standard. The micro-cell was capped and transferred to a Varian A-60 instrument for spectral analysis. A weak spectrum was obtained showing broad and unresolved peaks. Peaks at (τ) 4.71 and 6.0 were only just discernible above the background noise. As a computer system (C.A.T.) was not available on this instrument the micro-cell was transferred for analysis to a Varian XL-100 instrument. A much improved spectrum was obtained showing strong and well resolved peaks (Fig. VII.9). These peaks had shift positions of (τ) 9.03 (6H, doublet $ME_2.CH.$, J 6.3 Hz), ca. 9 (3H, triplet, aliphatic $ME.CH_2.-$), 6.01 (2H, triplet, $-CH_2.CH_2.O.$ J 6.0 Hz) and 4.71 (2H, multiplet, $CH_2.CH=CH.CH_2$).

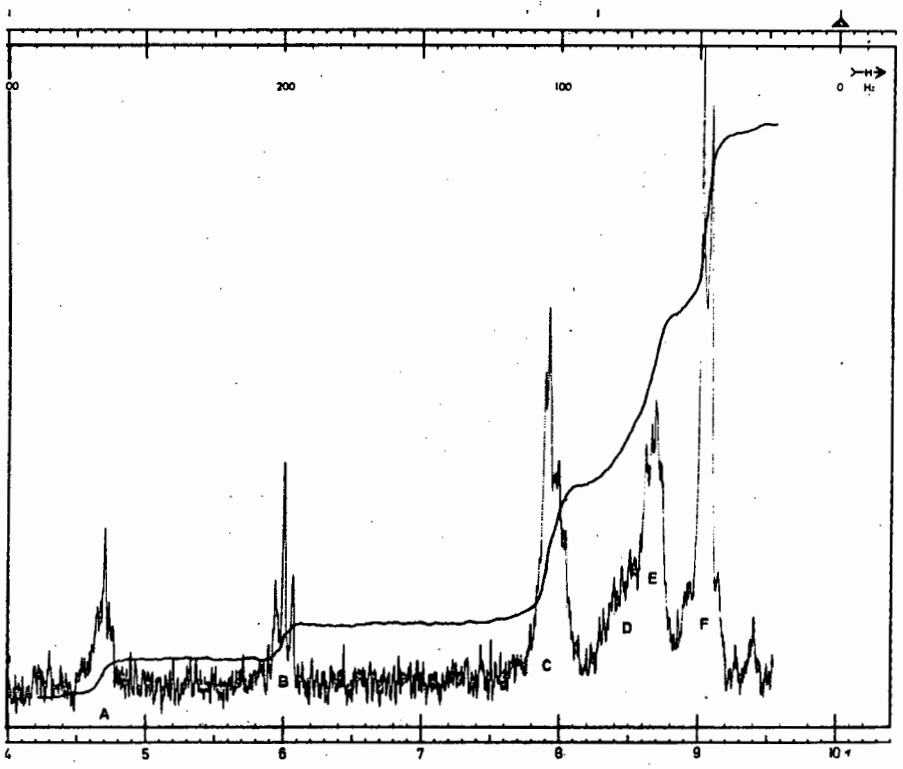

Figure VII.9. The n.m.r. spectrum of the isolated pheromone (concentrates P_1 and P_2, 320 μg). A Varian XL - 100 instrument was used with a micro-cell (120 μl; cylindrical bore) with carbon tetrachloride as solvent. Freq. response 5 Hz, sweeptime 1,000 sec., sweep width 1,000 Hz and spectrum amp. 3500/1.

In an attempt to improve the resolution, the C.A.T. was linked to the n.m.r. and the spectrum scanned 14 times. Apart from a better signal to noise ratio, the spectrum remained the same. Finally, decoupling studies were carried out, successfully, on peaks a, b, c and d.

4. Mass spectrometry

A portion of the pheromone solution (45 µl , ca. 100 µg) from the n.m.r. micro-cell was chromatographed in three runs (3 x 15µl) on the same 2.5% FFAP column, as used previously, under similar conditions (section 2.3.(iv)a) except that a carrier gas flow rate of 12 ml/min. was utilised. The stream splitter was constricted to conduct between 2 - 3% of the carrier gas to the detector. The pheromone emerged after 90 minutes as a single large peak and was trapped by passage of the effluent gas through a hypodermic needle (20 cm) the centre portion (5 cm) of which passed through a container filled with crushed dry ice. After three collections, the condensed material was washed on to the direct probe of the mass spectrometer by running 3 - 4 drops of dichloromethane through the needle.

The mass spectrum (Fig. VII.10) showed a molecular ion peak at m/e 240.2089, M^+, 0.1% ($C_{15}H_{28}O_2$ requires 240.2097) and two significant ions at m/e 138.1408, 90% ($C_{10}H_{18}$ requires 138.1395) and m/e 110 (100%, base peak).

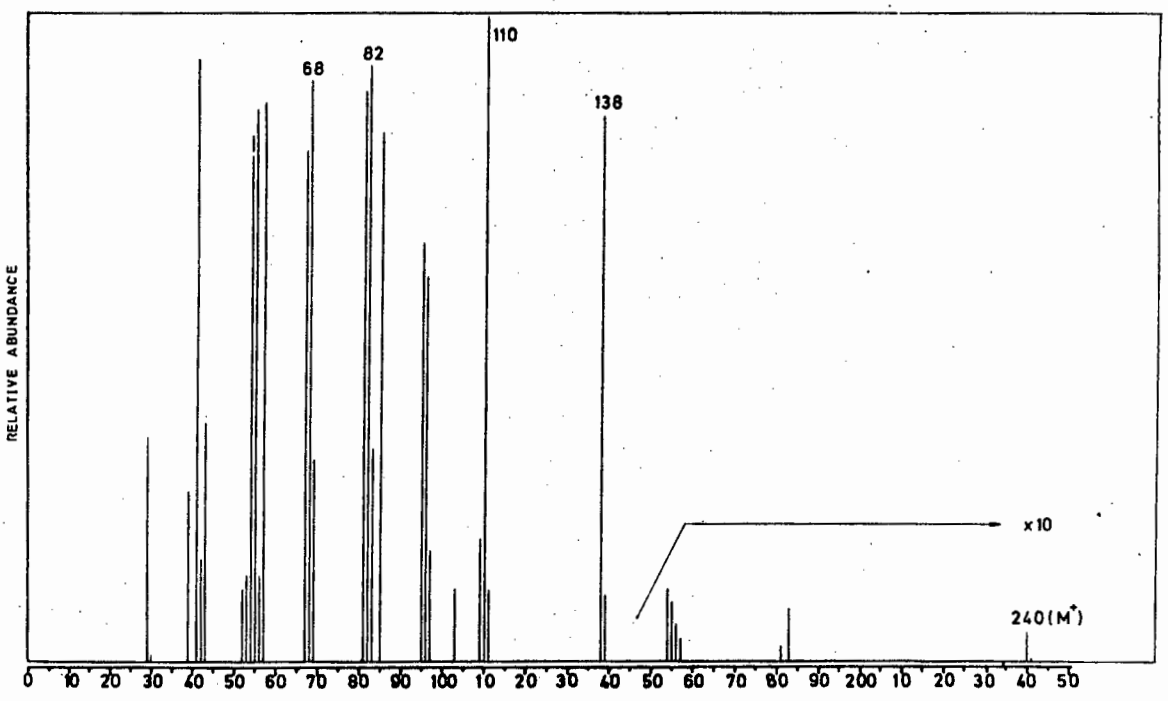

Figure VII.10. The mass spectrum of the natural pheromone (ca., 100 μg). An A.E.I. - MS 902 was used with the direct probe technique.

5. Infrared spectrophotometry

A portion of the pheromone solution (40 μl , 100 μg) from the n.m.r. micro-cell was concentrated under a stream of pure nitrogen to a volume of 10 μl and carefully transferred to an i.r. micro-cell (10 μl capacity, path length .1mm). The loaded micro-cell

was fitted into the condensed beam of a Beckman I.R.-7 instrument and the spectrum determined. A reference cell was not available on this instrument so the pheromone solution was removed from the micro-cell and replaced by pure carbon tetrachloride. The absorption bands in the spectrum of the pure solvent were then subtracted from the bands in the pheromone spectrum. The resulting spectrum showed absorptions at 2950 (CH), 1741 (C=O), 1387 and 1371 (Me_2.CH) and 1192 cm^{-1} (C.O.C).

6. Chemical studies on crude extracts from virgin female moths.

(i) Stability: Preliminary studies by other workers[48] showed that pheromone activity was not destroyed by heating at 100° for two hours in sealed tubes.

(ii) Saponification: A portion (200 ♀ ME) of a virgin female extract was refluxed in methanolic KOH (1 ml) for 15 minutes. The neutral products were extracted in ether (1 ml) and dried over anhydrous sodium sulphate (30 mg) to give a solution S_1. Bioassay(EAG) of an aliquot (50µl S_1, 10 ♀ ME) revealed no activity.

A portion (250µl , 50 ♀ ME) of S_1 was evaporated under a stream of nitrogen and acetylated by refluxing in pyridine (0.2 ml) and acetic anhydride (2 drops), for 10 minutes. Water (1 ml) was added and the acetylated products extracted in light petroleum (1 ml). The petroleum extract was washed twice with 5% sodium

bicarbonate (2 x 1 ml) and dried over anhydrous sodium sulphate (30 mg). Bioassay (EAG) of the entire solution revealed no activity. Similarly treatment with acetyl chloride in ether did not restore activity. The acidic compounds were extracted in ether (1 ml) and dried over anhydrous sodium sulphate (50 mg) to give a solution S_2. Bioassay (EAG) of an aliquot (50 µl S_2, 10 ♀ ME) revealed no activity. Methylation of the remainder of S_2 (ca.190 ♀ ME) with diazomethane in ether (1 ml) failed to regain activity.

(iii) Unsaturation: Hydrogenation - In the preliminary studies of Read and Hewitt [48] it was shown that the hydrogenation of female extracts over platinum oxide reduced activity in field tests.

Bromine (30 µl) was added to a portion (200 ♀ ME) of a virgin female extract in carbon tetrachloride (1 ml) and set aside for 1 hour. The excess bromine was boiled off at 50° and the residue assayed (EAG) for activity. No antennal response was obtained. A portion (10 ♀ ME) of a virgin female extract in chloroform (50 µl) was added at 0° to a solution of perbenzoic acid in chloroform (50 µl) and set aside for 30 minutes. The mixture was spotted on to a section of filter paper and bioassayed (EAG). No activity was found.

(iv) Reduction with $LiAlH_4$ A portion (10 ♀ ME) of a virgin female extract in a small test tube was taken up in 4 drops of sodium-dried diethyl ether. Powdered $LiAlH_4$ (50 mg) was added and the mixture allowed to stand for

20 minutes. Water (3 drops) was cautiously added and the residue extracted with diethyl ether (2 x .5 ml). The ether extracts were combined and concentrated to a volume of 0.1 ml. Bioassay (EAG) of this concentrate revealed that pheromone activity had been destroyed.

7. Synthesis of cis-dec-5-en-1-yl isovalerate and positional isomers.

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1. cis-Dec-5-en-1-yl isovalerate.

(i) 4-Chlorobutan-1-yloxytetrahydropyran: Dry dihydropyran (25.0 g, 0.3 mol) was added dropwise over a period of 20 minutes to a stirred mixture of 4-chlorobutan-1-ol (24.9 g, 0.23 mol) (Note 1) and conc. hydrochloric acid (0.5 ml). During the addition, the reaction temperature was kept below 30° by cooling the reaction flask in an ice bath. After being stirred for two hours the mixture was set aside, overnight, at room temperature. Sodium bicarbonate (0.5 g) was added and the mixture stirred for one hour. Filtration and distillation gave 4-chlorobutan-1-yloxytetrahydropyran (40.0 g ; b. p. 88 - 89°/3 mm) in 90.5% yield.

Found : C, 56.2 ; H, 8.9 ; Cl, 18.1.

Calc. for $C_9H_{17}ClO_2$: C, 56.1 ; H, 8.8 ; Cl, 18.4%

Notes:

1. 4-Chlorobutan-1-ol was prepared according to the method of Starr and Hixon (b. p. 64 - 65°/7 mm) in 45% yield.[100] (Found: C, 44.5 ; H, 8.3 ; Cl, 32.7. C_4H_9ClO requires C, 44.4 ; H, 8.4 ; Cl, 32.4%.

(ii) Hex-5-yn-1-yloxytetrahydropyran: A solution of 4-chlorobutan-1-yloxytetrahydropyran (40.0 g, 0.21 mol) in dry dimethyl sulphoxide (DMSO) (100 ml) (Note 1) was slowly added under nitrogen to a stirred mixture of lithium acetylide ethylenediamine complex (25.0 g, 0.27 mol) and dry DMSO (150 ml). The reaction temperature was kept below 30° throughout the addition by cooling in an ice bath. After one hour the ice bath was removed and the mixture stirred at room temperature for 18 hours. Water (200 ml) was cautiously added and the mixture extracted with ether (4 x 100 ml). The combined ether extracts were washed with 10% sodium chloride solution (2 x 100 ml), once with water (100 ml) and dried over anhydrous sodium sulphate.

Concentration by rotary evaporation and distillation gave, after a small forerun (3 g), hex-5-yn-1-yloxytetrahydropyran (27.7 g ; b. p. 106°/15 mm) in 73.1% yield. The infrared spectrum (liquid) showed absorptions at 3300 (terminal C≡C) and 1200 - 1040 cm^{-1} (tetrahydropyranyl).

Found : C, 72.3 ; H, 10.0.

Calc. for $C_{11}H_{18}O_2$: C, 72.4 ; H, 9.9%.

Notes:

1. DMSO was dried over sodium hydride using triphenyl methane (200 mg) as indicator.

(iii) Dec-5-yn-1-yloxytetrahydropyran: Freshly cut lithium metal (1.4 g, 0.2 mol) was added in small portions (ca. 0.25 g) to liquid ammonia (400 ml) in a 500 ml two necked flask equipped with a magnetic stirrer, a drying tube (potassium

hydroxide pellets) and a dropping funnel. To minimise the loss of ammonia, cottonwool was wrapped around the flask leaving a small gap through which the level of the ammonia could be observed. A short while after the addition of the last portion of lithium, conversion of the metal into lithamide was judged to be complete due to the disappearance of the blue colour of dissolved lithium. Hex-5-yn-1-yloxytetrahydropyran (27.7 g, 0.17 mol) was added, dropwise, over a period of 15 minutes. At this stage the ammonia lost during the reaction was replenished. After stirring for one hour, dry distilled 1-bromobutane (25.3 g, 0.18 mol) was added over a period of twenty minutes. Stirring was continued for three hours whereupon solid ammonium chloride (5.0 g) was added to remove unreacted lithamide and the flask allowed to stand overnight when the ammonia evaporated. Water (500 ml) was added and the mixture extracted with light petroleum (3 x 50 ml). The combined extracts were washed with water(2 x 150 ml) and dried over anhydrous sodium sulphate (5.0 g).

Concentration and fractional distillation at reduced pressure gave, after a small forerun (1.5 g ; b. p. 70 - 80°/0.4 mm) dec-5-yn-1-yl-oxytetrahydropyran (26.1 g; b. p. 110 - 113°/0.4 mm)69% yield.The infrared spectrum (liquid) showed absorptions at 2950 (-CH) and 1200 - 1040 cm^{-1} (tetrahydropyranyl).

Found : C, 75.1 ; H, 10.9.

$C_{15}H_{26}O_2$ requires : C, 75.5 ; H, 11.0%.

(iv) cis-Dec-5-en-1-yloxytetrahydropyran: Dec-5-yn-1-yloxytetrahydropyran (10.0 g, 0.042 mol) was hydrogenated in hexane (50 ml) over Lindlar catalyst (0.2 g) (Note 1) poisoned by the addition of 3 - 4 drops of quinoline. Hydrogenation was effected under slight positive pressure with agitation using a shaker. A large decrease in the rate of hydrogenation was observed when the calculated volume of hydrogen had been taken up. The flask was removed and the catalyst recovered by filtration.

Analysis of the product by thinlayer chromatography (t.l.c.) on silica gel impregnated (25%) with silver nitrate showed complete reduction of the alkyne to the cis olefin. (Note 2). The filtrate was therefore concentrated and used directly in the next step.

Notes:

1. The catalyst used (Pd-$CaCO_3$-PbO), was prepared according to the method described by Fieser and Fieser [101] which is a slight modification of Lindlar's original procedure.[86]
2. The plates were run in a benzene-light petroleum (80:20) solvent system and visualised by spraying with 1% dichlorofluroscein in ethanol. Subjection to ultraviolet radiation gave vivid green spots on a purple background.

(v) cis-Dec-5-en-1-ol

Ethanol was added to a mixture of cis-dec-5-en-1-yloxy-tetrahydropyran (10.0 g, 0.042 mol) and 2N-hydrochloric acid (100 ml) until it became homogeneous. The

solution was kept at 40° for eight hours and then poured into water (200 ml) and extracted with light petroleum (3 x 50 ml). The combined extracts were washed with water (50 ml), with a 5% solution of sodium bicarbonate (50 ml) and dried over anhydrous sodium sulphate (2 g).

Concentration and fractional distillation at reduced pressure gave, after a small forerun, cis-5-decen-1-ol (6.0 g; b. p. 67°/0.15 mm) in 90% yield.

The infrared spectrum (liquid) showed absorptions at 3350 (-OH) and 2930 cm^{-1} (-CH).

Found : C, 76.7 ; H, 12.9.

$C_{10}H_{20}O$ requires : C, 76.8 ; H, 12.9%.

(vi) cis-Dec-5-en-1-yl isovalerate: A mixture of cis-dec-5-en-1-ol (6.0 g, 0.039 mol), dry benzene (40 ml) distilled isovaleric acid (7.2 g, 0.07 mol) and conc. sulphuric acid (0.5 ml) was stirred at 55° for eight hours. Water (200 ml) was added and the mixture extracted with light petroleum (3 x 25 ml). The combined extracts were washed with a 5% sodium bicarbonate solution (2 x 30 ml) and dried over anhydrous sodium sulphate (5 g).

Concentration and fractional distillation at reduced pressure gave cis-dec-5-en-1-yl isovalerate (6.9 g ; b. p. 94°/0.3 mm) in 73% yield. A portion (2.0 g) was purified by preparative g.l.c. on the Varian Aerograph 1200 instrument using a 1.8 m x .6 mm i.d. glass column, packed with 10% SE 30 on Chromosorb P 60/100

mesh. A column temperature of 200° was employed with a carrier gas flow rate of 40 ml N_2/min.

The infrared spectrum of the purified ester showed absorptions at 2950 (-CH), 1740 (C=O) and 1190 cm^{-1} (C-O-C).

The n.m.r. spectrum (carbon tetrachloride) showed absorptions at (τ) : 9.03 and 9.09 (6H, d, Me_2.CH=, J 6.0 Hz), ca., 9 (3H, t, aliphatic Me-), 6.0 (2H, t, .CH_2.CH_2.O., J 6.0 Hz) and 4.71 (2H, m, -CH=CH-).

Found : C, 74.8 ; H, 11.7.

$C_{15}H_{28}O_2$ requires : C, 74.9 ; H, 11.8%.

2. trans-Dec-5-en-1-yl isovalerate.

(i) trans-Dec-5-en-1-yloxytetrahydropyran: Clean lithium metal (0.75 g, 0.12 mol) was added in two portions to stirred liquid ammonia (250 ml) in a cylindrical glass vessel. After five minutes dec-5-yn-1-yloxy-tetrahydropyran (14.0 g, 0.06 mol) was slowly added to the vessel by means of a pipette, the tip of which projected immediately below the surface of the liquid ammonia. This procedure minimised splashing and subsequent coating of the walls of the vessel with unreduced material. The mixture was stirred for two minutes and the vessel sealed in a cylindrical metal container fitted with a small tap and a safety valve, set to blow at 200 p.s.i. The reaction mixture was allowed to attain room temperature and then set aside for 24 hours. After slow release of the internal

pressure the apparatus was dismantled and water (200 ml) cautiously added to the residue. The whole was extracted with light petroleum (2 x 100 ml) and the combined extracts washed with water (2 x 50 ml) and dried over anhydrous sodium sulphate (5.0 g).

Concentration by rotary evaporation, and analysis of the product by thin layer chromatography (t.l.c.) on silica gel, impregnated (25%) with silver nitrate (Note 1) showed approximately 70% reduction to the trans isomer.

A further treatment, using lithium (0.5 g, 0.07 mol) in liquid ammonia (300 ml) and analysis by (t.l.c.) revealed the presence of trace amounts of unreduced material. A third reduction was deemed unnecessary. The product was, therefore, concentrated and used directly in the next step.

The infrared spectrum (liquid) showed absorptions at 2950 (-CH), 1200 - 1000 (tetrahydropyranyl) and 970 cm^{-1} (trans-CH=CH-).

Notes:

1. The t.l.c. plates were run in a benzene-light petroleum (80:20) solvent system and visualised by spraying with 1% dichlorofluroscein in ethanol. Subjection to u.v. radiation gave vivid green spots on a purple background.

(ii) trans-Dec-5-en-1-ol: Ethanol was added to a mixture of trans-dec-5-en-1-yloxytetrahydropyran (12.1 g, 0.05 mol) and 2N-hydrochloric acid (100 ml) until it became homogeneous. The solution was kept at 40^{o} for

eight hours, poured into water (200 ml) and extracted with light petroleum (3 x 50 ml). The combined extracts were washed with water (50 ml), with a 5% solution of sodium bicarbonate (50 ml) and dried over anhydrous sodium sulphate (3.0 g).

Concentration and fractional distillation at reduced pressure gave, after a small forerun (1.1 g ; b. p. 50 - 55°/0.1 mm)trans-dec-5-en-1-ol (6.3 g; b. p. 63 - 64°/0.1 mm) in 81% yield.

The infrared spectrum (liquid) showed absorptions at 3330 (-OH), 2930 (-CH) and 970 cm^{-1} (trans-CH=CH-).

Found : C, 76.8 ; H, 12.9.

$C_{10}H_{20}O$ requires : C, 76.9 ; H, 12.9%.

(iii) trans-Dec-5-en-1-yl isovalerate: A mixture of trans-dec-5-en-1-ol (6.3 g, 0.04 mol) dry benzene (30 ml), distilled isovaleric acid (7.1 g, 0.07 mole) and conc. sulphuric acid was stirred at 55° for eight hours. Water (200 ml) was added and the mixture extracted with light petroleum (2 x 30 ml). The combined extracts were washed with a 5% sodium bicarbonate solution (2 x 30 ml) and dried over anhydrous sodium sulphate (3 g).

Concentration and fractional distillation at reduced pressure gave, after a small forerun, trans-dec-5-en-1-yl isovalerate (7.1 g ; b. p. 84°/0.2 mm) in 74% yield.

A portion (2.0 g) was purified by preparative g.l.c. using the Varian Aerograph, 1200 series instrument on a 1.8 m x .6 mm i.d. glass column, packed with 10% SE 30 on Chromosorb P 60/100 mesh. The column was conditioned at 200° for two days before use. An oven temperature of 200° was employed with a carrier gas flow rate of 40 ml N_2/min.

The infrared spectrum (liquid) showed absorptions at 2930 (-CH), 1730 (C=O), 1175 (C-O-C) and 970 cm^{-1} (trans-CH=CH-).

The n.m.r. spectrum showed absorptions at (τ) :(CCl_4) 9.03 and 9.10 (6H, d, Me_2-CH-, J 6.03 Hz), ca., 9 (3H, t, aliphatic Me-), 6.01 (2H, t, $.CH_2.CH_2.O$, J 6.0 Hz) and 4.67 (2H, m, -CH=CH-).

Found : C, 74.9 ; H, 11.7.

$C_{15}H_{28}O_2$ requires : C, 74.9 ; H, 11.7%.

3. cis-Dec-4-en-1-yl isovalerate.

(i) Pent-4-yn-1-yloxytetrahydropyran: Pent-4-yn-1-yloxy-tetrahydropyran (43.1 g ; b. p. 65 - 68°/0.1 mm) was prepared from dihydropyran (37.8 g, 0.45 mol) and pent-4-yn-1-ol (24.8 g, 0.3 mol) (Note 1) in 85.5% yield, following a procedure described previously (exp. 1.(i)).

The infrared spectra (liquid) showed absorptions at 3240 and 2120 (-C≡CH) and 1200 - 1000 cm^{-1} (tetra-hydropyranyl).

Found : C, 71.5 ; H, 9.6.

$C_{10}H_{16}O_2$ requires : C, 71.4 ; H, 9.6%.

Notes:

1. Pent-4-yn-1-ol was prepared in 61.5% yield according to the method of Jones, Eglington and Whiting,[83]

(ii) Dec-4-yn-1-yloxytetrahydropyran: Dec-4-yn-1-yloxy-tetrahydropyran (25.5 g , b. p. 99 - 100°/0.4 mm) was prepared in 63.7% yield by reaction of the sodium salt of pent-4-yn-1-yloxyterahydropyran (27.0 g, 0.17 mol) with 1-bromopentane (25.3 g, 0.17 mol) in liquid ammonia, following a procedure described previously (exp. 1.(iii)).

The infrared spectrum (liquid) showed absorptions at 2930 (-CH) and 1200 - 1000 cm^{-1} (tetrahydropyranyl).

Found : C, 75.4 ; H, 11.1.

$C_{15}H_{26}O_2$ requires : C, 75.5 , H, 11.0%.

(iii) cis-Dec-4-en-1-yloxytetrahydropyran: cis-Dec-4-en-1-yloxytetrahydropyran (10.0 g, 0.042 mol) was hydrogenated over Lindlar catalyst, following a procedure described previously (exp. 1 (iv)).

Analysis of the product by t.l.c. showed complete reduction to the cis olefin.

(iv) cis-Dec-4-en-1-ol: cis-Dec-4-en-1-ol (6.1 g ; b. p. 83 - 84°/0.15 mm) was prepared in 90% yield by the hydrolysis of cis-dec-4-en-1-yloxytetrahydropyran (10.0 g , 0.042 mol) following a procedure described previously (exp. 1-(iv)).

The infrared spectrum (liquid) showed absorption at 3330

(-OH) and 2930) cm^{-1} (-CH).

Found : C, 76.8 ; H, 13.0.

$C_{10}H_{20}O$ requires : C, 76.9 ; H, 12.9%.

(v) cis-Dec-4-en-1-yl isovalerate: cis-Dec-4-en-1-yl isovalerate (6.8 g, b. p. 91 - 3°/1 mm) was prepared in 71% yield from cis-dec-4-en-1-ol (6.1 g ; 0.04 mol) and isovaleric acid (10.2 g, 0.1 mol) following a procedure described previously (exp. 1. (vi)).

A portion (2.0 g) was purified by preparative g.l.c. (exp. 1.(vi)).

The infrared spectrum (liquid) showed absorptions at 2930 (-CH), 1740 (C=O) and 1190 cm^{-1} (C-O-C).

Found : C, 75.1 ; H, 11.8.

$C_{15}H_{28}O_2$ requires : C, 74.9 ; H, 11.8%.

4. trans-Dec-4-en-1-yl isovalerate.

(i) trans-Dec-4-en-1-yloxytetrahydropyran: Dec-4-yn-1-yloxytetrahydropyran (14.0 g , 0.059 mol) was reduced to the trans olefin by two reductions with lithium/liquid ammonia, following a procedure described previously (exp. 2.(i)). T.l.c. analysis of the final reaction product revealed the presence of trace amounts of unreduced material.

(ii) trans-Dec-4-en-1-ol: trans-Dec-4-en-1-ol (7.1 g ; b. p. 67°/0.15 mm) was prepared in 77% yield by the acid hydrolysis of trans-dec-4-en-1-yloxytetrahydropyran (13.2 g, 0.055 mol), following a procedure described previously (exp. 1.(v)).

The infrared spectrum (liquid) showed absorptions at 3330 (-OH) and 2930 cm^{-1} (-CH).

Found : C, 76.2 ; H, 13.0.

$C_{10}H_{20}O$ requires : C, 76.9 ; H, 12.9%.

(iii) trans-Dec-4-en-1-yl isovalerate: trans-Dec-4-en-1-yl isovalerate (8.3 g ; b. p. 102 - 3° / 2.5 mm) was prepared in 75% yield from trans-dec-4-en-1-ol (7.1 g, 0.045 mol) and isovaleric acid (10.2 g, 0.1 mol) following a procedure described previously (exp. 1.(vi)). A portion (2.0 g) was purified by preparative g.l.c. (exp. 1.(vi)).

The infrared spectrum (liquid) showed absorptions at 2930 (-CH), 1735 (C=O), 1170 (C-O-C) and 970 cm^{-1} (trans -CH=CH-).

Found : C, 75.0 ; H, 11.8.

$C_{15}H_{28}O_2$ requires : C, 74.9 ; H, 11.8%.

5. cis-Dec-6-en-1-yl isovalerate.

(i) 5-Iodopentyloxytetrahydropyran: 5-Chloropentyloxytetrahydropyran (45.0 g , 0.22 mol) (Note 1) and sodium iodide (52.5 g, 0.35 mol) were refluxed in acetone (200 ml) for six hours. The solution was filtered, poured into water and extracted with light petroleum (2 x 100 ml). The combined extracts were washed with water (200 ml), with 5% sodium thiosulphate (50 ml) and dried over anhydrous sodium sulphate (5 g).

The bulk of the product was not distilled due to persistent decomposition during distillation (Note 2) and used

directly in the following condensation. A small portion was distilled and submitted for analysis.

Found : C, 40.0 ; H, 6.5 ; I, 41.1.

Calc. for $C_{10}H_{19}IO_2$: C, 40.3 ; H, 6.4 ; I, 42.6%.

Notes:

1. 5-Chloropentyloxytetrahydropyran was prepared in 65% yield according to the method of Ames and Islip.[78]
2. Compare the instability of 7-iodoheptyloxytetrahydropyran.[79]

(ii) Hept-6-yn-1-yloxytetrahydropyran: A two litre four necked flask was fitted with an efficient stirrer a gas inlet tube reaching the bottom of the flask, a length of flexible iron wire (25 cm) capable of being dipped into the flask or withdrawn as required and an outlet tube. A layer of cottonwool was wrapped around the outside to give some insulation. The flask was charged with liquid ammonia (1 litre) and purified acetylene (Note 1) bubbled through the liquid at a rate of five bubbles per second. After five minutes (Note 2) a portion (ca. 2.0 g) of sodium (6.9 g, 0.3 mol) was attached to the iron wire and slowly lowered below the surface of the liquid ammonia. The flow of acetylene was increased to 10 bubbles/second. The dissolving sodium formed a continuous blue streak which disappeared with the formation of sodium acetylide. Addition of the sodium was done at a rate such that the whole solution did not turn blue. This occasionally happened as small pieces of the main portion broke away. At

such times the wire was withdrawn and the sodium suspended above the surface until the blue colour was discharged. The remainder of the sodium was added in the same way. After complete conversion of the metal into sodium acetylide the acetylene bubbler was removed and 5-iodopentyloxytetrahydropyran (65.0 g, 0.22 mol) was added dropwise over thirty minutes. Stirring was continued for three hours whereupon the flask was allowed to stand overnight when the ammonia evaporated. Water (400 ml) was cautiously added and the whole extracted with light petroleum (3 x 100 ml). The combined extracts were washed with water until neutral and dried over anhydrous sodium sulphate (10 g).

Concentration and fractional distillation at reduced pressure gave hep-6-yn-1-yloxytetrahydropyran (22.1 g, b. p. $66^{o}/0.15$ mm) in 57% yield.

The infrared spectrum (liquid) showed absorptions at 3230 and 2105 (-C≡CH) and 1200 - 1000 cm^{-1} (tetrahydropyranyl).

Found : C, 73.1 ; H, 10.2.

Calc. for $C_{12}H_{20}O_2$: C, 73.4 ; H, 10.3%.

Notes:

1. The acetylene was purified by passage through a cold trap (acetone/dry ice), a simple mercury trap to prevent suck back, two bubblers of conc. sulphuric acid and finally through a soda lime column ($CaCl_2$ + 10% NaOH + 12% water).

2. The initial passage of acetylene, for five minutes, served to saturate the liquid ammonia before the addition of the sodium.

(iii) Dec-6-yn-1-yloxytetrahydropyran: Dec-6-yn-1-yloxytetrahydropyran (10.1 g ; b. p. 99 - 100°/0.4 mm) was prepared in 55% yield from the lithium salt of hept-6-yn-1-yloxytetrahydropyran (15.1 g, 0.077 mol) and 1-bromopropane (11.1 g, 0.09 mol), following a procedure described previously (exp. 1.(iii)).

The infrared spectrum (liquid) showed absorptions at 2930 (-CH) and 1200 - 1000 cm^{-1} (tetrahydropyranyl).

Found : C, 75.4 ; H, 10.9.

$C_{15}H_{28}O_2$ requires : C, 75.6 ; H, 11.0%.

(iv) cis-Dec-6-en-1-yloxytetrahydropyran: Dec-6-yn-1-yloxytetrahydropyran (5.0 g, 0.021 mol) was hydrogenated over Lindlar catalyst, following a procedure described previously (exp. 1.(iv)). Analysis by t.l.c. revealed complete reduction to the cis olefin. The product was therefore used directly in the next step.

(v) cis-Dec-6-en-1-ol: cis-Dec-6-en-1-ol (2.6 g ; b. p. 59°/0.1 mm) was prepared in 80% yield by the acid hydrolysis of cis-dec-6-en-1-yloxytetrahydropyran (5.0 g, 0.021 mol) following a procedure described previously (exp. 1.(v)).

The infrared spectrum (liquid) showed absorptions at 3330 (-OH) and 2930 cm^{-1} (-CH).

Found : C, 76.8 ; H, 12.9.

$C_{10}H_{20}O$ requires : C, 76.9 ; H, 12.9%.

(vi) cis-Dec-6-en-1-yl isovalerate: cis-Dec-6-en-1-yl isovalerate (3.2 g, b. p. 91 - 92°/0.1 mm) was prepared from cis-dec-6-en-1-ol (2.65 g ; 0.017 mol) and isovaleric acid (3.69 g ; 0.035 mol) following a procedure described previously (exp. 1.(vi)). A portion (2.0 g) was purified by preparative g.l.c. (exp. 1. (vi)). The infrared spectrum (liquid) showed absorptions at 2930 (-CH), 1740 (C=O) and 1190 cm^{-1} (C-O-C).

Found : C, 75.0 ; H, 11.7.

$C_{15}H_{28}O_2$ requires : C, 74.9 ; H, 11.7%.

6. trans-Dec-6-en-1-yl isovalerate.

(i) trans-Dec-6-en-yloxytetrahydropyran: Dec-6-yn-1-yloxy-tetrahydropyran (5.0 g, 0.21 mol) was converted to the trans olefin in high yield by two reductions by lithium/liquid ammonia, following a procedure described previously (exp. 2.(i)). T.l.c. on silica gel impregnated (25%) with silver nitrate showed only trace amounts of unreduced alkyne. The product was therefore used directly in the next step.

The infrared spectrum (liquid) showed absorptions at 2930 (-CH), 1200 - 1000 (tetrahydropyranyl) and 970 cm^{-1} (trans -CH=CH-).

(ii) trans-Dec-6-en-1-ol: trans-Dec-6-en-1-ol (2.6 g ; b. p. 58°/0.1 mm) was prepared in 87% yield by the acid hydrolysis of trans-dec-6-en-1-yloxytetrahydro-pyran (4.5 g, 0.019 mol), following a procedure described previously (exp. 1.(v)).

The infrared spectrum (liquid) showed absorptions at 3330 (-OH) and 2930 cm^{-1} (-CH).

Found : C, 76.7 ; H, 12.8.

$C_{10}H_{20}O$ requires : C, 76.8 ; H, 12.9%.

(iii) trans-Dec-6-en-1-yl isovalerate: trans-Dec-6-en-1-yl isovalerate (3.0 g ; b. p. 68°/0.05 mm) was prepared in 71.5% yield from trans-dec-6-en-1-ol (2.6 g, 0.017 mol) and isovaleric acid (3.1 g, 0.03 mol) following a procedure described previously (exp. 1.(vi)). A portion (2.0 g) was purified by preparative g. l. c. (exp. 1.(vi)).

The infrared spectrum (liquid) showed absorptions at 2930 (-CH), 1740 (C=O), 1190 (C-O-C) and 970 cm^{-1} (trans-CH=CH-).

Found : C, 74.8 ; H, 11.7.

$C_{15}H_{28}O_2$ requires : C, 74.9 ; H, 11.7%.

7. 8-Methyl-cis-non-4-en-1-yl valerate.

(i) 8-Methylnon-4-yn-1-yloxytetrahydropyran: 8-Methylnon-4-yn-1-yloxytetrahydropyran (12.1 g, b. p. 98 - 100°/3 mm) was prepared in 42.5% yield from the lithium salt of pent-4-yn-1-yloxytetrahydropyran (20.0 g , 0.12 mol) (Note 1) and 3-methyl-1-bromobutane (18.1 g, 0.12 mol) (Note 2) following a procedure described previously (exp. 1.(iii)).

Found : C, 75.4 ; H, 11.1.

$C_{15}H_{26}O_2$ requires : C, 75.6 ; H, 11.0%.

Notes:

1. Prepared from dihydropyran and pent-4-yn-1-ol in 85% yield (b. p. 65 - 68°/0.1 mm).
2. Prepared from isoamylalcohol and 48% hydrobromic acid in 65% yield (b. p. 160°/760 mm).

(ii) 8-Methylnon-4-en-1-yloxytetrahydropyran: 8-Methylnon-4-yn-1-yloxytetrahydropyran (8.0 g , 0.034 mol) was hydrogenated over Lindlar's catalyst (200 mg) following a procedure described previously (exp. 1.(iv)). Analysis of the product on t.l.c. using silica gel impregnated (25%) with silver nitrate revealed no unreduced material. The olefin was therefore used directly in the next step.

(iii) 8-Methyl-cis-non-4-en-1-ol: 8-Methyl-cis-non-4-en-1-ol (4.5 g, b. p. 95 - 7°/5 mm) was prepared in 87% yield by the acid hydrolysis of 8-methyl-cis-non-4-en-1-yloxytetrahydropyran (8.0 g , 0.034 mol) following a procedure described previously (exp.1.(v)).

The infrared spectrum (liquid) showed absorptions at 3330 (-OH) and 2930 cm^{-1} (-CH).

Found : C, 76.8 ; H, 12.9.

$C_{10}H_{20}O$ requires : C, 76.9 ; H, 12.9%.

(iv) 8-Methyl-cis-dec-4-en-1-yl valerate: 8-Methyl-cis-dec-4-en-1-yl valerate (4.9 g, b. p. 89°/0.1 mm) was prepared from 8-methyl-cis-dec-4-en-1-ol (4.5 g, 0.029 mol) and valeric acid (5.1 g, 0.05 mol), following a procedure described previously (exp.1.(vi)).

The infrared spectrum (liquid) showed absorptions at 2940 (CH), 1745 (C=O), 1375 and 1360 (Me_2.CH) and 1175 cm^{-1} (C-O-C).

The n.m.r. spectrum (carbon tetrachloride) showed absorptions at (τ) : 9.07 and 9.15 (6H, d, Me_2.CH., 5.5 Hz), ca., 9.08 (3H, t, aliphatic Me-), 6.0 (2H, t, $CH_2.CH_2.O$, 6.02 Hz) and 4.68 (2H, m, .CH=CH.).

Found : C, 74.8 ; H, 11.8.

$C_{15}H_{28}O_2$ requires : C, 74.9 ; H, 11.7%.

8. Decanyl isovalerate.

Decanyl isovalerate (5.3 g ; b. p. 96 - 97°/0.1 mm) was prepared in 69% yield from decanol (5.0 g, 0.032 mol) and isovaleric acid (5.1 g, 0.05 mol) following a procedure described previously (exp. 1.(vi)). A portion (2.0 g) was purified by preparative g.l.c. (exp. 1.(vi)).

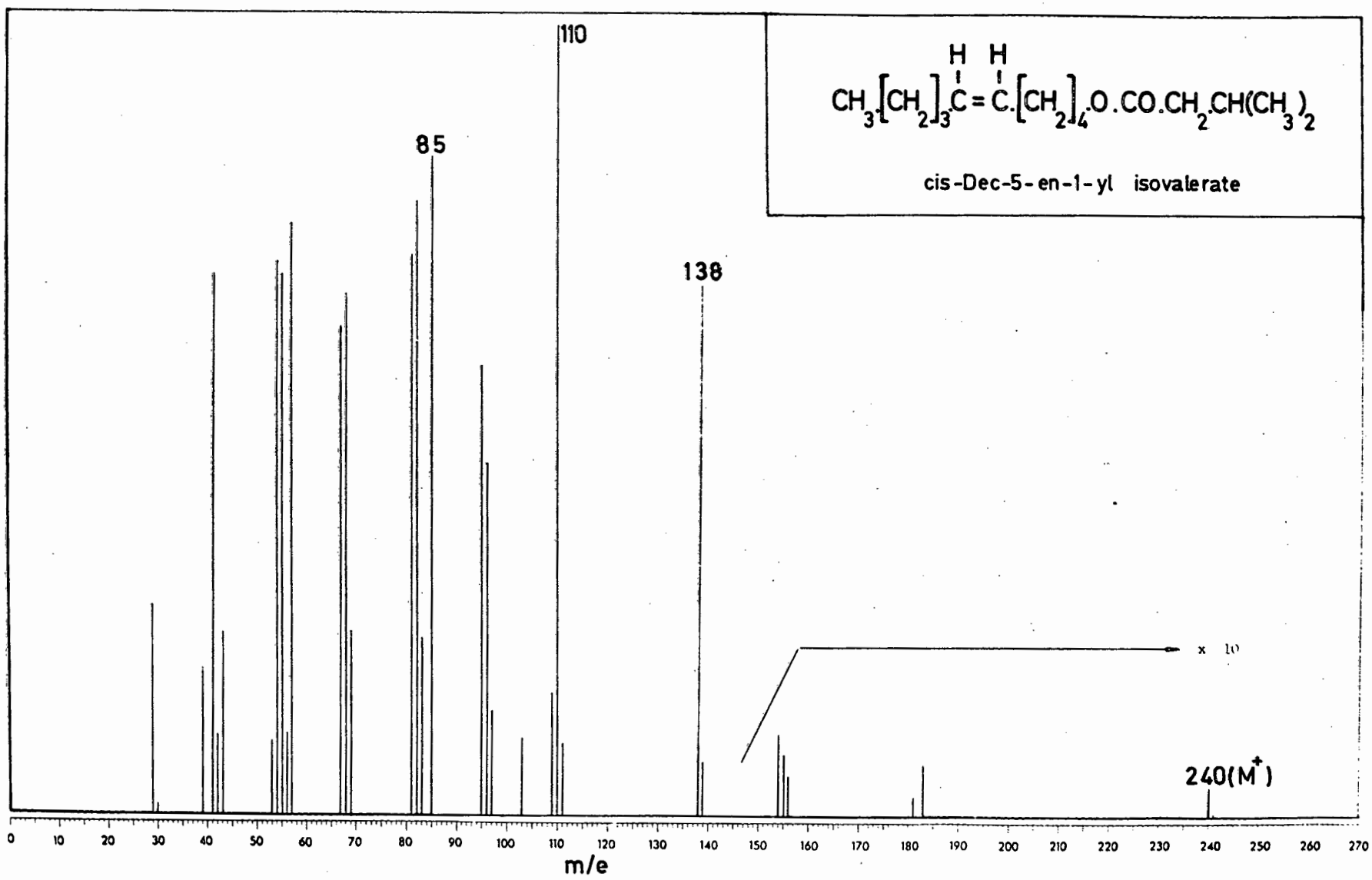
The infrared spectrum (liquid) showed absorption at 2930 (-CH), 1740 (C=O) and 1190 cm^{-1} (C-O-C).

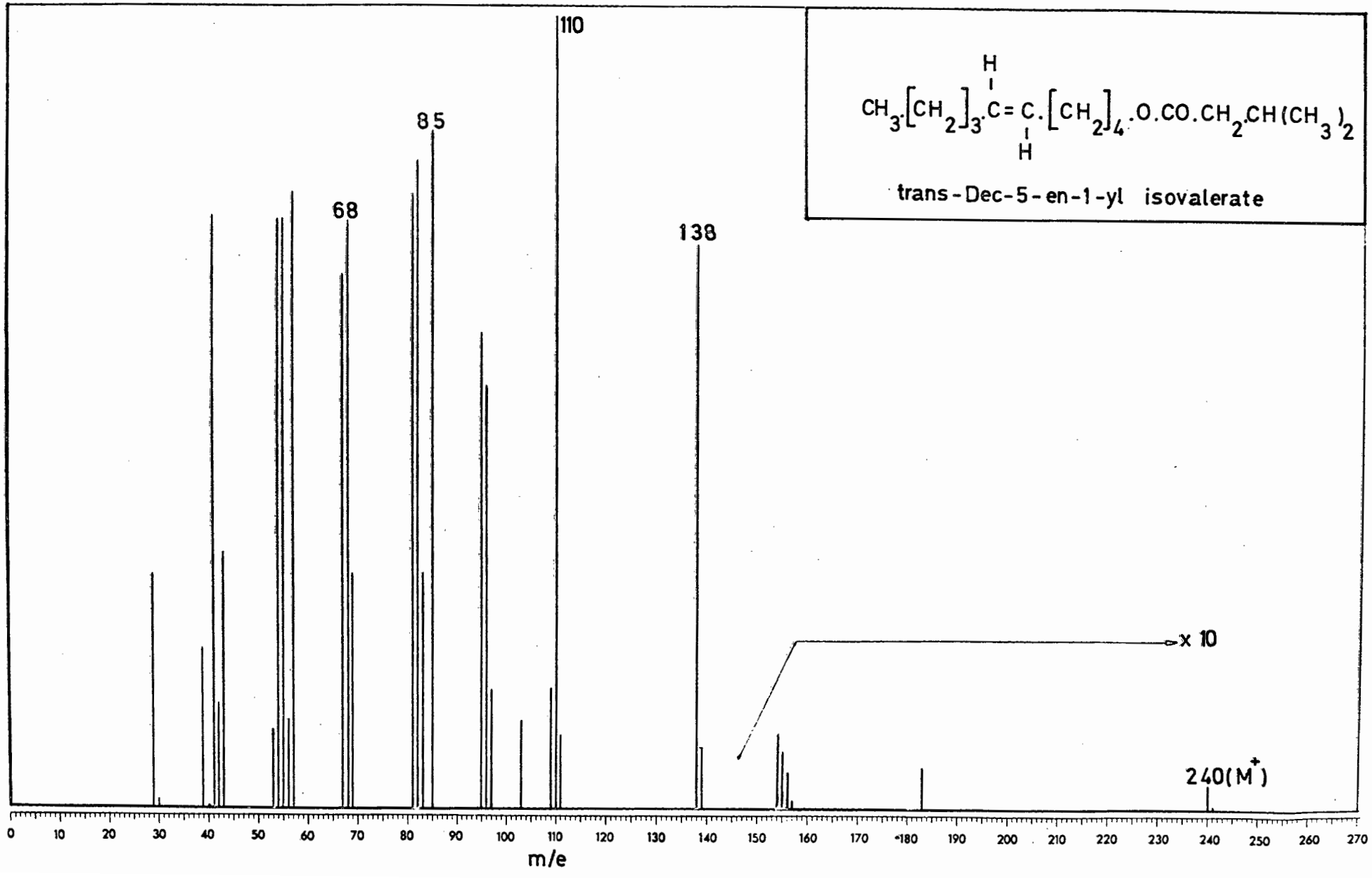
Found : C, 74.5 ; H, 12.6.

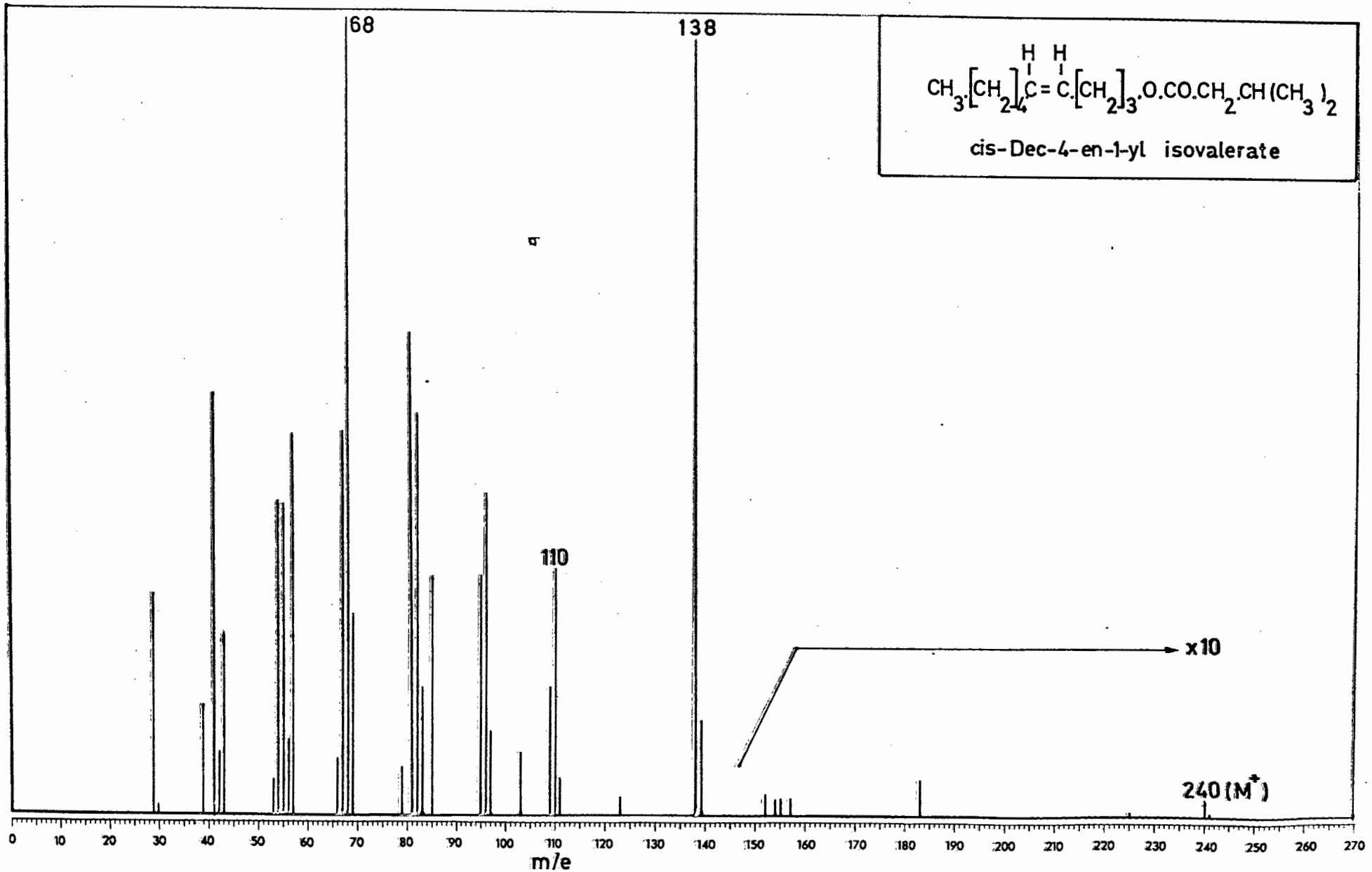
$C_{15}H_{30}O_2$ requires : C, 74.3 ; H, 12.5%.

APPENDIX

The NMR and Mass spectra obtained on the cis and trans isomers of Δ^4 and Δ^6 - decenyl isovalerate


110
85
138
240(M+)
x 10
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210 220 230 240 250 260 270
m/e
CH3.[CH2]3.C(H)=C(H).[CH2]4.O.CO.CH2.CH(CH3)2
cis-Dec-5-en-1-yl isovalerate


110
85
68
138
x 10
240(M+)
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210 220 230 240 250 260 270
m/e
CH3.[CH2]3.C(H)=C(H).[CH2]4.O.CO.CH2.CH(CH3)2
trans-Dec-5-en-1-yl isovalerate


68
138
110
x10
240 (M+)
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210 220 230 240 250 260 270
m/e
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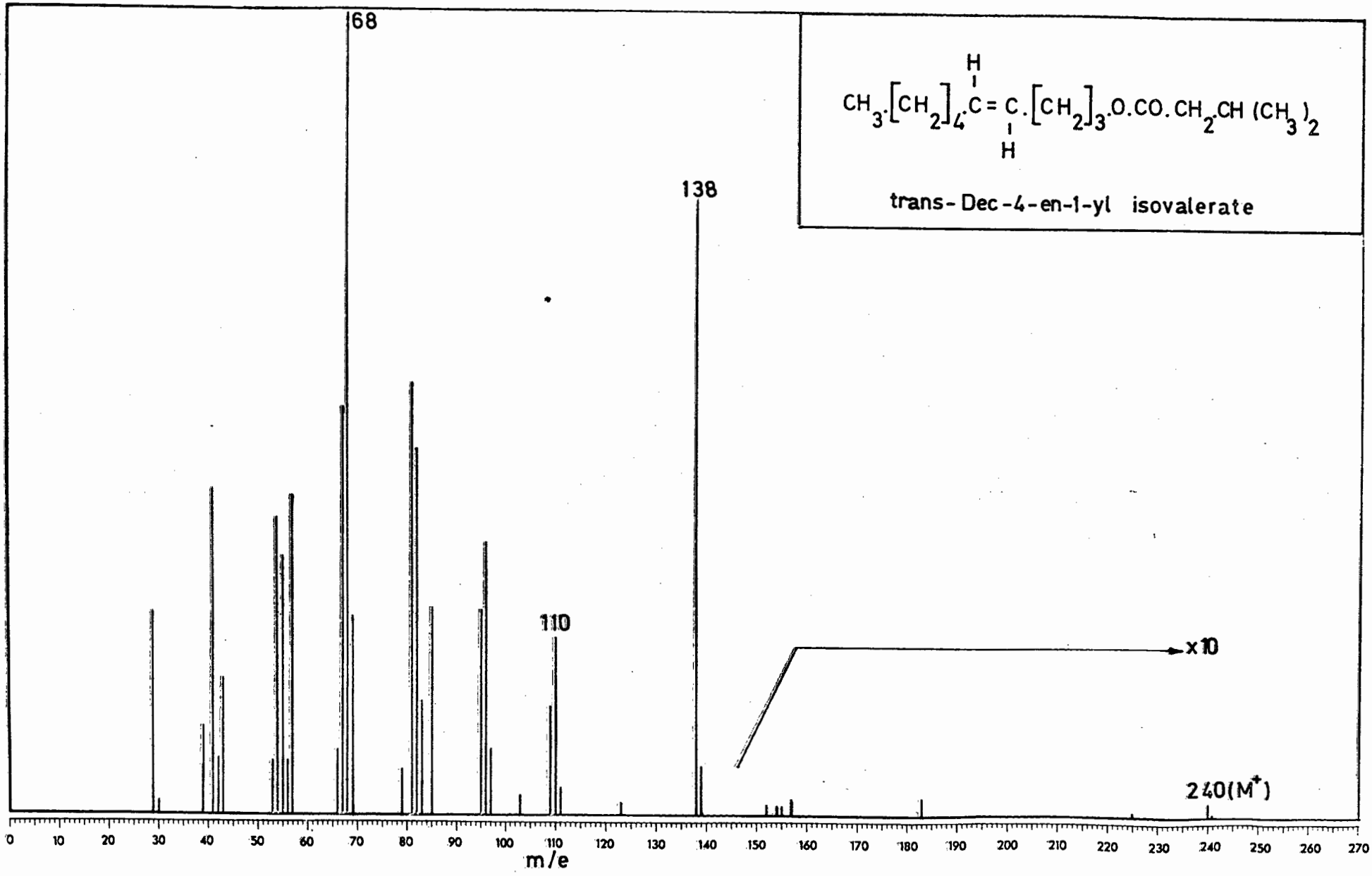
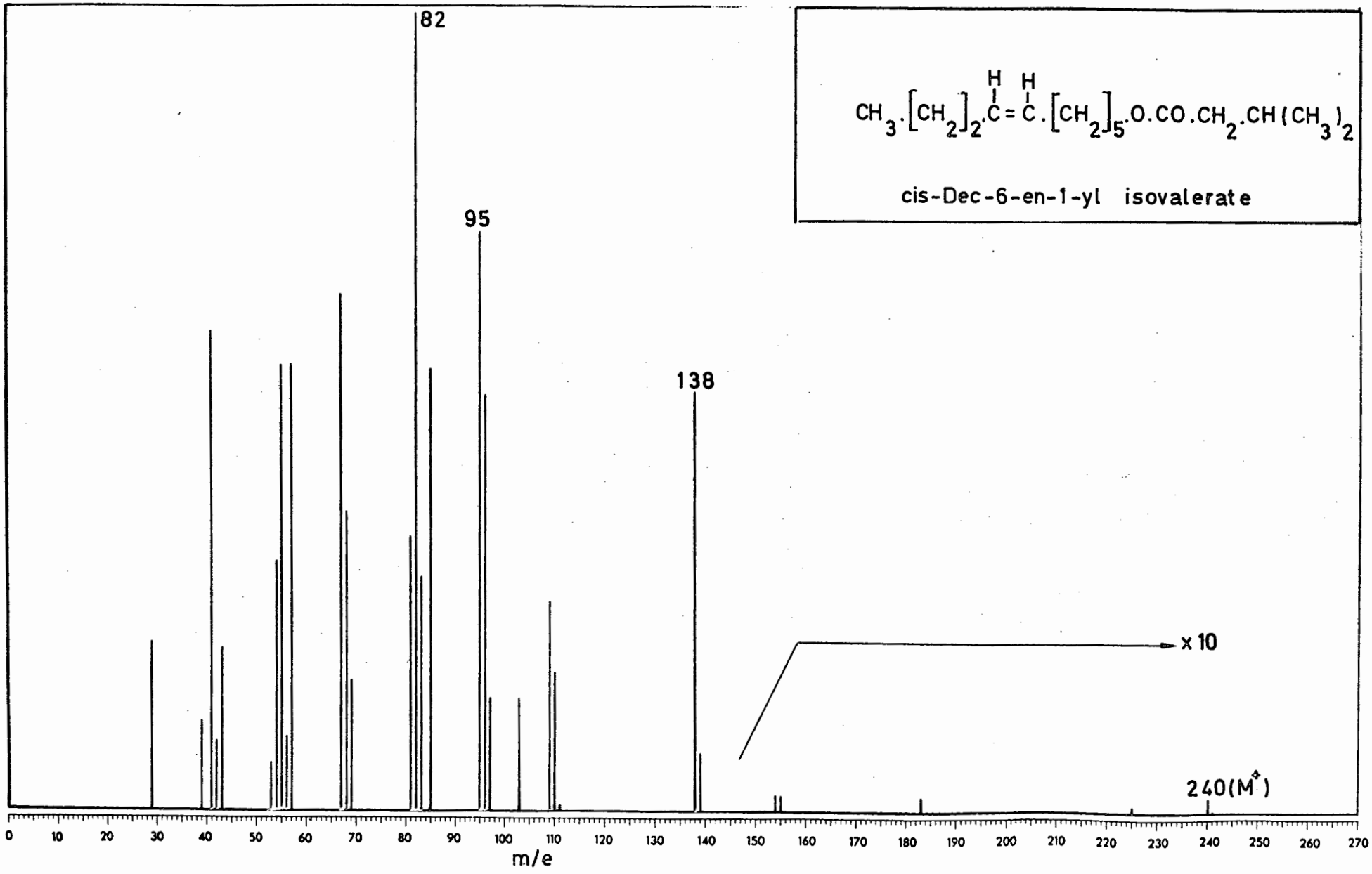
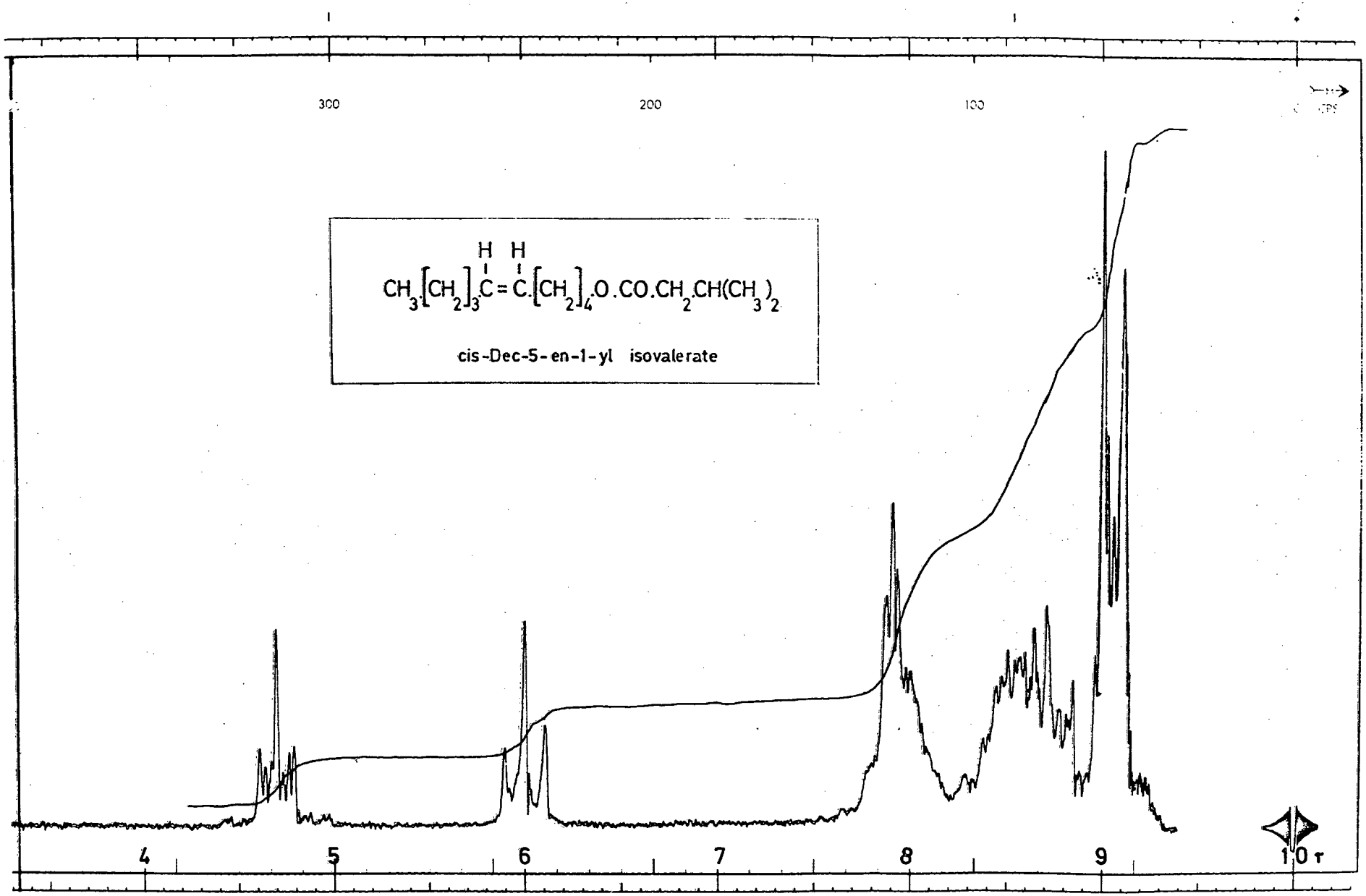
cis-Dec-4-en-1-yl isovalerate

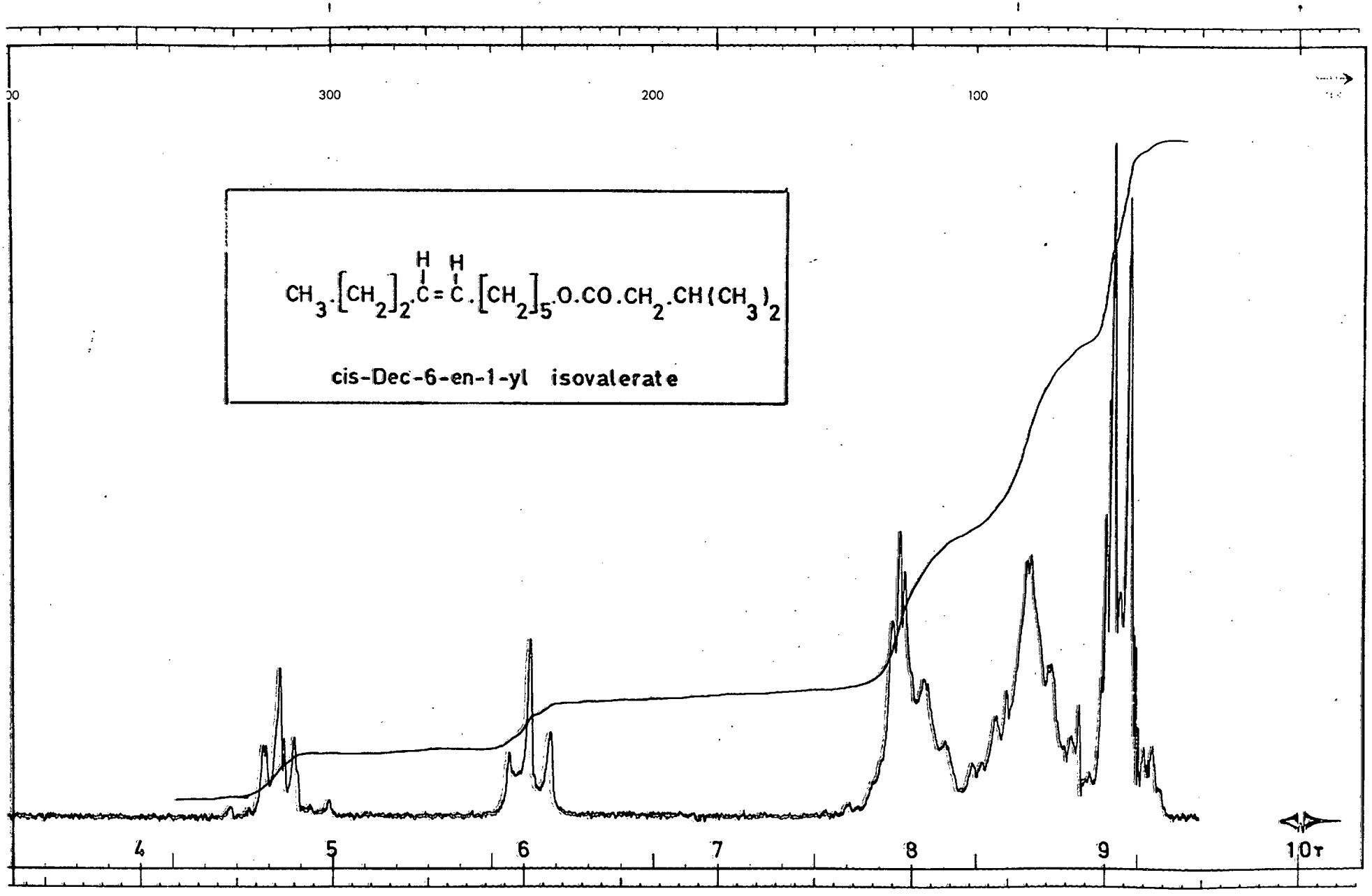

68
138
110
x10
240(M+)
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210 220 230 240 250 260 270
m/e
CH3.[CH2]4.C(H)=C(H).[CH2]3.O.CO.CH2.CH(CH3)2
trans-Dec-4-en-1-yl isovalerate

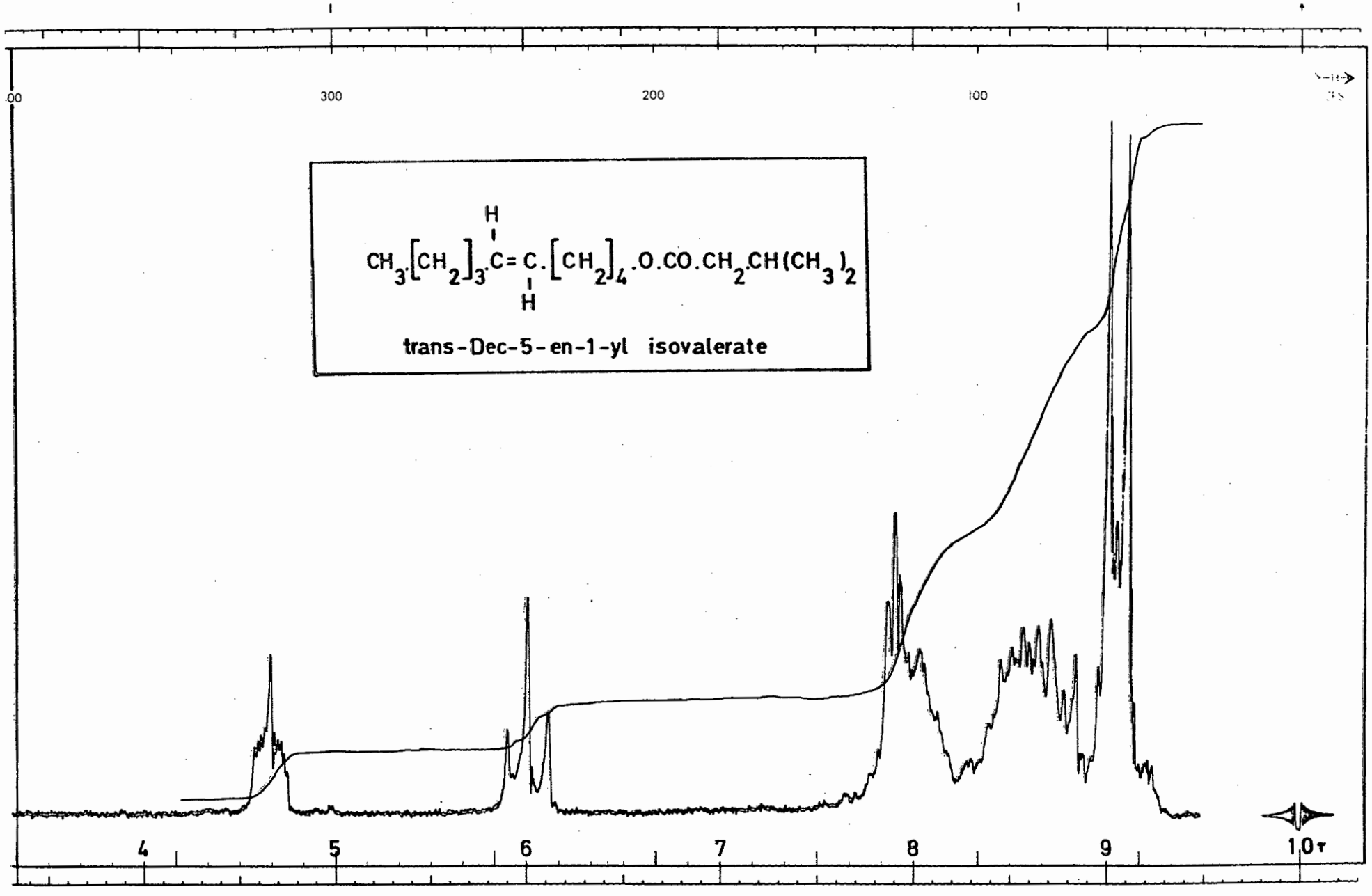


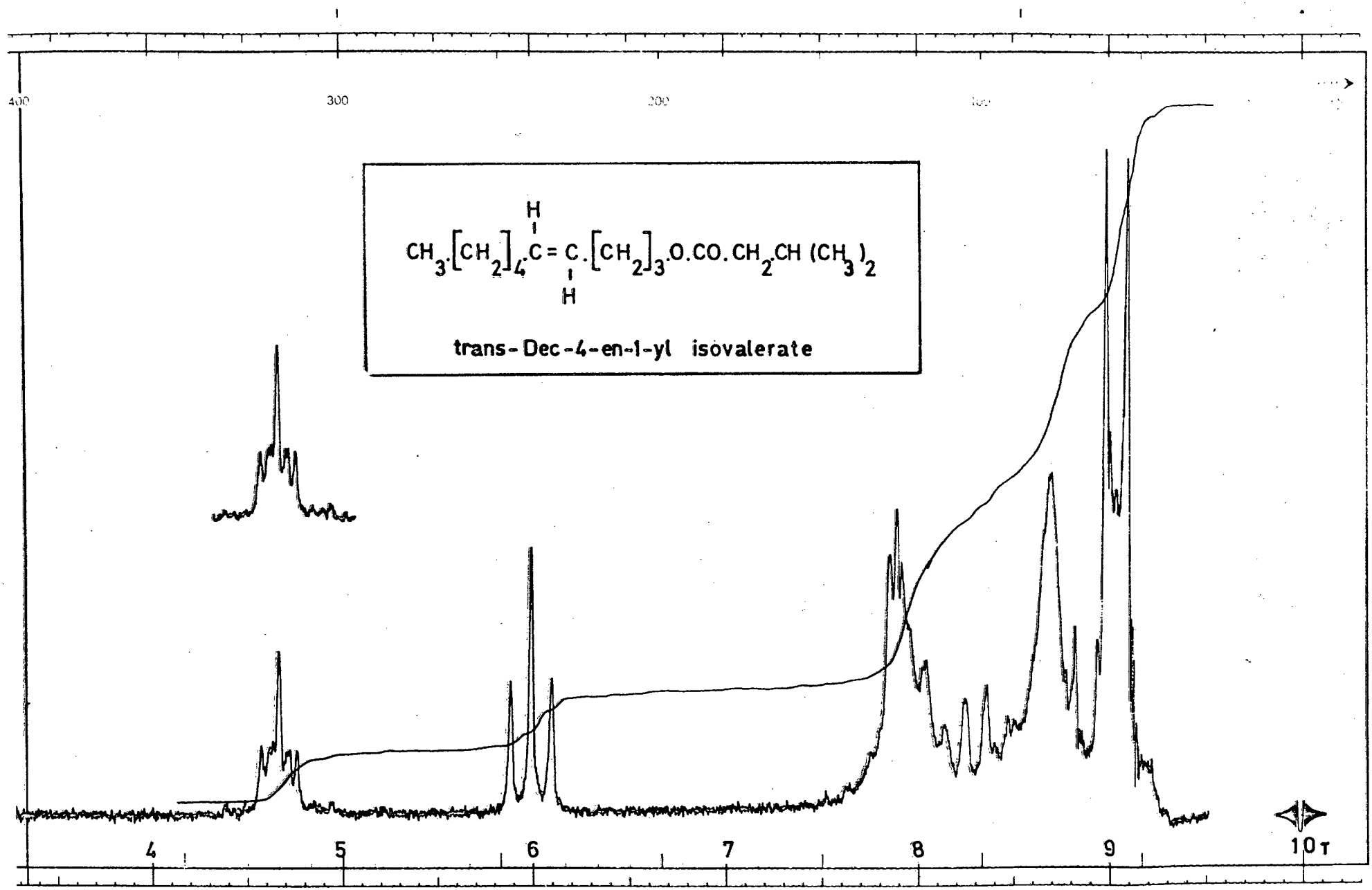
$CH_3.[CH_2]_2.\overset{H}{\underset{|}{C}}=\overset{H}{\underset{|}{C}}.[CH_2]_5.O.CO.CH_2.CH(CH_3)_2$

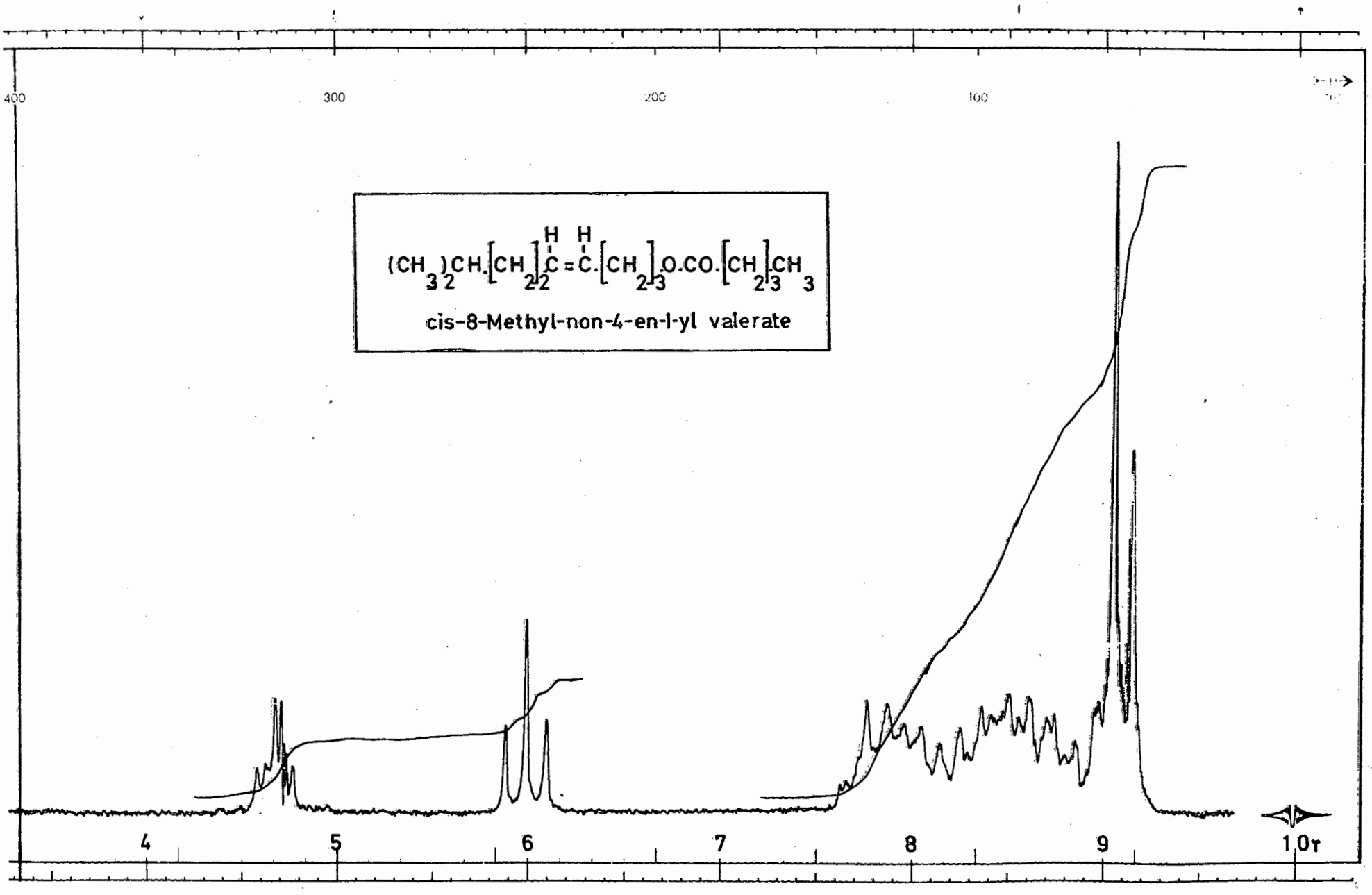
cis-Dec-6-en-1-yl isovalerate


300
200
100
CPS
$CH_3[CH_2]_3\overset{H}{C}=\overset{H}{C}[CH_2]_4.O.CO.CH_2.CH(CH_3)_2$
cis-Dec-5-en-1-yl isovalerate
4
5
6
7
8
9
10 τ


300
200
100
$CH_3.[CH_2]_2.\overset{H}{\underset{|}{C}}=\overset{H}{\underset{|}{C}}.[CH_2]_5.O.CO.CH_2.CH(CH_3)_2$
cis-Dec-6-en-1-yl isovalerate
4
5
6
7
8
9
10τ


300
200
100
4
5
6
7
8
9
10τ
trans-Dec-5-en-1-yl isovalerate


400
300
200
100
$CH_3.[CH_2]_4.\overset{H}{\underset{H}{C}}=\overset{H}{\underset{H}{C}}.[CH_2]_3.O.CO.CH_2.CH(CH_3)_2$
trans-Dec-4-en-1-yl isovalerate
4
5
6
7
8
9
10 τ


400
300
200
100
$(CH_3)_2CH.[CH_2]_2\overset{H}{\underset{|}{C}}=\overset{H}{\underset{|}{C}}.[CH_2]_3O.CO.[CH_2]_3CH_3$
cis-8-Methyl-non-4-en-1-yl valerate
4
5
6
7
8
9
10τ

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