INSECT PHEROMONES

THE SEX PHEROMONE

OF

ARGYROPLOCE LEUCOTRETA MEYR.

by

John Simcoe Read B.Sc.Agric.(Natal).

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Department of Chemistry, University of Cape Town.

C.S.I.R. Natural Products Research Unit.
University of Cape Town.

April, 1968.

ACKNOWLEDGEMENTS

The author is pleased to acknowledge the contribution of others in this investigation that involved a range of disciplines.

The co-operation of Dr. P.H. Hewitt (Entomologist, N.P.R.U.), Dr. T. Bosman and Dr. D. Fletcher (Department of Entomology, Faculty of Agriculture, University of Natal) which helped to overcome many of the biological problems encountered in the earlier part of the project.

Thanks go to Dr. R. Nel (Director), Dr. A. Myburgh (Chief Entomologist) and Dr. V. Whitehead of the Fruit and Food Technology Research and Institute, Department of Agriculture, Stellenbosch for the rearing of 1,700,000 moths. The assistance of Miss J.Y. Zoutendyk (Graduate Research Assistant N.P.R.U.) in the tedious separation of male and female pupae was invaluable throughout the project.

To Dr. M. Jacobson, Dr. R.S. Berger, Dr. A. Sekul and Dr. A. Sparks of the U.S. Department of Agriculture for a gift of small quantities of synthetic pheromones.

To Professor J.F. Brock and Dr. L. Krut of the Department of Medicine, University of Cape Town, for the exclusive use of their Barber Colman gas chromatograph over a period of several months, and to Mr. M. Wells for keeping it in working order.

To Dr. S.H. Eggers (C.S.I.R. Laboratories, Pretoria) and Professor W. Whalley (The London School of Pharmacy) for mass spectra.

To Professor A. Butenandt and Dr. D. Schneider (Max-Planck Institute for Biochemistry, Münich) for the hospitality shown to a representative of this laboratory.

To my colleague Mr. D.L. Morgan for helpful discussions throughout the project.

To Mrs. R. de Kock and Mrs. P. Ritchie for typing.

The award of bursaries and scholarships by the Beit Trust, Charelick Saloman Scholarship Board and the Council for Scientific and Industrial Research are gratefully acknowledged by the author.

Acknowledgement is made of the financial assistance from the Department of Agriculture, Consolidated Citrus Estates and African Explosives and Chemical Industries for the support of this project.

Gratitude is due to these and others, but most particularly to Professor F.L. Warren who supervised the project and whose imagination and enthusiasm made this project both exciting and fruitful.

Finally I acknowledge assistance by my wife Marianne in assembling the manuscript.

SUMMARY

The female false codling moth, *Argyroploce leucotreta* Meyr. (Lepidoptera, Eucosmidae), is shown to possess a sex pheromone which causes copulatory behaviour in adult males. The pheromone probably originates from glandular cells situated dorsally in the intersegmental membrane between the eighth and ninth segments of the female's abdomen. Extracts of whole virgin females, or their terminal abdominal segments are found to be active.

A novel method of extraction is described and a male biased inhibition of activity noted. Extracts of mixed populations of sexually immature males and females were inactive until fractionated by column chromatography. Steam distillation was found to be a satisfactory method of purification.

Partial purification was achieved by column chromatography on alumina, silicic acid and silver nitrate impregnated silicic acid. Gas liquid chromatography was used as the final step in the purification.

A possible structure of the pheromone was deduced from reactions on the crude extracts, analysis by gas liquid chromatography, and mass spectrometry, and this was confirmed by comparison with synthetic acetates.

Bioassay, mass spectrometry, oxidation and gas chromatographic analysis showed the pheromone to be *trans*-dodec-7-en-1-yl acetate, the geometrical isomer of the sex pheromone isolated from the cabbage looper

moth, Trichoplusia ni Hubner (Lepidoptera, Noctuidae). The synthetic pheromone of T.ni was shown to be inactive and did not inhibit male response to the active isomer..

The mass spectra of alkenyl acetates and the syntheses of the known pheromones are discussed.

CONTENTS

Chapter 1

INSECT SEX PHEROMONES

1.1 Introduction

One night, Jean Henri Fabre, the great French naturalist, was amazed at the appearance of a large number of male great peacock moths (*Saturnia pyri*) attracted to a female moth trapped in his house (Fabre, 1904). This observation led to the study of sex attraction and although his experiments strongly indicated attraction by odour over long distances, Fabre was not convinced. He expressed his incredulity saying "as well might one expect to tint a lake with a drop of carmine". This attraction has been proved to be by odour, but these potent chemicals are present in microgram amounts only. It required twenty years of elegant and painstaking work before the silkworm moth (*Bombyx mori*) sex pheromone was isolated and identified in Butenandt's laboratory (Butenandt *et al.*, 1959). Recently refined physical methods of separation and identification have resulted in a notable increase in the number of known sex pheromones.

It is now known that many insects depend on a chemical means of communication, and this principle may also apply to certain other forms of animal life. For chemists and entomologists it has become a task of deciphering the 'grammar' of some of the systems of chemical communication, and discovering the explanations

for many of the instinctive behaviour patterns. The social insects eg. termites, ants, wasps and bees may be considered to form a series of super-organisms in which the individual, regulated by these chemicals, takes the place of the cells regulated by hormones, the 'brain' is the queen of the nest. The queen substance of the honey bee (Apis mellifera) is found to contain trans-9-oxodec-2-enoic acid, which acts as caste control pheromone, and also a sex attractant for the drones earlier in the queen's life (Butler and Fairy, 1964; Callow et al., 1964).

The nest of the social insect is also co-ordinated by trail substances, alarm substances, odours for colony recognition and for recognition of the dead.

Research into the chemical language of insects, broadly considered, should help explain not only instinctive behaviour, but provide basic information in chemoreception and insect phylogeny. There are interesting possibilities for pest control since the specificity of the sex attractants, which are natural compounds, offers the possibility of specific pest control with little chance of the insect developing an immunity.

1.2 Pheromones and the Problem of Definition.

These physiologically active substances, secreted externally to help regulate the organisms external environment by influencing

other individuals, have been termed "pheromones" derived from the Greek words "pherein" (to carry) and "hormon" (to excite, stimulate). Karlson and Lüscher (1959) proposed the term to include substances "secreted by an individual and received by a second individual of the same species in which they release a specific reaction, for example, a definite behaviour or developmental process. The principle of minute amounts being effective holds. They function as chemical messengers among individuals".

In another publication, Karlson and Butenandt (1959) suggested the necessity for distinguishing between substances which act sensorily, for which the term "telomones" was suggested, and those which act biochemically, to which the terms pheromones would be restricted.

However Novak (1966) makes a sharp distinction between exohormones and pheromones. The exohormones act biochemically and have an activity which is phylogenetically primary, from which a more complicated secondary function has developed; whereas pheromones have developed secondarily to compliment a primary function, e.g. mating, and these act indirectly by way of the senses.

It is possible that a pheromone acting through the senses could cause a physiological change on the individual as opposed to behavioural reaction. This would be possible through neurosecretorily active scents. Such an effect could be termed a primer effect, as opposed to the releaser effect of a sex attractant or alarm substance.

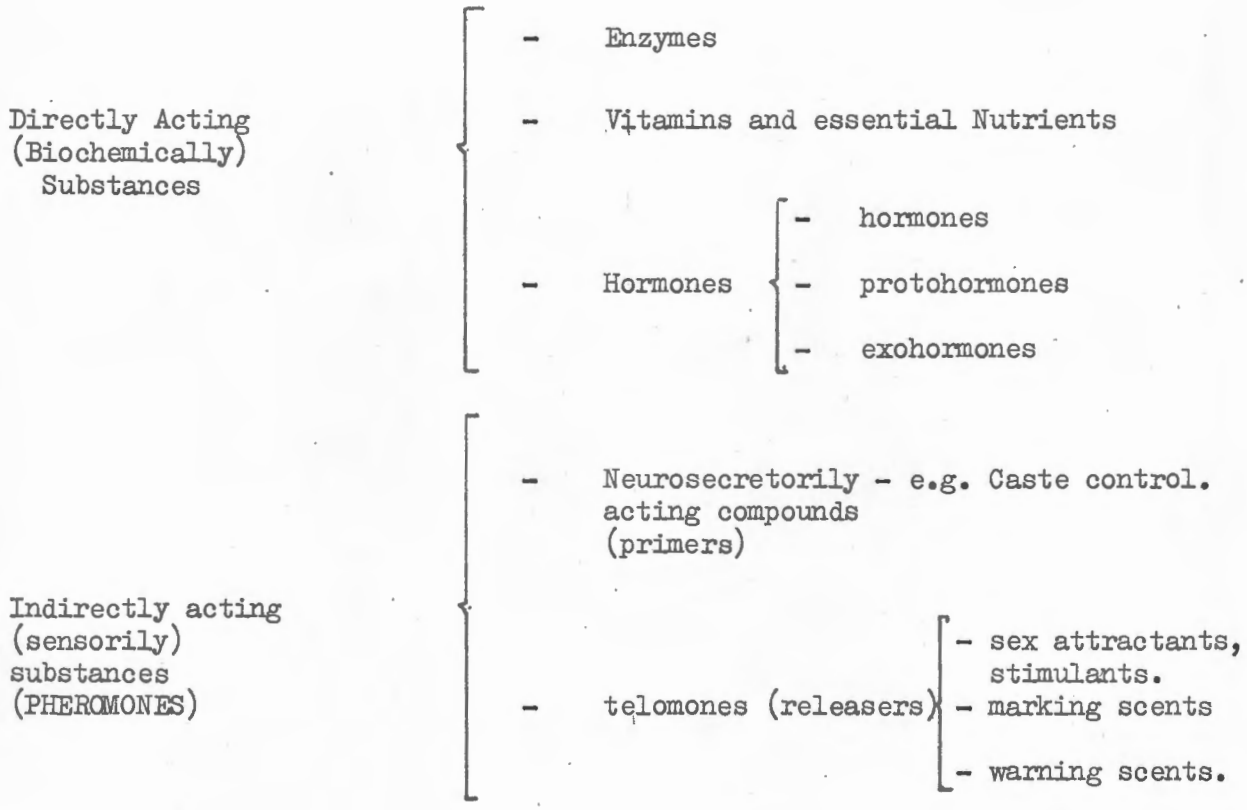


Fig. 1 Classification of Biologically Active Compounds and the Definition of Pheromones, adapted from Novak (1966).

Only one such primer effect pheromone has been identified in insects, in the queen substance of the honey bee.

These physiologically active compounds, including pheromones, may be classified as shown in Figure 1.

Another name that has been suggested instead of 'pheromones' is 'telergones', from the Greek 'tele' (afar) and 'ergon' (action) by Kirschenblatt (1962). The term "metarchon" (Wright, 1964) relates particularly to pest control. Wright specifies: "an external stimulus artificially introduced into the environment of an organism for the purpose of modifying its behaviour, by eliciting an inappropriate response or inhibiting an appropriate one".

This differentiation of sex attractants or stimulants referred to in Figure 1 is necessary for although in some species scents may cause the male to seek the female over some distance and mate, other species may utilise a sex stimulant only, as the potential mate may be within close range, on the host plant for instance. This difference in range may be manifested by difference in molecular size. The shorter range pheromones do tend to be of smaller size, 6-12 carbon chain.

On the borderline of pheromone investigations, but nevertheless part of the field of chemoreception and behaviour are the food lures, phagostimulants and ovipository stimuli which manifest the relationship between the host plant and the insect. A specific phagostimulant has also been the subject of investigations by the author. It was found

that (-)quebrachitol, 2-methyl ether of levo-inositol, was the natural phagostimulant for the fruit sucking moth larvae (Serrodes inara), isolated from the leaves of wild plum (Pappea capensis). Recently a sex pheromone-stimulant has been isolated from oak tree leaves (Riddiford, 1967). It is found that trans-hex-2-en-1-al stimulates the female polyphemus moth (Antheraea polyphemus) to release her sex pheromone.

1.3 The Occurence of Sex Pheromones

There are widespread demonstrations of occurence of sex pheromones in the Insecta that are emitted by the female to attract the male. The majority of these have been demonstrated in the order Lepidoptera, whether this is phylogenetically characteristic, or merely due to experimental availability is not certain. There are, however, many cases in which the adult's only role is reproduction by way of sex attractants. Since Jacobson (1965) gives an excellent summary in his book it is sufficient simply to note the numbers of insects.

Orthoptera : Ten species with demonstrated pheromones. Of these only the American Cockroach (Periplaneta americana) has been investigated chemically, unfortunately an unsuccessful investigation.

Lepidoptera : One hundred and eleven, or more, species possess demonstrated pheromones, and five of these are known chemically.

Hymenoptera : Seventeen species.

Diptera : Four species, including the ubiquitous housefly have been shown to possess some form of male attractant.

Isoptera : Two species.

There are also sex pheromones secreted by the male to attract the female. In some cases the males pheromone serves to compliment the female's, making her more receptive for copulation, and these have been termed aphrodisiacs. Many of the Lepidoptera that are shown to possess these are the butterflies. The following is a summary of the number of species, with demonstrated pheromones, per order :-

Orthoptera	:	three species
Hemiptera	:	two species
Lepidoptera	:	forty species
Coleoptera	:	two species
Hymenoptera	:	one species*
Diptera	:	three species
Meloptera	:	two species
Neuroptera	:	one species.

*The Bumble Bee (Bombus terrestris) pheromone has been identified as farnesol (Stein, 1963).

As well as the above classes of pheromones there are the so called assembling scents which are emitted by one sex, causing both sexes to assemble for mating, these have been found in the order Coleoptera. Nine species (consisting of three families) have an

assembling scent produced by the male and in one species it is produced by the female. These scents are usually found in the frass (excreta) of a pioneering individual male that has attacked a particular host plant. This obviously leads to mass attack of that particular plant. The frass of one of these, Ips confusus, has been successfully investigated chemically. It is a blend of three monoterpenoid alcohols, which show no activity when separated. (Silverstein, 1966).

1.4 The Sex Pheromones in the Order Lepidoptera

In the order Lepidoptera are found some of the best documented and most spectacular cases of large range chemical communication. In this order are also found many examples of the different stages and paths of development. At one extreme are the diurnal, nectar feeding butterflies that appear to have evolved in step with flowering plants. Here visual perception is important and acute and it mediates in flower recognition and courtship. Conversely pheromones are poorly developed and are apparently limited to sex stimulants produced in the special wing scales (androconia) of the males.

Many moths are also nectar feeders but most are twilight or nocturnal forms. With these species, for example A. leucotreta, olfactory response assume an increasing importance and the visual element in food plant recognition declines. In these species short range attractants and stimulants are found.

Finally there are species where feeding is no longer possible in the adult stage, and significantly it is here that the highest development of specific attractants is found. In some cases the females are quite flightless and immobile, e.g. the S. African wattle bag worm (Acanthopsyche junodi), and dependance upon a sophisticated lure-and-receptor system is then complete. The males of these species are characterized by their very feathery antennae, the seat of numerous olfactory receptors. The sensitivity of these receptors is quite remarkable, it is estimated by the author that ten to one hundred molecules per cubic centimetre of air of the pink bollworm moth pheromone can elicit a male response in the field. That is about one part in 10^{17} parts of air that is being detected. A similar striking case has been observed in the field by the author with pine emperor moth (Nudaurelia cytheria). The degree of specificity attained is not total, but such that only males of closely related species respond. These species are kept apart by seasonal and ecological differences.

1.4.1 Identification and Structure

The sex pheromones in this order have been isolated in the neutral lipid fractions of gland extracts, and often lose activity on alkaline hydrolysis. The pheromones of five different moth species have been isolated and synthesised. They are all long chain unsaturated primary alcohols or their acetate derivatives (Fig. 2). The details of isolation are not of significance here;

but the different procedures for following the isolation and the novel methods applied in the determination of structures are of interest and will be mentioned.

1.4.1.1 Bombyx mori, silkworm moth.

This pheromone was established, in the preliminary work, as a primary alcohol of twelve to sixteen carbon atoms, (Butenandt, 1941; Makino et al., 1956; Hecker, 1956).

In 1959 the pheromone was isolated in the pure form from 500,000 virgin females, as a 4'-nitroazobenzenecarboxylic acid ester (12 mg.), identified as hexadeca-10,12-dien-1-ol (Butenandt et al., 1959) and designated "bombycol" (Butenandt, 1963). The particular isomeric form was established from synthetic hexadecadienols as the trans-10, cis-12 form. The other three isomers were not very active (Butenandt and Hecker, 1961; Hecker, 1960; Truscheit and Eiter, 1962). This active isomer gave an electrical response at $10^{-10}\mu g$ on the electroantennogram (Schneider, 1963).

1.4.1.2 Porthetria dispar, gypsy moth.

This moth's sex pheromone had been investigated as early as 1925 by Bloor (Jacobson, 1965). During thirty years of study this attractant was shown to be a long chain alcohol and various degrees of purification achieved by esterification and column chromatography (Haller et al., 1944, Acree, 1953 a, 1953 b, 1954). Jacobson's group of the United States Department of Agriculture finally reported the isolation, identification and synthesis (Jacobson et al., 1960,

1961) of the major attractant 20 mg, isolated from 500,000 females. The pheromone was shown to be (+)-10-acetoxy-*cis*-hexadec-7-en-1-ol. All optical forms were active at $10^{-12}\mu g$ in the laboratory and at 10^{-6}-$10^{-7}\mu g$ in the field (Jacobson, 1965).

1.4.1.3 *Pectinophora gossypiella*, pink bollworm moth.

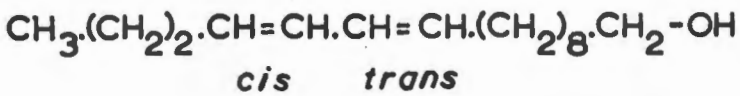
Early work by Berger and co-workers (1964) showed the pheromone to have unsaturation and a saponifiable ester group, and by gas chromatography to be probably a 18-carbon ester.

Jacobson's group (Jones *et al.*, 1966) however showed it to be an acetate of a branched chain, 16-carbon, primary alcohol with two double bonds, 10-propyl-*trans*-trideca-5, 9-dien-1-yl acetate, after isolation of 1.6 mg from 850,000 females. This was confirmed by synthesis. The branch chain was determined by the method of hydrogenolytic gas chromatography (Beroza and Sarmiento, 1963), which gives the parent paraffin chain immediately prior to gas chromatography, which can then be compared with well documented standards. Confirmation of the total structure was obtained with n.m.r. spectroscopy.

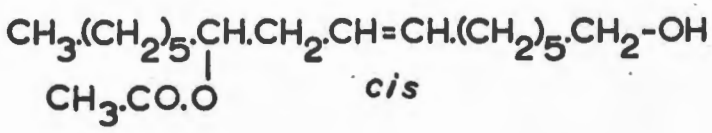
1.4.1.4 *Trichoplusia ni*, cabbage looper moth.

Berger, (1966) after column and preparative gas chromatography was able to show that the pheromone was a simple straight chain alkenyl acetate, *cis*-dodec-7-en-1-yl acetate and confirmed this by synthesis.

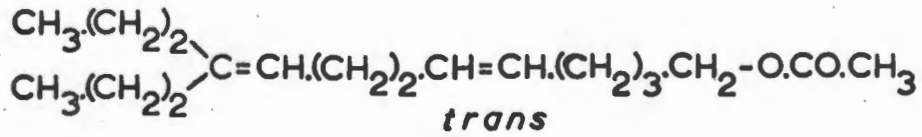
Silkworm moth



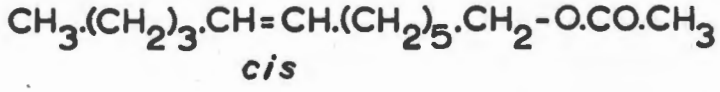
Gypsy moth



Pink Bollworm moth



Cabbage Looper moth



Fall Army Worm moth

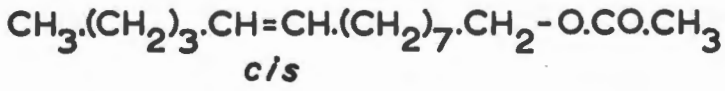


Fig. 2. The sex pheromones of known structure from species in the order Lepidoptera.

1.4.1.5 Spodoptera frugiperda, fall army worm moth.

Whilst this thesis was in preparation Sekul and Sparks (1967) isolated 900 μg of attractant material from 135,000 virgin female moths, after purification by column chromatography, thin layer chromatography and preparative gas chromatography. They identified it as cis-tetradec-9-en-1-yl acetate and confirmed by synthesis of both geometrical isomers from methyl myristoleate.

These pheromones occur in moths belonging to very different families (Bomycidae, Lymantriidae, Gelechiidae and Noctuidae respectively), however, they show an intriguing similarity of structure (Fig. 2). In the family Noctuidae, of which T.ni and S. frugiperda are members, the two pheromones are homologous long chain alcohol acetates.

1.5 Anatomy and Physiology of the Pheromone Glands in the Lepidoptera.

Generally the female insects are found to have glandular scent producing organs in the neighbourhood of the sexual opening. These are particularly well developed in the females of such Lepidoptera as the Bomycidae, Lasiocampidae and Saturniidae, in which the eggs of the female are ripe for laying at the time of emergence from the pupae.

Of the thirty or more species investigated for the female sex pheromone production glands all the glands are found to be situated near the terminal segments of the abdomen (Jacobson, 1965). In most species these are found in the last intersegmental membrane

which has become adapted as a glandular area. In the Argynnis species the glandular area occurs between the seventh and eighth segments. In this species the male is shown to secrete a chemical odour, from organs situated on the wings, that excites the female in preparation for mating.

The form of the glandular membrane varies in different species. It may be in the form of a saddle, ring or hose, or in the form of paired lateral sacs (sacculi laterales). In order to release the scent the abdomen is stretched and the intersegmental fold is simultaneously stretched outwards (Steinbrecht, 1964a). Special muscles may also be present to aid its function. In the family Noctuidae, differences are found in gland structure amongst its members. Deep ring shaped glands are reported for the noctuids Cucullia verbasci and C. argentea (Gotz, B. 1951). While in the female Egyptian cotton leafworm (Prodenia litura) the organs are tufts of modified scales or hairs with gland cells at their base, covering the ninth abdominal segment (Hammad and Jarczyk, 1958). During the resting period these are withdrawn into the eighth segment. No special secretory ducts are found in the glandular epthelial cells and the hairs serve to increase the surface area for evaporation of the secretion. There is no similarity found between P. litura and other noctuids. The pheromone gland of the female codling moth (Carpocapsa pomonella), of the family Tortricidae, has been shown to lie dorsally in the intersegmental fold between the last two segments

of the abdomen (Barnes et al., 1966). The gland consists of columnar cells lining the invaginated area. The cells are characterized by large nuclei at their bases. The gland can be protruded to show as a reddish hump. The width of the area is 0.30 - 0.33 mm., and the depth of the invaginated area actually lined by glandular cells is also about .33 mm.

The paired scent organs of the female silkworm moth (B. mori) have been closely investigated by Steinbrecht (1964b) using the electron microscope and histochemical techniques. He was able to show that the last intersegmental membrane, although still remaining a basic unicellular layer of interlocked cells, was differentiated from the normal insect epidermis in the following manner.

The cells contained large polymorphous nuclei with diffuse chromatin. During the development of the gland the endoplasmic reticulum in the cytoplasm changes from a granular to an agranular form. The golgi apparatus was not distinct and mitochondria were available in large numbers. At the beginning of glandular activity lipid droplets began to appear in increasing numbers in the gland epithelium. Histochemically these were shown to possibly contain an attractant precursor. The gland epithelium has greater activity of the enzyme NADP-Tetrazolium - Reductase (E.C. No. 1.6.99.1) compared to the normal intersegmental membrane. This may be required for the reduction of a possible carbonyl precursor to an alcohol. The surface of the glandular cells are increased by a

factor of 30 to 60 by lamellae like intrusions or villi. These increase in number with increase in the attractant activity of the gland.

Connected with this is the fact that the glands do not store any amount of attractant, it is only found on the surface. (This has been inferred experimentally in other species). It seems that the production takes place through the membrane, by single molecules.

Chapter 2

THE FALSE CODLING MOTH

2.1 Description and Life Cycle

The false codling moth, Argyroploce leucotreta (Lepidoptera, Eucosmidae. Meyr.) has been a serious fruit pest in the orchards of the Cape, Transvaal and Natal for many years. Unlike the codling moth, Carpocapsa pomonella, A. leucotreta is indigenous to Southern Africa and can exist without hardship on its natural host plants, e.g. custard apple and sour plum. Fortunately the moth has natural enemies to keep it in check most seasons. It has five or six generations per year, but as the eggs are laid at irregular intervals the generations overlap. The spoilage of fruit in orchards is normally about two percent but on occasions when the natural biological control breaks down it can arise to 15 percent. The unpredictability of this moth makes it a difficult pest to control with insecticide applications. The fruits liable to attack are many, pomogranates, apricots, guavas, avocadoes, peaches, walnuts and especially all varieties of citrus.

The pest may be controlled by the judicious use of insecticides, but the fact that fruit may be contaminated with the larvae, prevents the export of South African citrus to many countries for fear of its introduction. It is for this reason that new pest control measures are being sought and the use of the sex pheromones presents a possible method of control.

It is a dark grey moth about three quarters of an inch across the wings (Fig. 3). The eggs are laid singly on the fruit and after a week the minute larvae bore in to eat the pulp leaving masses of granular excretion. This may last a month, and the attacked fruit colours prematurely and soon drops and rots. The larvae pupates in the ground for about three weeks after first spinning itself a silken cocoon with soil particles.

In the intersegmental membrane between the last two segments of the adult female, there is a red hump of suspected glandular cells found exactly as described for the pheromone gland of C. pomonella (Barnes et al., 1966). Swart (1966) expressed some doubt on the classification of A. leucotreta in the family Eucosmidae. He said that it exhibited a morphological characteristic found in the Tortricid moths. The sex pheromone may provide further evidence for the classification, when pheromones from species in the two families are known.

2.2 Rearing of False Codling Moth on Artificial Media.

The false codling moth larvae were reared at the Fruit and Food Technology Research Station, by the Entomology Unit under Dr. A.C. Myburgh. They are reared by placing the externally sterile eggs in a sterilized maize meal medium innoculated with a common bread mould. This medium is found to be satisfactory for the larvae, which are allowed to pupate in the cotton wool

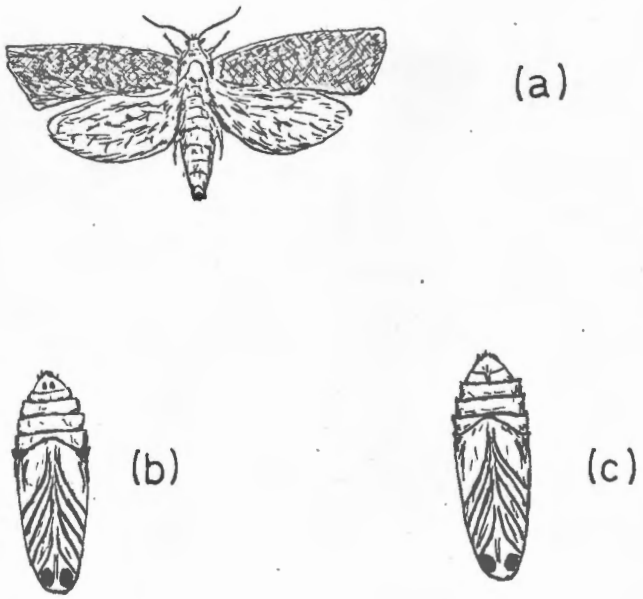


Fig. 3. Illustrations of the adult false codling moth (a), and the male (b) and female (c) pupae, which differ in the last two terminal segments.

plugs of the vessels. The maize meal may be digested by the larvae, if treated with acid previously, however the bread mould is preferred.

The pupae of A.leucotreta measure approximately a quarter inch in length and sexes can be separated only with the aid of a microscope (Fig. 3).

The adult moths can be conveniently anaethetized, for handling, in a CO_2 atmosphere. Ethyl acetate vapour was used before extraction.

Chapter 3

BIOLOGICAL TESTING

3.1 Possible Methods

The testing of compounds eliciting an immediately apparent behavioural response presents, at first glance, no immediate problem of bioassay. However it is methods that have reasonable consis - tency and quantitative results that are necessary for any isolation and identification of a sex attractant. These may be behavioural or physiological in basis.

If the sex attractant has sufficiently long range, as in the case of the gypsy moth, pink bollworm moth and the pine emperor moth, field trapping procedures can be designed to give an unequivocal result. This method suffers from the drawback that it requires a large population in an infested area throughout the year, a condition rarely found. The method that the author used successfully for the pine emperor moth was an adaptation from a design supplied by the Gatooma Cotton Research Station, Rhodesia.

A large sheet iron tray (3' x 3' x 3") was filled with an alkaline solution of nicotine sulphate and detergent. Over this a cylinder with gauze bottom was placed containing either live virgin females or extracts. This method gave excellent results and effectively wiped out the area of the infestation.

Trapping procedures in the citrus orchards were not successful for the assay of the false codling moth sex attractant. It was

supposed that the attractant was a short range attractant and an alternative laboratory trapping procedure was designed on a smaller scale to that of the pine emperor moth.

Another method that was considered for the bioassay of the false codling moth was the electrophysiological method perfected by Schneider, the electroantennogram. (Schneider, 1957; Schneider, 1963 a, 1963 b, and Boeckh et al., 1963).

This method was used successfully by the author to assay for the pine emperor moth sex attractant. Briefly it consists of an isolated male antenna mounted between two electrodes which record slow changes in the nerve receptor potential. The sex attractant is tested by passing short puffs of air over an impregnated filter paper onto the large feathery antenna. The response is shown on an oscilloscope. This method was found to be mechanically difficult and tedious with the small thin thread-like antennae of the false codling moth.

Many types of olfactometers have been designed that depend on the behavioural response of the moth. These may depend on giving the moth a choice between two particular extracts and counting their flight across a photoelectric beam. A method was used that depended on choice but was found to be inadequate in the response and the number of samples that could be tested.

3.2 The Bioassay Method

The method used was a semi-quantitative adaptation of the

method used by Shorey and Gaston (1964) for the quantitative bioassay of the sex pheromone of Trichoplusia ni. It is essentially a visual count of male moths responding to the pheromone. Briefly, it consists of exposing 10 male moths, contained in a cylindrical plastic vessel closed at either end with gauze, to a stream of air that has been passed through a filter paper impregnated with the extract under test. This was carried out in a fume cupboard to remove the contaminated air. The number of male moths that exhibited the characteristic "whirling dance" with their claspers extended, were counted over a period of 30 secs/cage. Appropriate controls were always conducted. Unfortunately facilities for temperature and humidity control were unavailable so optimum conditions could not be defined. All assays were conducted between 7 and 11 p.m., at approximately 0.2 ft-C illumination. The effects of seasonal temperature variations on pheromone response were noted. These observations showed that the male moths responded to the pheromone in the range of 17-22^{o}C and 45% - 75% R.H. Tests to find the most convenient times for the assay, and a suitable age of male moths, were carried out. The results given below show that the adult male moths can respond to the pheromone two days after emergence, and do so after sun-down. Shorey and co-workers (1965) were able to alter the 24 hour circadian rythym of the moth by reversing the light dark period and were able to elicit responses during daytime, but no

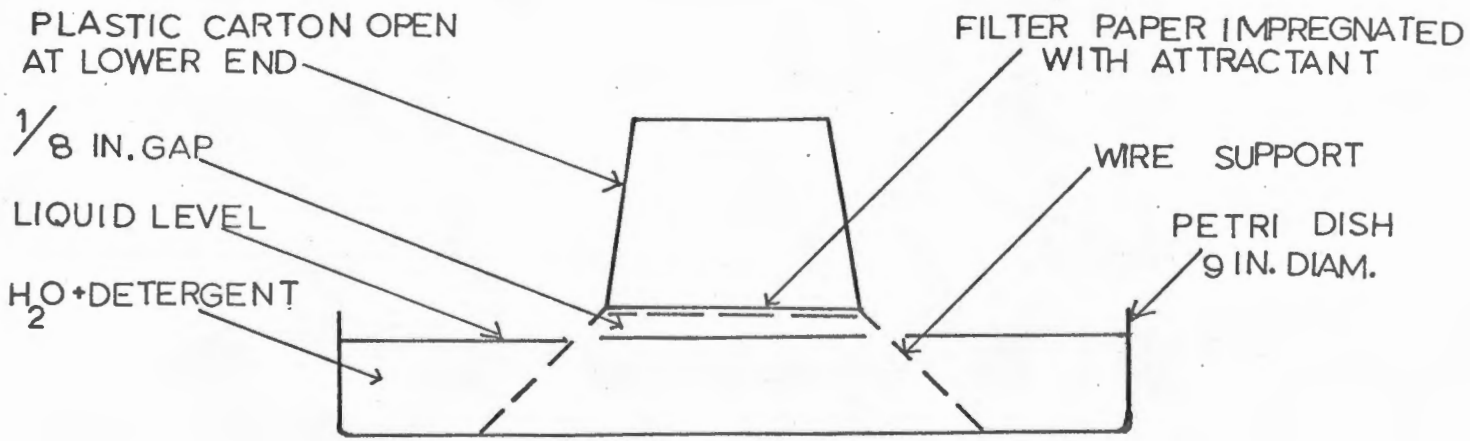


Fig. 4 Trap used to confirm behavioural assay for the false codling moth sex pheromone.

similar attempt was made by the author with the false codling moth for daytime testing.

A trapping technique was used to confirm the results of the behavioural assay, depending on the availability of male moths. The traps were constructed as illustrated (Fig. 4). Moths approaching the source of the pheromone were invariably trapped in the liquid. One hundred to two hundred male moths were released in a room measuring 45 ft x 18 ft containing two or three traps spaced at equal intervals.

Experimental

3E.1 Sex specificity of the assay.

Two hundred male abdominal segments and two hundred female abdominal segments were extracted separately with ethanol. One fortieth of each concentrate (five moth equivalents, ME) was tested for pheromone activity. (Table 1)

TABLE I

Behavioural Response to Male and Female Abdominal Extracts

	Average % male moth response in eight cages			
	Expt. 1	Expt. 2	Expt. 3	Expt. 4
Solvent (control)	7.5	10	5.0	10
5 ♂ ME	11	20	10	10
5 ♀ ME	84	84	73	70

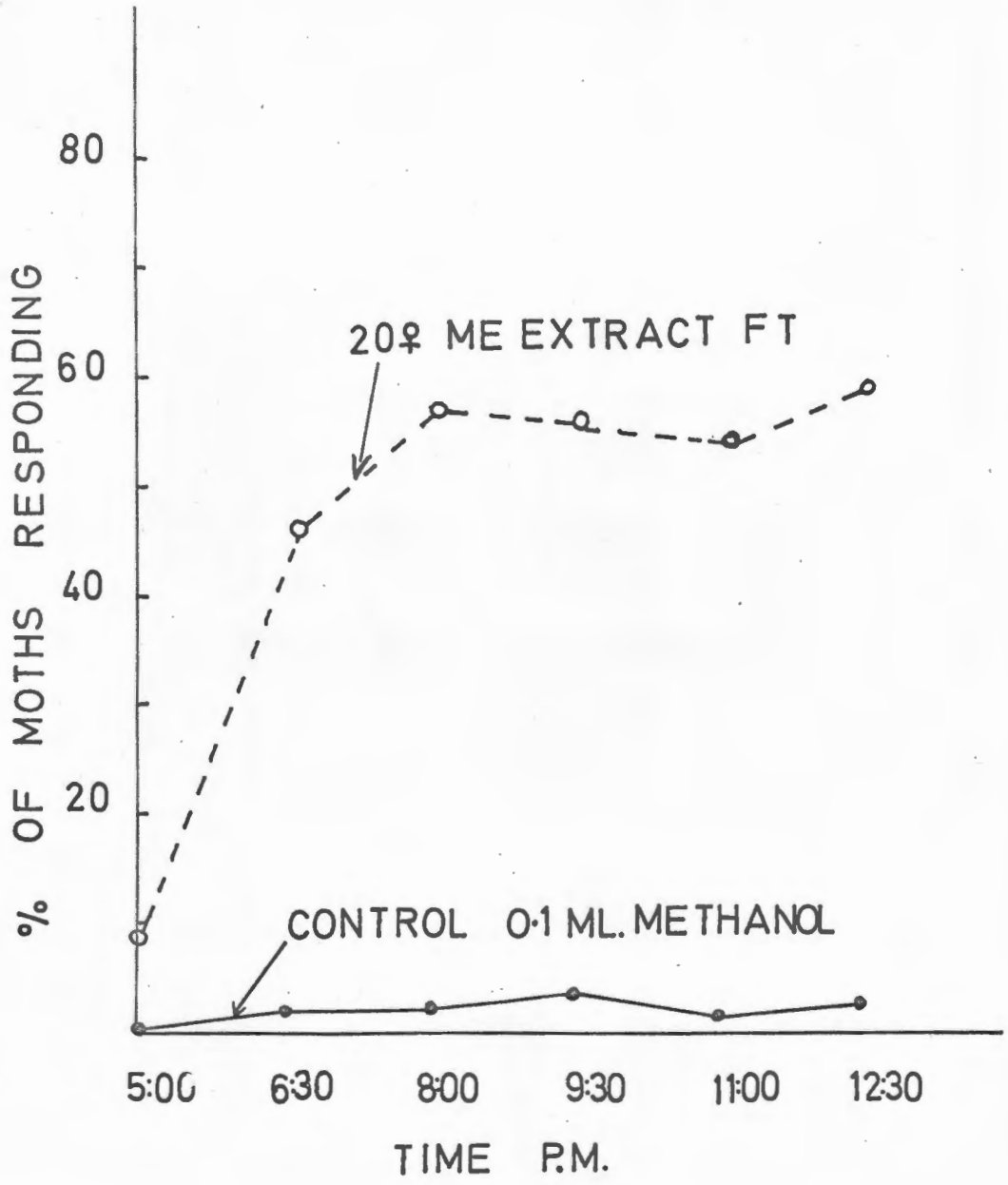


Fig. 5 Response of male moths, to the female sex pheromone, during the evening hours.

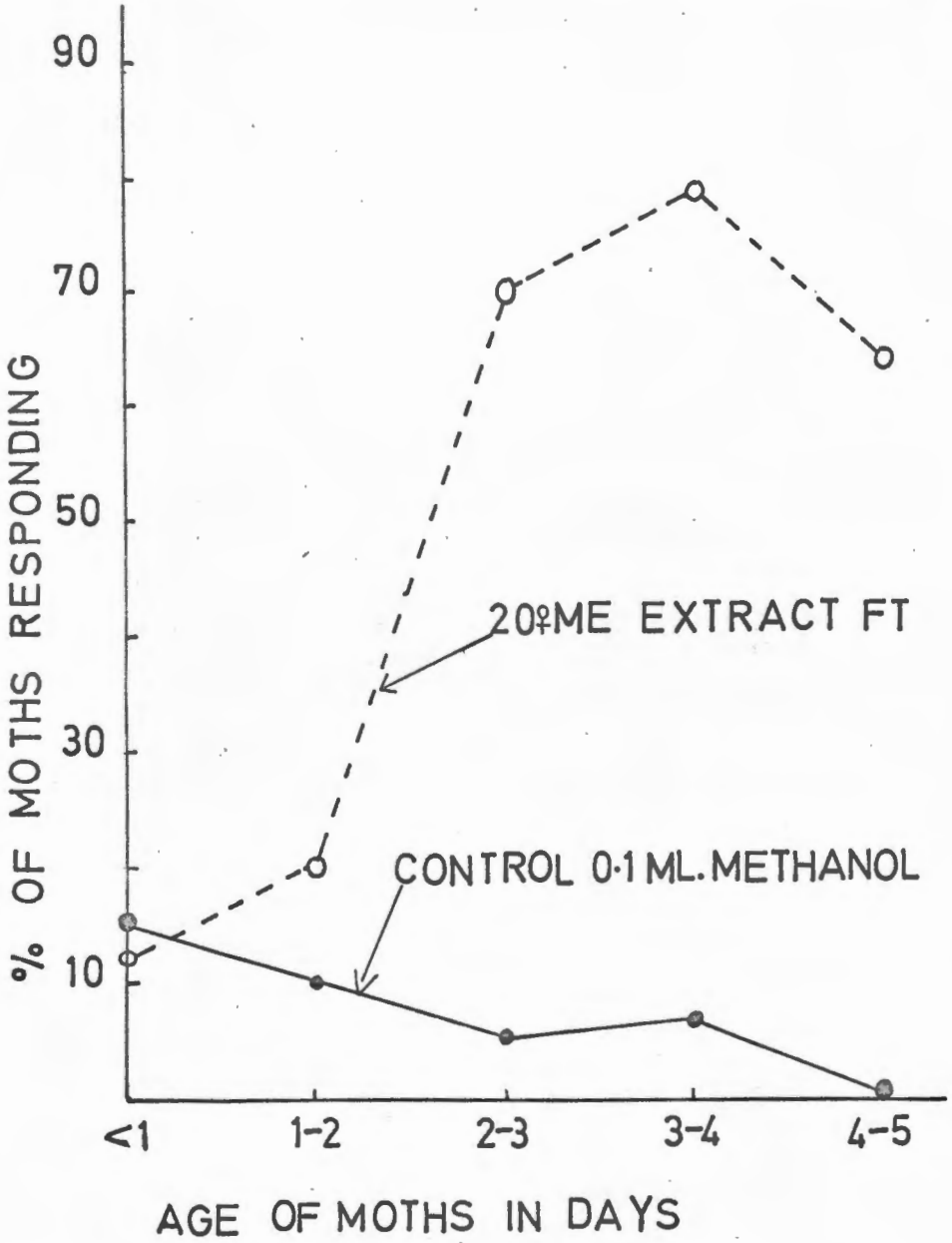


Fig. 6. Response of male moths, to the female sex pheromone, with increasing maturity.

3E. 2 The effect of time of day on male moth response

Twenty cages of moths were exposed to an extract of the terminal abdominal segments of the female, equivalent to 20 moths (20ME of Extract FT, section 4E.1) at 90 minute intervals starting at 8.00 a.m. No response was observed during daylight hours, but during the period 6.30 p.m. to 12.30 a.m., when testing ended, approximately 50% of the moths were responsive (Fig. 5). All subsequent assays were conducted between 7 p.m. and midnight.

3E.3 The effect of age of male moth on response

Commencing with moths less than one day old the response to 20ME of extract FT. was determined at daily intervals until the sixth day (Fig. 6). The male moths were found to respond fully two to three days after emergence.

Chapter 4

EXTRACTION AND INITIAL PURIFICATION

The sex pheromone glands of adult moths, from the order Lepidoptera, are usually found in the terminal segments of the female abdomen and activity is lost soon after mating. Therefore most extracts that have been made have been from the abdominal terminal segments cut from the adult virgin females. The choice of solvents and methods have differed from one investigation to another.

Butenandt (1955) reports that the following general scheme was used to isolate bombycol from a petroleum extract of virgin female terminal segments. The neutral fraction was isolated and purified by alumina chromatography and counter current distribution. The attractant was finally isolated as the ester of 4'-nitroazo-benzenecarboxylic acid. A similar method has been used in the extraction of the pheromone from P. litura (Zayed et al., 1963). Flaschenträger and Amin (1950) reported that steam distillation could be used as a purification procedure for the P. litura pheromone.

Jacobson and co-workers (1961) took benzene extracts of terminal segments from virgin P. dispar females and isolated an active neutral fraction which was purified by repeated precipitation of inactive solids from a cold acetone extract. The final purification was carried out by paper chromatography. In earlier

experiments with P. dispar extracts, Stefanovic (1959) was able to steam distil an active fraction.

Jacobson's group (Jones et al., 1966) used whole P. gossypiella female moths and prepared a dichloromethane extract. Precipitation from cold acetone followed by column chromatography on Flourosil and silver nitrate impregnated silica gel, and gas chromatography were the purification procedures used. Berger et al. (1964) found that not all the activity could be carried over with steam distillation.

Berger (1966) used dichloromethane to extract the attractant from T. ni female abdominal segments and purified the extract by silicic acid chromatography and gas chromatography. Sekul and Sparks (1967) made an ether extract of S. frugiperda female abdominal segments and followed this with cold acetone precipitation. This layer and column chromatography with silicic acid, and silver nitrate impregnated silic gel were followed by a final purification with gas chromatography.

The method used by the author to achieve an active extract of A. leucotreta female sex pheromone was evolved using mixed male and female populations of whole moths. The sexing of pupae under a microscope and the removal of the terminal abdominal segments from CO_2 anaethetized females, was a time and labour consuming procedure. However since mating in mixed populations was not observed until two days after emergence, these populations were extracted during this inactive period. These extracts were inactive but activity

was recovered after column chromatography. Recombination of fractions did not diminish the pheromone activity.

This column chromatography was adopted as a standard initial purification procedure. A later development described in the following chapter was the use of a silver nitrate impregnated silicic acid column for further purification.

The extracts were prepared in ethanol and the solution kept cold over night and the inactive precipitate discarded. Steam distillation was successfully used to purify the ethanol extract, but did not enhance the activity of the inactive extracts from mixed populations.

Preparative gas chromatography was carried out on material derived from extract MF and an active fraction collected each time. Unfortunately reinjection of the collected active fraction showed two peaks. This was taken as an indication that substantial breakdown had occurred either on the column or on the hot wire of the detector. So no further collection of the active compound was carried on this instrument.

Furthermore, when this gas chromatography analysis was repeated from freshly prepared extract MFS (see also section 5.4.1), activity no longer occurred at the same retention time (i.e. after methyl stearate). Instead it had a far shorter retention time. The extract MF was made from moths that had been left standing in ethanol for three to four months, and the GLC results were taken as an indication of the desirability of obtaining fresh extracts.

Experimental

All solvents were redistilled and dried before use. Light petroleum (b.p. 40° - 50°C) was used throughout. Initially concentrations of all bulk solutions were effected at 40° - 45°C/8-10 mm in a rotary evaporator. Later the bulk ethanol extracts were more satisfactorily concentrated by a steam heated flash evaporator.

4E.1 Extraction Procedures

Nomenclature of the Extracts: Various extraction procedures were used and the following abbreviations apply. Extract FT - tips of female moths; Extract F - whole female moths; Extract MF - mixed populations of whole male and female moths; Extract MFS - extract MF steam distilled.

Extract FT - This extract was kindly donated by African Explosives and Chemical Industries Ltd. and was prepared as follows. The terminal four segments of 3-4 day old virgin female moths were cut off, and extracted in a soxhlet with peroxide free ethyl ether for three hours. The ether was dried over sodium sulphate, and evaporated under reduced nitrogen pressure. The oily residue was extracted with methanol which dissolved the attractant material. The final concentration was 20 moth equivalents (ME)/0.1 ml methanol.

Extract MF - Visual observations showed that virtually no mating occurs in mixed populations of moths less than two days old. Whole moths (two day old male and female) were homogenized in five

times their weight of ethanol in a Waring blender, filtered, and the filtrate concentrated to a third of its original volume. The concentrate, held overnight at 0^{o}, and filtered (filtrate X) from the precipitated solid was poured into an equal volume of half saturated aqueous sodium sulphate and repeatedly extracted with petroleum until this phase was colourless. The combined petroleum extracts were washed twice with water, dried over sodium sulphate and concentrated.

Extract MFS - The procedure was identical to that used for preparation of extract MF as far as filtrate (X) which was diluted with an equal volume of water and steam distilled until the distillate was no longer cloudy. An equal volume of saturated sodium sulphate was added to the distillate and the mixture extracted twice with half its volume of light petroleum. The petroleum layer was dried over sodium sulphate and concentrated.

4E.2 Steam distillation of the pheromone from extract FT

Five ml. of extract FT were steam distilled as previously described. The light petroleum extracts of the distillate and the residue remaining after distillation were adjusted to give a concentration of 20 ME (female) 0.1 ml. assuming that the attractant is either completely distillable or completely non-distillable. The extracts were then assayed for activity. (Table 2).

TABLE 2

Steam Distillation of Extract FT

	Average % male moth response in sixteen cages	
	Expt. 1	Expt. 2
0.1 ml. petroleum control	7.5	4.0
20 ♀ ME undistilled control	73	72
20 ♀ ME distillate	67	68
20 ♀ ME residue	2.5	3.0

The results clearly show that the attractant material is completely steam volatile. These results were confirmed by trapping (Table 3).

TABLE 3

Attractiveness of Fractions from Steam Distillation of Extract FT Assayed by Trapping Method

	No. of male moths caught	
	Expt. 1	Expt. 2
0.1 ml. petroleum control	0	0
20 ♀ ME distillate	10	11
20 ♀ ME residue	0	0

4E.3 Column Chromatography

Columns of 3 x 34 cm and 12 x 1 cm were prepared by slurrying Woelm alumina (Grade 1 neutral) in petroleum. Thirty ml. of extract MF containing 30,000 ME were chromatographed on the larger columns using 500 ml each of petroleum, benzene, benzene-ethyl acetate (1:1 v/v), ethyl acetate and methanol in that order. Twenty thousand ME of extract MFS were fractionated on the smaller columns using 40 ml, each of the above solvents for development.

4E.3.1 Attractiveness of extract MF before and after alumina chromatography

Even at a concentration of 260 ME (male and female) this extract elicited little response (Table 4).

TABLE 4

Attractiveness of 260 ME, (Male and Female) of Extract MF

	Average % male moth response in eight cages	
	Expt. 1	Expt. 2
0.2 ml. petroleum control	3.0	1.8
260 ME (♂ and ♀) extract MF	9.5	10

Following alumina chromatography the fractions were tested for activity (Fig. 7).

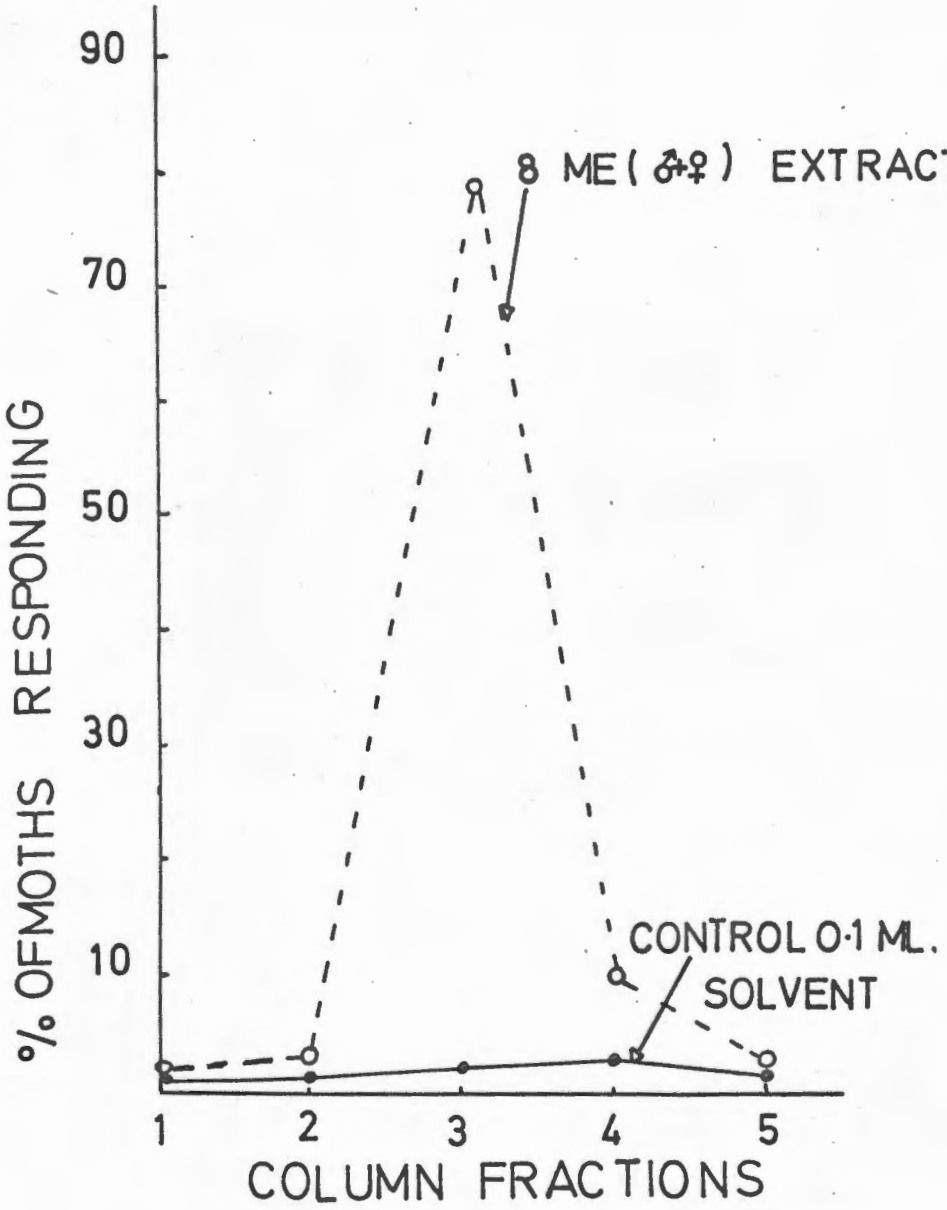


Fig. 7 Response of male moths to extract MF following alumina chromatography.

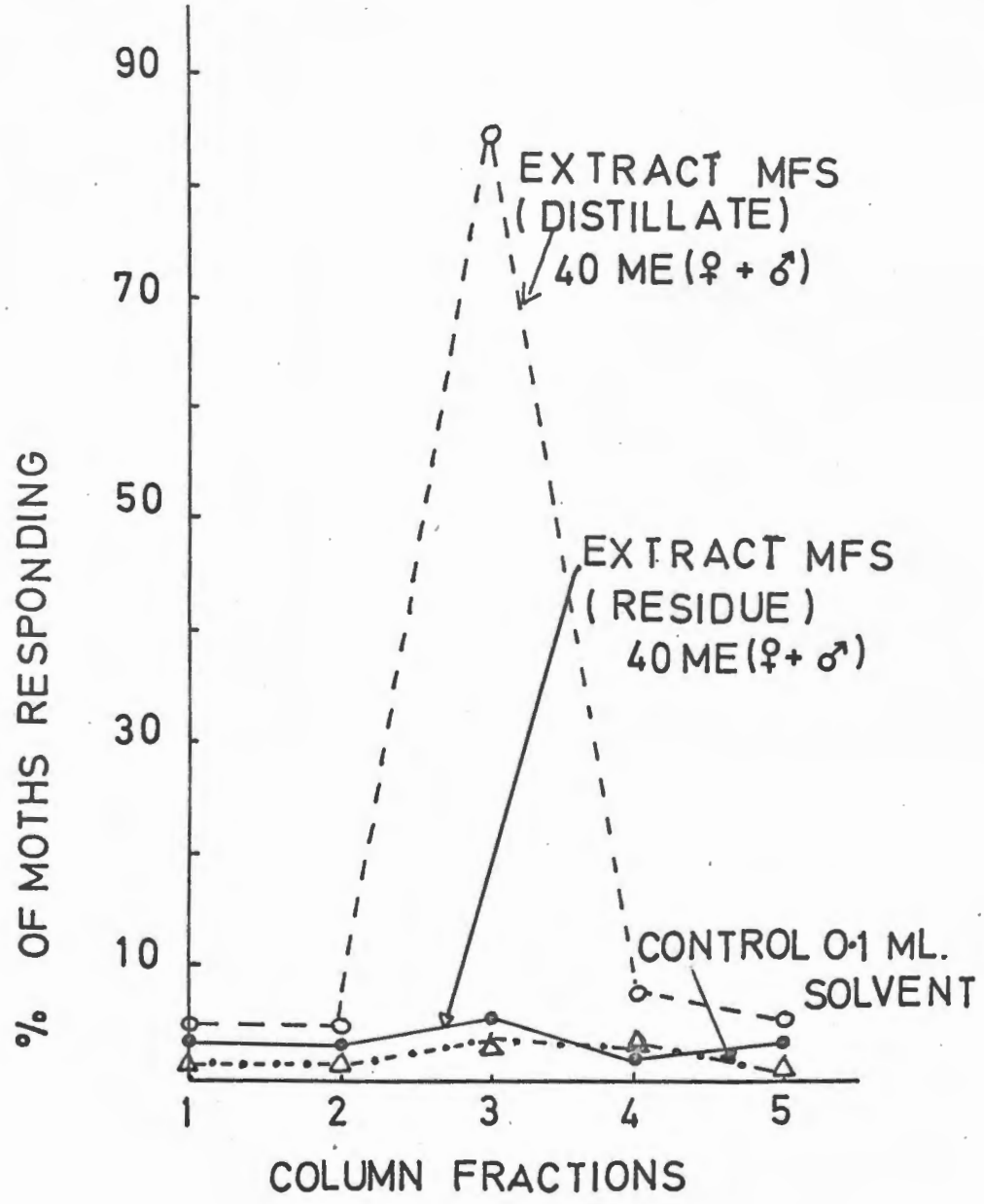


Fig. 8 The effect of alumina fractionation on pheromone potency of extract MFS and the petrol extract of the steam distillation residue.

4E.3.2 Attractiveness of extract MFS before and after alumina chromatography

Extract MFS (0.1 ml.) containing 40 ME (male and female) was bioassayed in the usual manner (Table 5). In addition the residue remaining after steam distillation was extracted with light petroleum and assayed for activity (Table 5).

TABLE 5

Attractiveness of Extract MFS and the Petroleum Ether Extract of the Steam Distillation Residue

	Average % male moth response in ten cages	
	Expt. 1	Expt. 2
0.1 ml. petroleum control	4	4
40 ME (♂ and ♀) residue	2	3
40 ME (♂ and ♀) extract MFS	16	7

Three traps, each baited with extract MFS (0.1 ml.), accounted for only three male moths confirming the results of the behavioural assay technique. Extract MFS and the light petroleum extract of the residue were fractionated separately on small alumina columns, and the fractions bioassayed (Fig. 8).

A dramatic recovery of attractant potency is evident in the benzene-ethyl acetate fraction of extract MFS chromatograph (Fig. 8).

Traps baited with 40 ME (male and female) benzene-ethyl acetate fraction, 40 ME (male and female) ethyl acetate fraction and light petroleum (0.2 ml.) caught 14, two and zero moths respectively, confirming the results of the behavioural response assay.

Fractionation does not enhance the attractiveness of the petroleum ether extract of the steam distillation residue. This confirms the earlier conclusion that the attractant is completely steam volatile.

4E.4 Attractiveness of extract F of whole female moths

The results show that attractant potency can be recovered from mixed extracts by alumina chromatography either before or after steam distillation (Figs. 7 & 8). However, chromatography was not a prerequisite for demonstration of activity with extract FT (female moths only). It was therefore of interest to see if extracts of whole virgin female moths prepared by the method described for extract MF elicited a response.

Two thousand whole female moths were extracted and 40 ME assayed in the usual fashion using 14 cages of moths. A suitable control was included. The mean percent response in the former case was 59% and in the latter only 8.0%.

4E.5 Preparative Gas Liquid Chromatography

An aluminium column (8 ft x 3/8 in) containing 30% SE 30 (Microtek

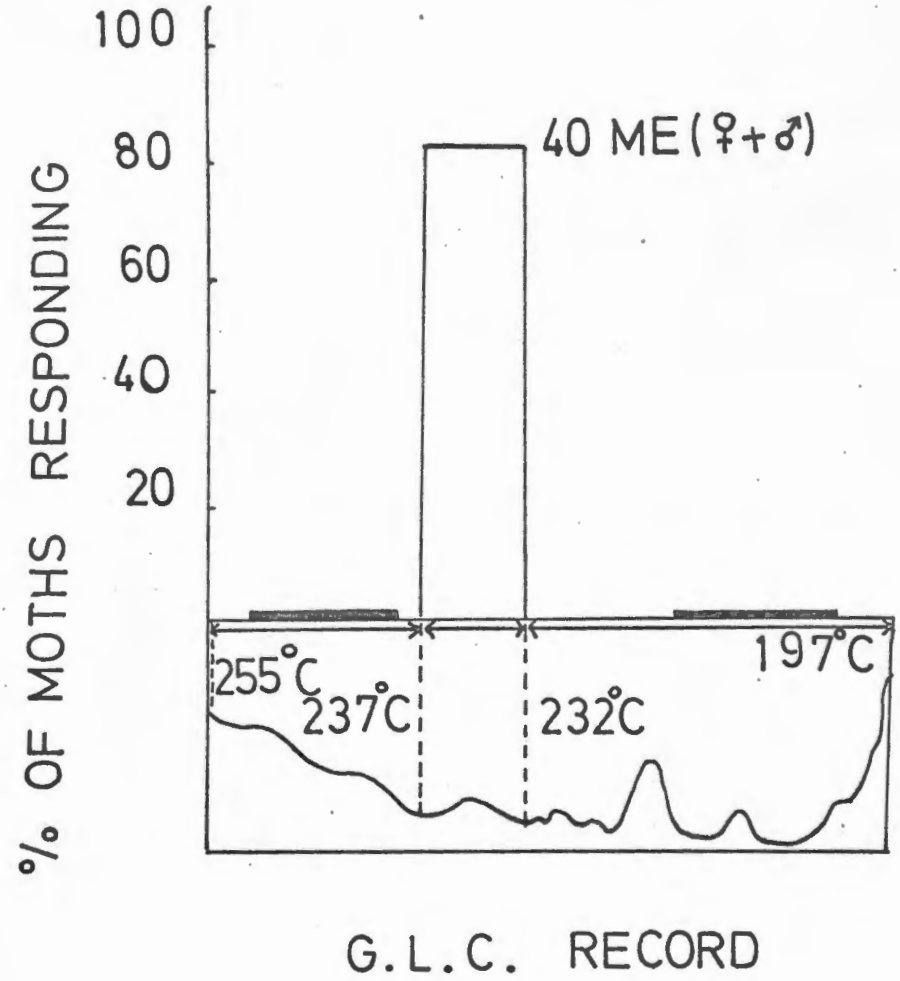


Fig. 9 A partial trace of the gas chromatographic separation of components in the steam distillate of extract MF. Male moth response is shown.

Instruments Inc.) on Chromosorb P, 45-60 mesh (Applied Science Laboratories, State College, Pennsylvania) was used in an Aerograph A 700 gas chromatograph with a thermal conductivity detector. The attractant material in petroleum (0.1 ml.) was injected under the following conditions: carrier gas, helium 200 ml./min.; injector temperature 300°; detector filament current 200 m.A; column temperature 130°. Five minutes after injection the column temperature was increased at the rate of 6°/min. to a maximum of 260°. Samples were trapped at the exit port in collector bottles containing petrol cooled in liquid nitrogen.

The benzene-ethyl acetate fractions from alumina chromatography of extract MF were concentrated to one third of their original volume, steam distilled and the distillate extracted with light petroleum. The petroleum extract (0.1 ml.) containing 1000 ME (male and female) was used for each injection. Fig. 9 is a tracing of a typical chromatograph record with corresponding male moth response indicated.

Chapter 5

STRUCTURAL STUDIES AND FINAL PURIFICATION

5.1 Gas Liquid Chromatography of Alkyl Esters

Gas liquid chromatography has proved an indispensible technique for research with lipids, and a brief outline is given of its applications. Alkyl esters may be successfully separated with non-polar or polar stationary phases. With non-polar phases unsaturated and branched components emerge before the corresponding saturated ones, whereas with polar phases the unsaturated esters emerge after the saturated ones. The non-polar liquid phases that find popular use are the Apeizon greases and silicone gum rubbers, the polar liquid phases used are usually the polyesters; ethylene glycol adipate (EGA), diethylene glycol succinate and neopentyl glycol adipate.

The efficiency of the column separation is the result of at least two factors, column efficiency and solvent efficiency. The column efficiency is expressed as the number of theoretical plates (n). This concept is carried over from distillation practices and is somewhat ambiguous but useful in gas chromatography. Basically the formula compares the narrowness of a peak (W) to the length of time the component has been in the column (t_R).

$$N = 16\ (t_R/W)$$

The efficient column keeps the peaks narrow and this may be effected practically by decreasing the column diameter and the absorption by the solid support, which is of small and even mesh size. Preparative

columns of large diameter are not satisfactory for analysis requiring high resolution, as the column efficiency is low.

In a comparison of column types the maximum number of plates per foot of column is given as 300 for ¾ in. diameter, and 800 for ⅛ in. diameter. (Baumann and Gill, 1966). The solvent efficiency depends on the distribution coefficients between the liquid and the gas phases. It is expressed by the ratio of the retention times of two peaks of t_{R1} and t_{R2}. The separation (α) is given by the equation, $\alpha = t_{R2}/t_{R1}$, while the resolution (R) depends on the peak width and is given by the equation,

$R = 2 \cdot t_R/(W_1 + W_2)$

5.2 Analysis by Comparison with Standard Compounds

With homologous compounds the graph of the logarithm of retention times against chain length, or degree of unsaturation, is reported to be a straight line. This has been shown to hold true for fatty acid esters (Farquhar *et al.*, 1959; Haken, 1967) and acetates, alcohols and hydrocarbons (Jamieson and Reid, 1967).

A useful concept, introduced by Miwa and co-workers (1960), is the Equivalent Chain Length (ECL). In a particular series of compounds, such as methyl esters, the logarithm values of the retention times of the standard saturated esters is plotted against the length of the carbon chain. The particular compounds are then accorded their equivalent chain length from this plot.

The E.C.L. constants are for a specific column packing and a given carrier gass, independent of experimental conditions, and have the advantage of being readily recognised as representing the number of carbon atoms or equivalent, in a compound. The ECL of a saturated straight chain ester having n carbon atoms is exactly n in all liquid phases, e.g. methyl stearate n = 18.0. In a comparison of esters it will be seen that dodecanyl acetate, $CH_3.(CH_2)_{10}.CH_2\text{-}O.CO.CH_3$, will have an ECL constant close to 13, the value for methyl tridecanoate, $CH_3.(CH_2)_{10}CH_2.CO.OCH_3$.

5.3 Mass Spectra of Alkenyl Acetates.

5.3.1 Fragmentation due to the Acetate Group

The acetate group undergoes a rearrangement by migration of a hydrogen atom to the carbonyl oxygen by way of a six membered cyclic transition state. This undergoes fragmentation by the elimination of a charged olefin fragment, (M-60), (Fig. 10). The positive charge seems to be stabilised on the acetate fragment only if it abstracts another hydrogen and becomes the protonated form, $[CH_3CO_2H_2]^+$, m/e 61. This is similar to the rearrangement and fragmentation of methyl esters of fatty acids (McLafferty, 1958).

An additional fragmentation undergone by alkyl acetates results in a fragment, m/e 116. This is not a very intense peak in the general fragmentation pattern but also occurs with propionates (m/e 140) and higher esters (Ryhage and Stenhagen, 1958). It appears that these

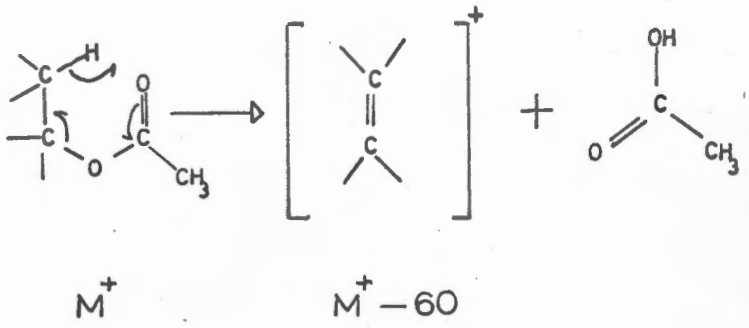


Fig. 10 Loss of acetic acid via a six membered cyclic transition state.

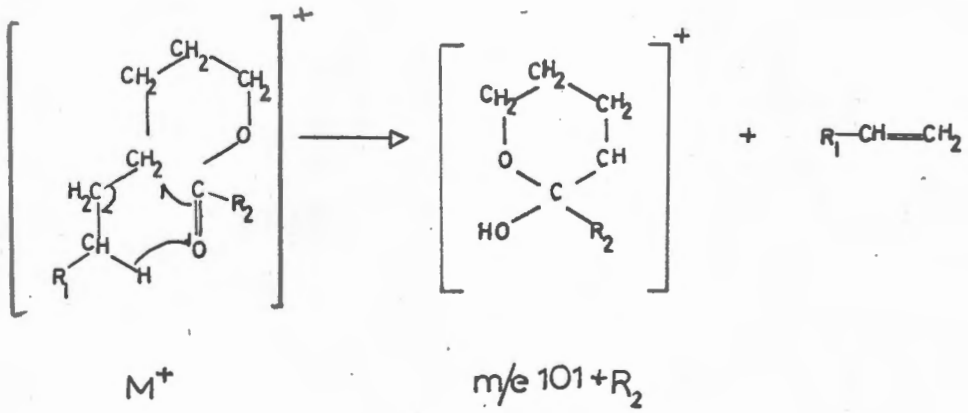


Fig. 11 Formation of six membered lactone ion by long chain alcohol esters.

ions are formed by cleavage between carbon atoms 4 and 5 of the alkyl chain, with rearrangement of one hydrogen atom. Ryhage and Stenhagen suggested tentatively that this transfer of hydrogen probably takes place from carbon atom 6, with the formation of a stable olefin and cyclic charged ion (lactone) as indicated in Fig. 11.

The base peak in the acetate mass spectrum is found at m/e 43. This ion can arise from the fission of the ester group to give ion $[CH_3C = O]^+$, and from the fragmentation of the alkyl chain to give the propyl action, $[CH_3CH_2CH_2]^+$.

5.3.2 Fragmentation due to the Olefinic Bond

The resonance stabilization of an alkyl cation leads to the increased probability of the fragmentation of a carbon-carbon bond to a double bond. This however seems to be of little analytical value as the olefinic bond in the molecular ion is found to be mobile if it is not in close proximity to a functional group. It is able to migrate easily by successive hydrogen radical or hydride ion shifts.

However the mass spectrum of mono-unsaturated acetates and methyl esters do not give the same pattern of increasring intensity to the C_3 and C_4 fragment as do the saturated components. Instead the C_5 and C_6 fragments (m/e 82 and 67 for dodecenyl acetate) are particularly intense (Ryhage and Stenhagen, 1960).

The double bond also effects the formation of the fragments m/e 61 and 116. The intensity of the fragment $[CH_3COOH_2]^+$ is

greatly reduced. This could be due to the fact that the positive charge is now more easily stabilized on the olefin fragment and that due to the double bond migration it is less easy to abstract a hydrogen atom. The migration of the olefinic bond must certainly negate the formation of ion m/e 116 which requires a hydrogen from carbon 6, right next to the olefin bond in dodec-7-en-1-yl acetate. (See Appendix 2 , Mass Spectra).

5.3.3 Determination of the Olefinic Bond Position

There are two techniques that have been used with success to detect the position of the double bond, reduction with deuterium and the formation of an acetal after reaction with osmium tetroxide.

5.3.3.1 Reduction with Deuterium.

Catalytic reduction with deuterium is of no value due to exchange of hydrogen at unsaturated centres. However it has been found that diimide, N_2H_2, as the active reagent from anthracene biimine will stereospecifically reduce by cis - addition to a double bond as shown in Fig. 12 (Corey and Mock, 1962). This may be done in deuterium oxide-dioxane solution to give the dideutero reduced product which can be analysed by the isotope increased fragments in the mass spectrum.

5.3.3.2 Formation of Erythro- and Threo- Acetal Derivatives

McCloskey and McClelland (1965) used osmium tetroxide to produce the erythro and threo-diol derivatives of mono-unsaturated

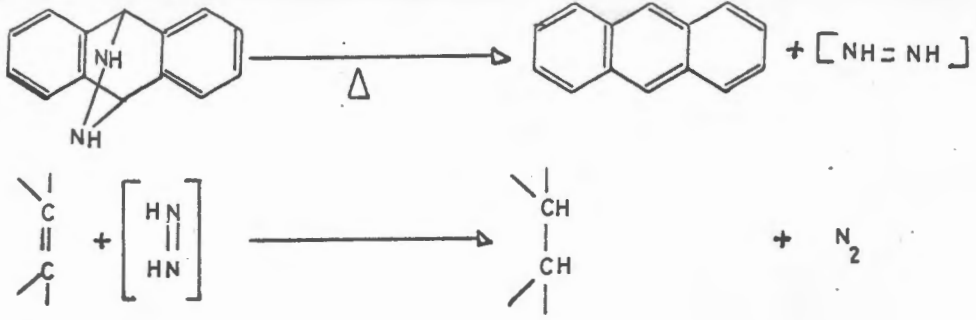


Fig. 12 Reduction of olefinic bonds by diimide from anthracine biamine.

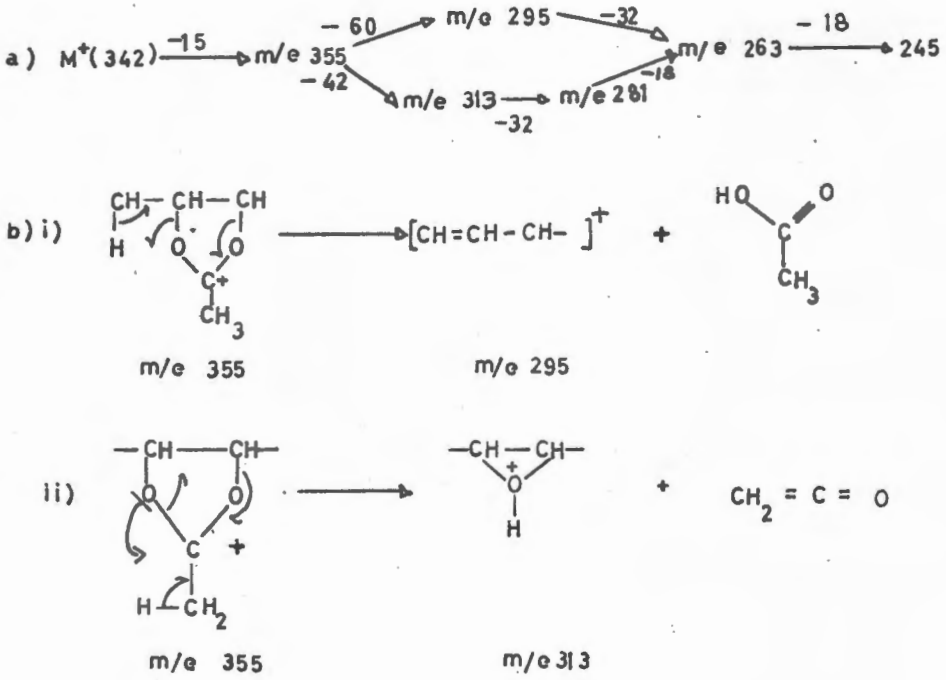


Fig. 13 Fragmentation of isopropylidene derivatives of methyl esters.

fatty acids, which were taken to their isopropylidene derivatives. The mass spectra of fatty acids so treated showed differences corresponding to the position and configuration of the original double bond. The differences that arose from the erythro and threo derivatives were differences of intensity and not mass.

the fragmentation pattern due to the dioxolane ring of methyl erythro-O-isopropylidene-9, 10-dihydroxyoctadecanoate derived from methyl oleate is shown in Fig. 13 a, and may be explained by the equations also shown in Fig. 13b. This explanation was derived after isotope labelling of the isopropylidene dioxolane ring with Oxygen-18 and deuterium.

They found that the intensity of ion m/e 281 (after loss of "ester" methanol from m/e 313) was at least twice as great in the erythro derivative from methyl oleate than in the case of the threo derivative from methyl elaidate. However the intensity of the ion also varied according to chain length and any diagnosis would require a closely related compound for comparison.

5.3.3.3 The mass spectrometric analysis of dihydrosterculate (natural) by Polacheck and co-workers (1966) suggests a further possible way of detecting the position of the double bond. The synthetic cyclopropane acid was prepared by the treatment of oleic acid with the Simmons-Smith reagent, iodomethylzinc, then catalytically reduced to the branched chain (methyl) compounds. Since the mass spectra of

intact cyclopropane esters revealed no characteristic peaks, the formation of a cyclopropane derivative from a carbene of the nature of dichlorocarbene, or a carboalkoxycarbene, may be useful.

5.4 Discussion of Results.

The determination of the sex pheromone structure was carried out in three parts; reactions on crude extract, GLC analysis on impure extract accompanied by bioassay, physicochemical analysis of the pure attractant and comparison with synthetic acetates.

The chemical tests on the crude extract showed that the pheromone was stable to heat and to steam distillation. Catalytic hydrogenation using platinum oxide catalyst resulted in loss of activity. Furthermore alkaline hydrolysis caused loss of activity which, however, was restored on treating the neutral fraction with acetyl chloride. These experiments were indicative of the pheromone being an unsaturated acetate ester.

5.4.1 GLC Analysis of Impure Extracts

Accordingly it was felt that the GLC methods used in the analysis of fatty acid methyl esters would be pertinent to the author's problem. The product obtained by steam distillation by (Extract MFS) was fractionated on alumina and the benzene-ethyl acetate fraction analysed with the Aerograph 700 instrument as described above (section Only two peaks were shown. While this in itself was an indication of

the improved procedure of extraction, it was doubtful that the sex pheromone was present in such quantities. Analytical GLC, on a Barber Colman argon ionization instrument with polyester liquid phase revealed this in fact to be so. Numerous smaller peaks were shown and the major peaks resolved into four peaks.

A further quantity of extract MFS was chromatographed on alumina to give a series of small fractions each of which was analysed by GLC. All the active fractions (MFS(a)) from the aluminium column showed a small peak of lower retention volume compared to the major peaks (Fig. 14). It was thought at this stage that this small peak was the active pheromone peak.

High vacuum distillation of a fraction, MFS(a), gave two fractions. GLC analysis of these showed that the first fraction was active and contained many compounds of lower volatility that were absent in the second distillate fraction, which was inactive (Fig. 14). These peaks were in the area of the suspected peak but no real purification was achieved, probably due to the similarity of the compounds.

The active distillate was mixed with a standard mixture of methyl esters of saturated acids (C_{15} through C_{19}) and the relative retention time of the suspected peak noted. On hydrogenation of the distillate, the suspected peak no longer occured at this relative retention time. (Fig. 15).. This would be expected if the pheromone was unsaturated, for on the polar phase, its relative

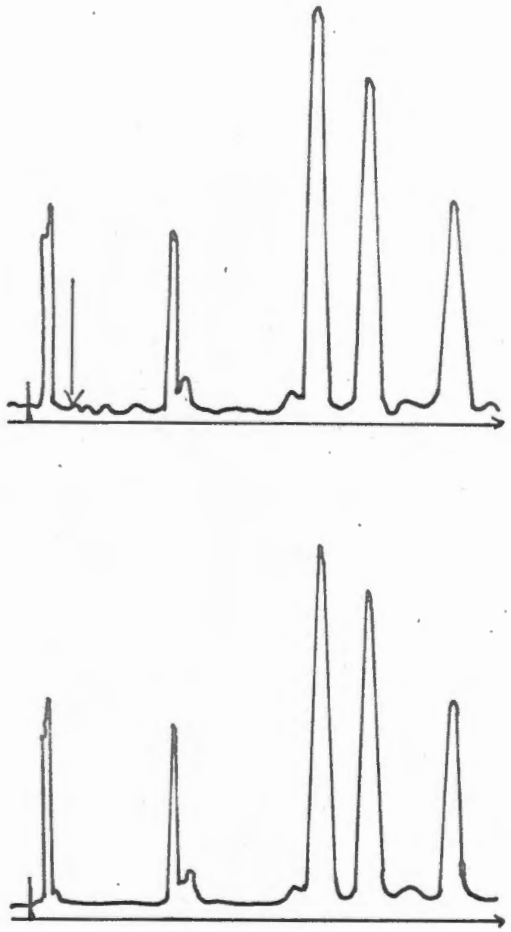


Fig. 14 Traces showing gas chromatographic separations of an active chromotagraphy fraction (A), and an inactive high vacuum distillate of the same fraction (B). The suspected pheromone is shown by the arrow,

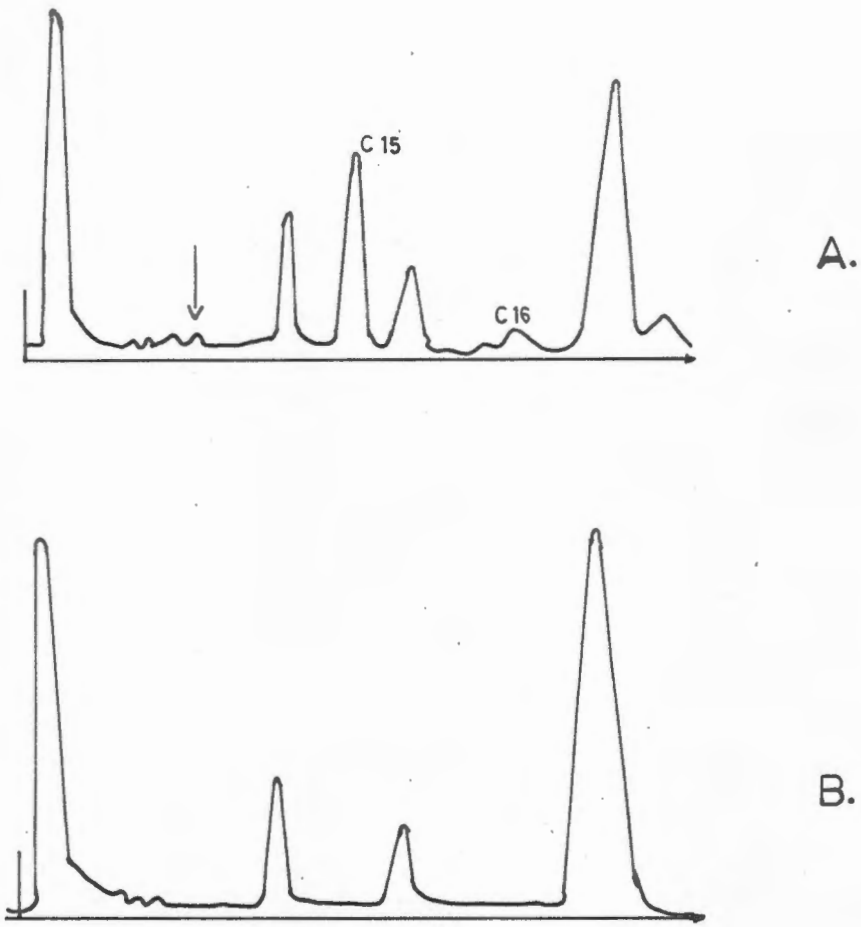


Fig. 15 Partial traces showing gas chromatographic separations of an active high vacuum distillate, with methyl esters standards (A), and the distillate after hydrogenation (B, inactive). The suspected pheromone peak is indicated.

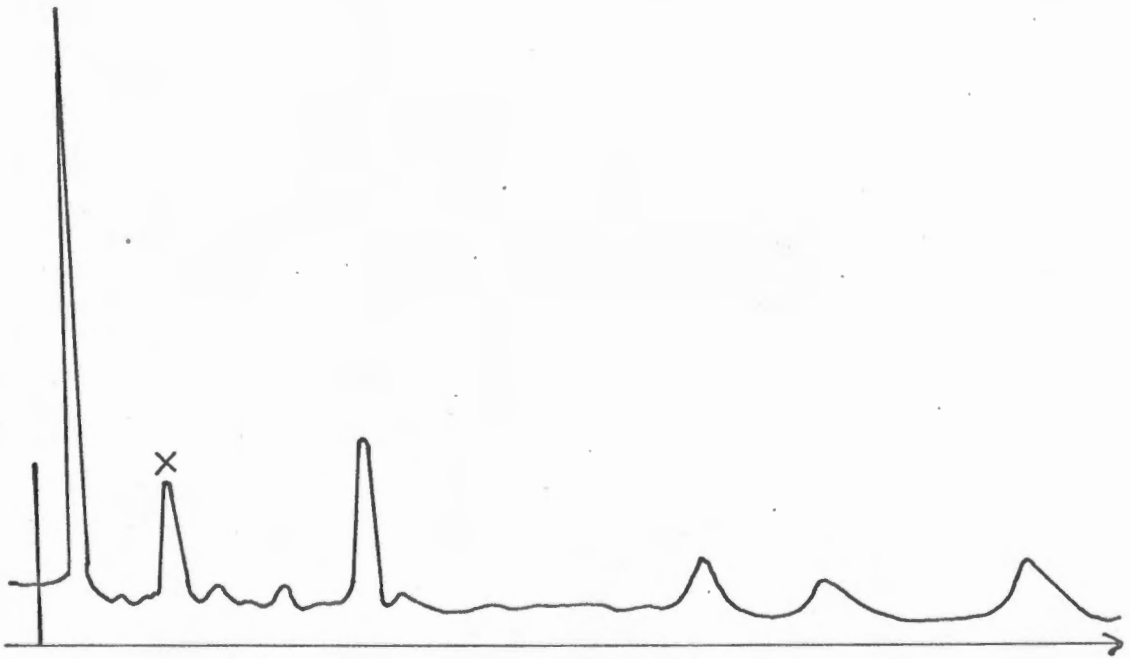


Fig. 16 A trace of a chromotographic separation of an active fraction (B6), showing increased purification of the pheromone (X).

retention time would decrease after hydrogenation.

Further purification of the active fractions by alumina chromatography followed by GLC showed a greatly increased peak in the area thought to be active (Fig. 16). On collection over cooled petrol, this peak was unequivocally identified as the active peak. It had a retention time almost midway between the C_{12} and C_{14} methyl esters, suggesting an alcohol chain length of approximately twelve carbon atoms. A preliminary report by Berger (1963) gave an identical GLC analysis of the cabbage looper moth (T. ni.) pheromone.

The behaviour of the pheromone with polar (EGA) and non-polar (silicone gum rubber) liquid phases was observed and the ECL constants calculated with reference to the saturated methyl esters. They were found to be 13.2 (EGA) and 12.8 (silicone gum rubber) (Fig.17). These results would be expected from a mono-unsaturated straight chain ester.

5.4.2 Preparative Gas Chromatography

In the original experiments use was made of an Autoprep 700 GLC apparatus with a hot wire detector (thermal conductivity) detector. (Section 4E.5) Although separation could be effected, the sensitivity was not sufficient, even when helium carrier gas was used, to permit extending the studies to very small quantities. Accordingly these studies were continued with an analytical GLC (Varian Aerograph 1200), using a flame ionization detector. The apparatus was modified

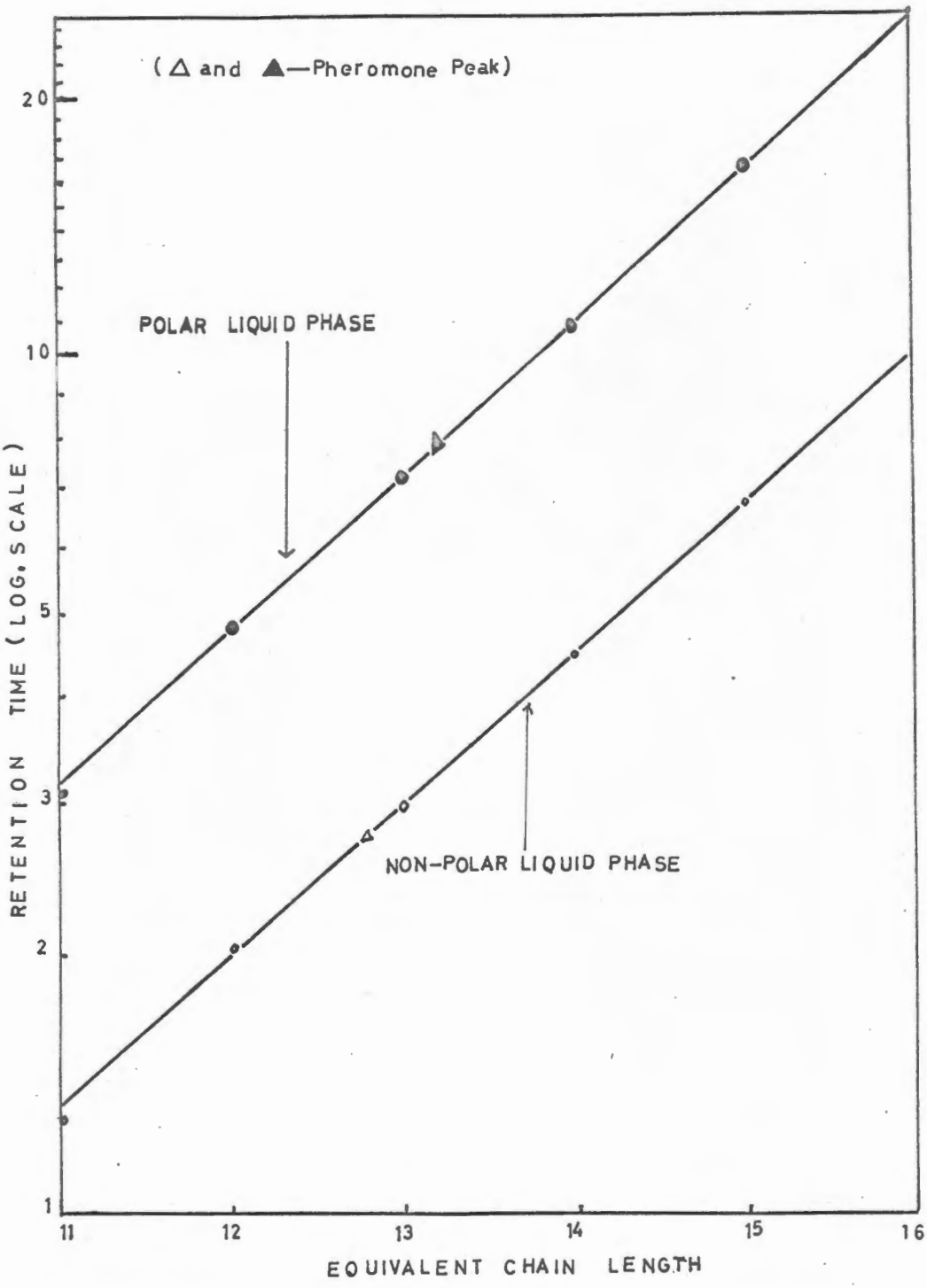


Fig. 17 The equivalent chain length of the pheromone on polar (EGA) and non-polar (SE-30) phases.

by a gas stream splitter (1:10) permitting the micropreparative collection of the major portion of the sample.

The flame detector was found to be sensitive to gas flow changes, so any collection method affecting the gas flow caused bad recording. The method adopted was a cooled glass U-tube fitted with teflon tubing. Since liquid nitrogen caused the organic vapours to be swept out as a "snow", ice water was the coolant. The tubes were washed with solvent later.

5.4.3 Physico-Chemical Analysis and Comparison with Synthetic Acetates

The mass spectrum of a collected active fraction showed fragments at m/e 43 (base peak), 61 ($CH_3CO_2H_2^+$, low intensity), 166(M^+ -60) and 226(M^+), and C_6 fragment (m/e 82) showed the highest intensity of the carbon fragments. There was no fragment at m/e 116. This indicated an alkenyl acetate and the formula, $C_{12}H_{25}$ O.O C.CH_3, fitted the molecular mass. With hydrogenation and GLC data this indicated that the pheromone was a dodecenyl acetate.

In 1966, Berger showed conclusively that the sex pheromone of the cabbage looper moth (T. ni.) was cis-dodec-7-en-1-yl acetate, and he kindly supplied a sample for comparison. It was identical on GLC (Fig.19+20) and gave the same fragmentation pattern, including the low intensity of the protonated acetic acid ion (m/e 61) (Fig.18). Bioassay showed that the synthetic sample elicited the full biological

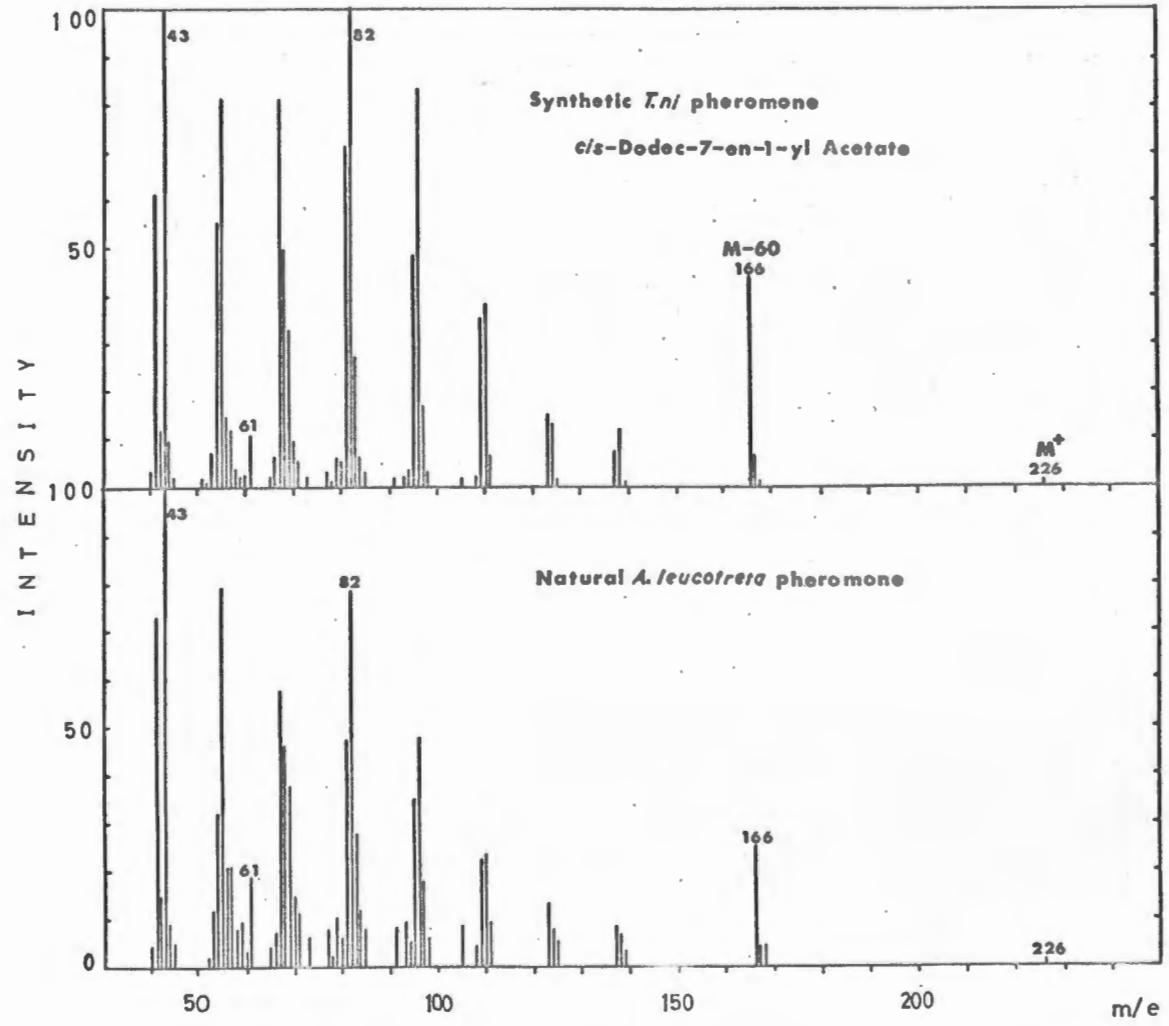


Fig. 18 Comparison of the mass spectra of cis-dodec-7-en-1-yl acetate and the natural sex pheromone of A. leucotreta females.

response, including mating, at a level of 1 μg. The identity of the A. leucotreta pheromone as a dodecenyl acetate was accepted but the position, and geometry, of the double bond remained to be shown.

Jacobson's group (Green et al., 1967) published the synthesis of the cis and trans isomers of dodec-7-en-1-yl acetate and kindly supplied samples for comparison. Sekul and Sparks (1967) published the identification and synthesis of the fall armyworm moth (S. frugiperda) sex pheromone. It was homologous to the T. ni pheromone, cis-tetradec-9-en-1-yl acetate. The pure cis-isomer was kindly supplied.

Bioassay of these synthetic acetates was carried out at the 1 μg level and it was shown that the cis-tetradec-9-en-1-yl acetate was inactive and the cis-dodec-7-en-1-yl acetate was active. However the latter contained at least 10% of the trans isomer (by infra red) and was nearly two thirds less active than the trans-dodec-7-en-1-yl acetate. (Table 6).

Thin layer chromatography (TLC) with silver nitrate (25%) impregnated silica gel showed the presence of a trans (higher R_f) compound in the cis-dodecenyl acetate preparation. (Fig. 22). This was repeated on a preparative scale to achieve purification of the two compounds. Elution of these acetates from the silica gel with ether or benzene proved unsuccessful, but steam distillation resulted in their recovery. The trans-dodec-7-en-1-yl acetate was shown to be completely active and the cis-isomer inactive.

Analysis of a semi-pure extract, by the same method, showed two major spots. The one of high R_f would be saturated compounds, and the other had a retention time identical to the trans-dodecenyl acetate.

To obtain pure pheromone for the determination of the double bond position, the following purification procedure was followed. A portion of crude extract MFS was purified by column chromatography. The column of alumina was eluted as before, but another was packed with silicic acid and eluted with petrol/ether mixtures. It was found that activity could be recovered from the latter column. The active fractions from these two columns were combined and hydrolysed in alcoholic potassium hydroxide. The neutral fraction was acetylated with acetyl chloride and found to be active. The acetylated mixture was then separated on a silicic acid column impregnated with five per cent silver nitrate, eluted with cyclohexane/ether mixtures and activity recovered. The active fractions were combined and the pheromone collected after GLC as before.

The methods required for the determination by mass spectroscopy required further purification after the reactions and this was felt to be too hazardous to attempt on the small quantities available. The Lemieux oxidation with periodate/permanganate solution had been used on the P. dispar pheromone (Jacobson et al., 1961), and had been carried out successfully on oleyl acetate previously by the author.
The reaction is suitable for small quantities as the products are analysed

by GLC after methylation with diazomethane.

trans-Dodec-7-en-1-yl acetate and the pheromone were oxidised and the methylated products analysed by GLC. The oxidation products were in low yield; but the GLC showed equivalent peaks from the two oxidised compounds, and one of the peaks corresponded to that of methyl pentanoate in retention time indicative of a double bond in the $C_{(7)}$- $C_{(8)}$ position in the pheromone.

Experimental

5E.1 Reactions on Crude Extract

5E.1.1 Hydrogenation

An aliquot of crude extract FT (100 ME) in methanol (0.5 ml.) was hydrogenated for five hours with finely divided platinum oxide catalyst.

One half of the solution was tested against the active extract (20ME). The experiment was done in duplicate and in both cases complete loss of activity was found after hydrogenation.

5E.1.2 Reaction with 2,4-dinitrophenylhydrazine

One aliquot of extract FT (100ME, 0.5 ml.) was added to 2,4-dinitrophenylhydrazine reagent (2 ml.), and a similar aliquot was added to 8.5% methanolic sulphuric acid (2 ml.). The latter was a control reaction, lacking the reagent.

The solutions were heated on a water bath (75^{o}) for two hours and then extracted with petrol (5 ml.) after the addition of water (5 ml.) a control extraction was carried out on just the reagent and water. No activity could be elicited from the extract reaction, but neither did either control show any pheromone activity. This test was done in duplicate.

The same procedure was repeated, but without heating the solutions. No loss of activity could be shown from the solutions containing active extract.

5E.1.3 Alkaline Hydrolysis

An aliquot (100 ME, 0.5 ml.) of extract FT was dissolved in 1N ethanolic potassium hydroxide solution (2 ml.) and refluxed for an hour in a water bath. The solution was extracted with petrol (4 ml.) after the addition of water (2 ml.) and the petrol extract tested for activity. This was carried out in duplicate and complete loss of activity resulted after hydrolysis.

5E.1.4 Acetylation and Methylation of the Hydrolysed Extract

An aliquot (100 ME) of extract FT was hydrolysed as above and extracted with petrol (6 ml.). Acetyl chloride (0.5 ml) was added to the concentrated petrol extract (0.5 ml.) and warmed for one minute. Water (4 ml.) and petrol (2 ml.) were added and the petrol extract washed with dilute bicarbonate solution and tested for activity.

The base soluble fraction was extracted with petrol (5 ml.) after acidification with concentrated hydrochloric acid (4 ml.) and the extract tested for activity.

The concentrated petrol extract was refluxed with boron trifluoride (14%) in methanol (1 ml.) for five minutes and extracted. The extract was tested for activity.

The only extract found to be active was the one from the acetylated products.

5E.1.5 Stability of the Pheromone to Heating in Methanol/Water

Three sealed tubes containing active methanol extract FT (100 ME, 0.5 ml. each) and water (0.5 ml.) were heated at 100°C for three, six and nine hours respectively. The contents were extracted with petrol and tested for activity. No loss of activity was recorded and the nine hour heated material showed undiminished activity even at the level 0.2 ME. This gave an indication of the pheromone's stability to heat and steam distillation.

5E.2 GLC Analysis of Impure Extract

A quantity of extract MFS (482,101 ♂ and ♀ ME) was purified by alumina chromatography as described in Section 4E.3, with two modifications. Twice the amount of benzene (i.e. 1200 ml.) was used and small fractions (50 ml.) collected.

The solvents evaporated and petrol (5 ml.) added to each fraction and the fractions were assayed for activity and activity found in the

latter benzene elution (1 μl of soln.per assay).

The active fractions (MFS (a)) were analysed by GLC on a Barber Colman argon ionization model 192 instrument. Each fraction (5 μl aliquots) was injected under the following conditions. The glass column (4' x $\frac{1}{4}$") was packed with 15% ethylene glycol adipate on acid washed Chromosorb W (80-100 mesh). The operating temperatures were 184.5° (column), 250° (flash heater) and 225° (detector cell). The argon flowed at 50 ml./min. and radium-226 was the ionization source. Relative gain of the amplifier was 10X and the chart speed, 15" per hour.

A chromatogram trace of an active fraction (MFS(a)) is shown in Fig. 14. The peak retention times of all the fractions were plotted on a linear scale and inspected for any correlation of activity with a particular peak. This peak is indicated in Fig.14.

One active fraction (MFS(a)) was distilled at high vacuum (5×10^{-4}mm Hg) and two fractions collected (b.p. 124° - 135° and 135° - 165°), both were light yellow oils. The lower b.p. fraction was active and the other inactive.

The GLC analysis of the inactive distillate is shown in Fig.14 and compared with the active fraction (MFS(a)). The lack of compounds of lower retention time agreed with the preliminary survey of fractions and peak-activity correlation. The active distillate was found to contain these compounds of lower boiling point and retention time, as well as the rest of the compounds found in the inactive distillate.

The active distillate was mixed with methyl ester standards (Applied Science Laboratories standard mixture L203) on injection with GLC analysis (column temp. 153°). The retention times of the standards (C_{15} through C_{19} fatty acid esters) were plotted on a logarithmic scale against carbon chain length and the retention times of the C_{10} through C_{14} fatty acid esters estimated. The retention time of the suspected peak was close to the estimated C_{13} methyl ester retention time.

Two aliquots of the distillate were hydrogenated with Pt_2O in methanol, and the totals injected on the GLC. One was mixed with standards for reference. The suspected peak was absent and this is shown in Fig. 15. All active extracts were combined and purified twice by alumina chromatography as described at the beginning of the section. Activity was found in benzene fractions 5 and 6 (B5 and B6), a more rapid elution than usual probably due to the presence of water in the benzene. The active fractions were analysed by GLC (column temp. 175°) and an increased peak found near the estimated C_{13} methyl ester retention time.

A collection device was fitted to the Barber Colman instrument consisting of a heated metal tube with taper. A glass tube with a ground glass joint (B10) was fitted to a test tube containing petrol. The tube was cooled in liquid nitrogen. Collection and reinjection of methyl stearate showed an estimated 25% collection with this apparatus.

The fraction B6 was collected in three fractions, the second fraction being the collection of the suspected peak (Fig. 16). The pheromone activity was found in second fraction, 48% and 51% response in two experiments. The rest of the fraction B6 was inactive, even after collection to the estimated retention time of C_{22} methyl ester.

The pheromone peak was analysed with reference to methyl ester standards (C_{11} - C_{15}, Applied Science Laboratories standard mixture L 202) on the Barber Colman with ethylene glycol adipate (polar phase) and the silicone gum rubber, SE-30 (non-polar phase). The equivalent chain length (ECL) was calculated from the logarithmic plot of the ester retention times. The ECL on polar phase was 13.2, and 12.8 on non polar phase (Fig.17).

Throughout this section, equivalent amounts (200-250♀ME) were injected for GLC analysis.

5E.3 Small Scale Preparative Gas Chromatography

Collections of the pheromone was carried out on a Varian Aerograph 1200, single column instrument with a flame ionisation detector, modified for collection by a effluent stream splitter. The final version used of the stream splitter was supplied by Varian and gave a 10:1 splitting ratio, only when gas flow was unhindered during collection. A glass 'U' tube fitted with a length of teflon tubing, cooled with ice water was used to trap the compounds as they emerged. The pheromone was dissolved in petrol for bioassay and redistilled dichloromethane for analysis. The collection of methyl laurate by this method was found to give 60-70% recovery of material.

The collection of the pheromone from fraction B6 was carried out on two different liquid phases, neopentyl glycol adipate (H1 - EFF 3A Applied Science Laboratories) and silicone gum rubber (DD-041, Perkin Elmer) in a column packing of 5% on acid washed, hexamethyl disilane (HMDS) treated Chromosorb W (80-100 mesh, Applied Science Laboratories). These were packed in stainless steel columns (10' x ¼") with a flow of 30 ml. nitrogen per min. at temperatures ranging from 140° to 170°. The injector port temperature was kept at 220°, and the detector at 250°. It was found that sharp separation of the pheromone, on collection, could be achieved if an external heating coil (180°) was placed at the exit port of the stream splitter.

The results of collection, and bioassay, were in agreement with the results given in Section 5E.2. Neopentyl glycol adipate is a less polar phase than ethylene glycol adipate and the ECL was determined at 13.1 on this phase.

5E.4 Collection for Mass Spectrometry

It was shown, by collection of lauryl acetate, that activated charcoal absorbed material too strongly for use in the mass spectrometer. Spectrographic graphite was therefore used for the preparation of samples.

The samples were prepared by gently evaporating the dichloromethane solution onto graphite (2mg.) with heat from a hair dryer. Originally this was done in a small test tube and the tube sealed immediately, but no logical mass spectra of the pheromone could be obtained from a sample prepared in this manner and posted for analysis.

Thereafter all samples were sealed in small melting point tubes, if postage was required. The mass spectra given in Fig. 18 was run from a sample freshly prepared before analysis, containing an estimated 20 μg of material (approx. 5,000 ♀ ME).

All spectra were run on AEI MS 9 mass spectrometers with direct insertion into the electron beam (70 eV.).

5E.5 GLC Analysis with cis-Dodec-7-en-1-yl acetate

An aliquot (1 μl.) of cis-dodec-7-en-1-yl acetate was dissolved in dichloromethane (1 ml.). This solution (5 μl.) was injected onto the column containing neopentyl glycol adipate at a temperature of 170°. This was compared with injections of methyl ester standards and fraction B5 (500 ♀ ME injection). The relative retention times of the two pheromones were identical (4.2 min.). Combination of the two solutions in the syringe (2.5 μl and 250 ♀ ME respectively) showed that two pheromones were not separated by the GLC analysis (Column Temp. 171°C), as shown in Fig. 19.

This experiment was repeated on the column containing silicone gum rubber. Methyl myristate (C_{14}) was included for comparison of relative retention times. The compounds again showed identity of retention times (3.9 mins., relative to methyl myristate, .65) as shown in Fig. 20. Combination of the pheromones and subsequent analysis showed that no separation could be achieved even at a column temperature as low as 140°.

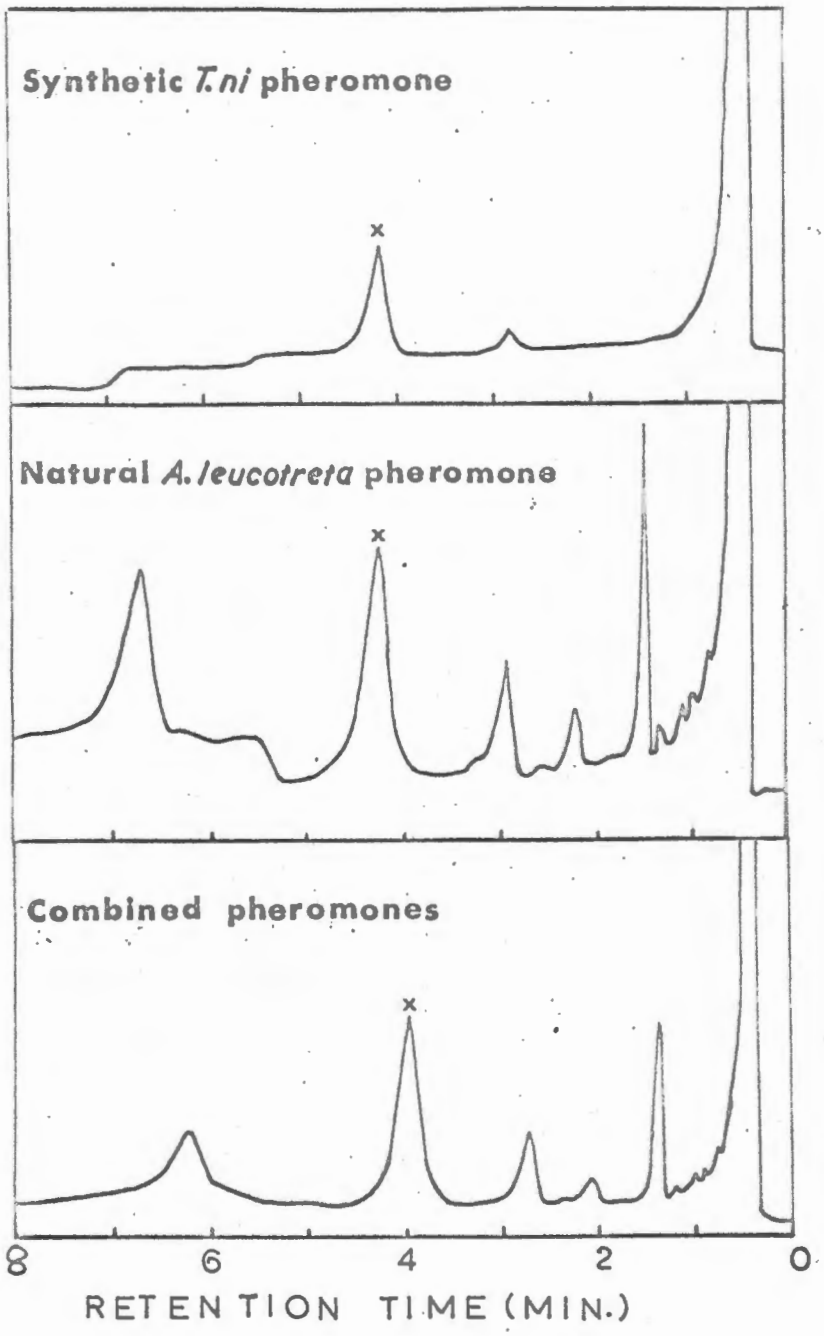


Fig. 19 Comparison by GLC analysis, on polar liquid phase, of the synthetic T. ni sex pheromone and the sex pheromone of A. leucotreta.

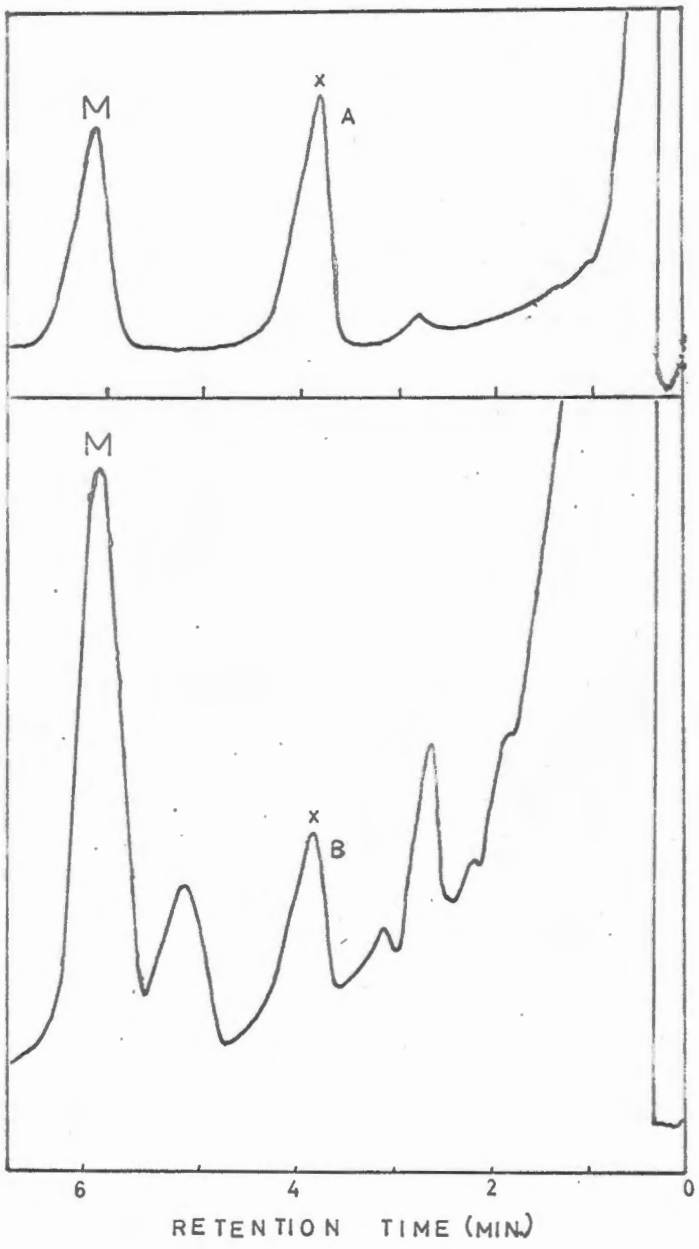


Fig. 20 Comparison by GLC analysis, on non-polar liquid phase, of the synthetic T. ni sex pheromone (A) and the A. leucotreta sex pheromone (B), relative to methyl myristate (M).

5E.6 Bioassay of Synthetic Acetates

Aliquots (1 μl each) of cis and trans-dodec-7-en-1-yl acetates and cis-tetradec-9-en-1-yl acetate were dissolved in petrol (1 ml.) and aliquots (1 μl, i.e. approx. 1 μg solute) of the solutions were tested for pheromone activity. (Table 6).

TABLE 6

Pheromone Activity of Synthetic Acetates

	Average % male moth response	
	Expt. 1	Expt. 2[x]
0.1 ml. petrol control	5	1
cis-tetradecenyl acetate	8	-
cis-dodecenyl acetate	25	6
trans-dodecenyl acetate	54	15

([x]Reversed order of testing; room temperature was high: 23.5°)

The infra-red spectra of the acetates were run in chloroform (1 μl solute/200μl solvent) in a microcell (path length 1 mm.) and the absorption band at 970 $cm.^{-1}$ (10.3μ) measured. The ratio of the strength of absorption in the cis and trans dodecenyl acetates was 5:37 (by area) respectively. The cis-tetradecenyl acetate showed no

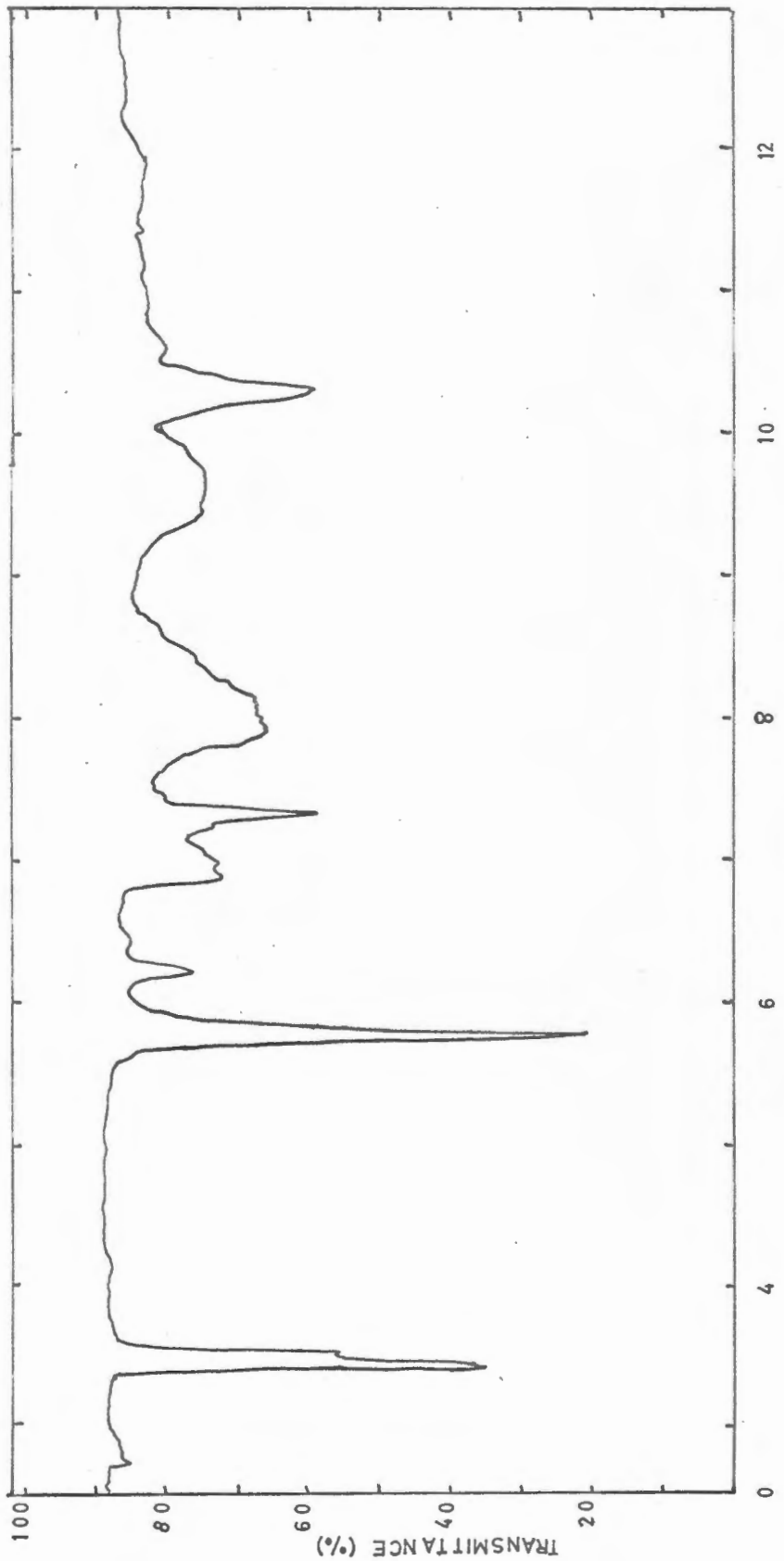


Fig. 21 The infra-red spectrum of trans-dodec-7-en-1-yl acetate.

absorption at 970 cm.$^{-1}$. The infra-red spectrumof trans-dodec-7-en-1-yl acetate is shown in Fig. 21.

5E.7 Purification of cis- and trans-Dodec-7-en-1-yl Acetates and Bioassay

Aliquots (0.1 μl for bioassay, 0.2 μl for visualizing) of cis and trans-dodec-7-en-1-yl acetate were spotted on a fresh silver nitrate (25%) impregnated silica gel thin layer plate (300 μ) (James and Morris, 1964). The plate was developed in cyclohexane/ether (90:10) for two hours in the dark. (The solvent front was allowed to overreach the end). Peroxide free ether and spectroscopically pure cyclohexane gave minimal blackening of the plate.

It was found that the best visualization could be achieved by first spraying the plate with dichlorofluorescein (1% ethanolic solution) and then exposing it to iodine vapours, instead of ultra violet light. This procedure gave yellow spots on a pink background. Examination of the visualized components showed the presence of a "trans" component in the cis-dodecenyl acetate (Fig. 22).

The unvisualized portion of the plate was divided into portions, as shown in Fig. 22 , the absorbent scraped into test tubes and each portion washed successively with ether (2 ml.) and benzene (2 ml.). Aliquots (1 ml.) of each solution were tested for activity but no activity could be shown in any fraction.

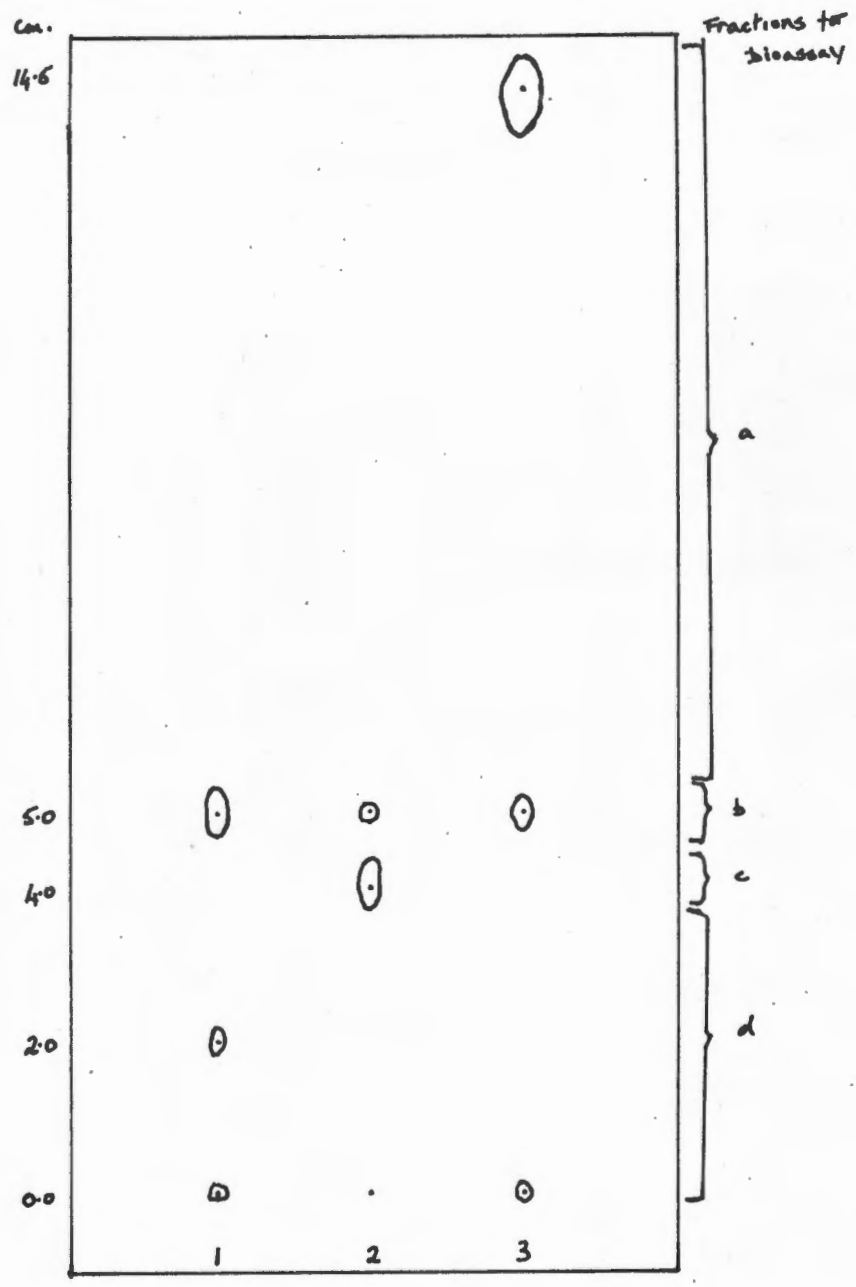


Fig. 22 Thin layer chromatography on silica gel impregnated with silver nitrate (25%), of trans-dodec-7-en-1-yl acetate(1), cis-dodec-7-en-1-yl acetate(2) and a semi-pure fraction of natural pheromone extract (3).

The separation was repeated as before, and only the two portions containing the major components were scraped off. Both portions were treated with water (2 ml.), vigorously boiled and the distillate collected into petrol (2 ml.). Aliquots (1 ml.) of the petrol solutions of pure cis and trans dodec-7-en-1-yl acetate were tested for activity. (Table 7).

TABLE 7

The Pheromone Activity of Pure cis and trans Dodec-7-en-1-yl Acetates

	Average % male moth response	
	Expt. 1	Expt. 2
1 ml. petrol control	6.25	8.0
cis-dodecenyl acetate	7.5	7.0
trans-dodecenyl acetate	41.0	62.5

5E.8 Recovery of Pheromone Activity from Extract MFS by Silicic Acid Chromatography

Crude extract MFS (10 mg. 800 ♀ ME) a yellow oil was fractionated on a silicic acid column (4g., 14 cm. x 6 mm.), slurried in petrol and eluted with increasing strength of ether (2.5%, 5%, 10%, 20%, 30%, 40%, 50%, 100%, 10 ml. each) and fractions (10 ml.) collected.

Since lauryl acetate elutes fairly rapidly with 2 to 5% ether it was felt that similar elution behaviour could be expected for the pheromone if it was recovered from the column. The fractions were tested for activity, only the last fraction (100% ether) however, elicited any response from the male moths (86.6%). Elution with more ether, or methanol, did not give further active fractions. Analysis of the active fraction (8 ME) by GLC showed the presence of the pheromone peak. The bioassay and GLC analysis were found to correlate well with a similar active fraction (800 ♀ ME) from an alumina column.

5E.9 Purification of Extracts on Silicic Acid Impregnated with Silver Nitrate

The active fractions (1,600 ME) from the column chromatography, dedcribed in section 5E.8 above, were hydrolysed by refluxing in ethanolic KOH (10 ml.) and the neutral fraction acetylated with acetyl chloride (3 ml.) as described in section 5E.1.3 and 5E.1.4 above. GLC analysis before and after treatment showed the lack of the major peaks as appeared in Fig. 14 & 15. This would indicate their fatty acid ester nature, confirming an infra red spectra of impure fraction collected by preparative GLC.

The cyclohexane solution was concentrated (1 ml.) and an aliquot (2 µl) was separated by thin layer chromatography silver nitrate/silica gel as described in section 5E .7 above. Two spots were present on

visualization a major spot of high R_f and a small one with a R_f equivalent to trans-dodec-7-en-1-yl acetate. It is assumed that the major spot consisted of saturated compounds.

This good separation encouraged the author to attempt the purification of the pheromone on a silver nitrate impregnated silicic acid column, with a lower percentage (5%) of silver nitrate. The silicic acid was impregnated by slurrying in a methanolic silver nitrate solution and the material reacted at 105° in the dark after evaporation of the methanol.

A test column (5g, 14 cm.x 1 cm.) was prepared in cyclohexane and eluted (10 ml. fractions) with ether (10%, 20%, 30%, 50% and 100%) in cyclohexane (50 ml. each) and trans-dodec-7-en-1-yl acetate was found in the second fraction, shown by bioassay and infra red spectra. The column was developed in the dark, with an aluminium for wrapping.

A similar column was prepared for the separation of the acetylated natural product (1,600 ♀ ME) and eluted with ether (1%, 3%, 6%, 12%, 25%, 50%) in cyclohexane (50 ml. each) and fractions collected (10 ml.). The activity eluted with 3% ether, fractions 7,8 and 9. These were combined and analysed by GLC on the SE-30 column. The pheromone peak was present in highest concentration, but other compounds of retention times approximately equivalent to C_{11}, C_{17} and C_{19} methyl esters were also present.

5E.10 Lemieux Oxidation of Oleyl Acetate

The following procedure described is a combination of the methods

described by Jacobson et al. (1961) and Kuemmel (1964), to give a homogeneous oxidation solution.

Oleyl acetate (4 µl) in tert-butanol (6ml.) was added to oxidation stock solution (3 ml., 0.0195 M potassium periodate and 0.001M potassium permanganate) diluted with water (3 ml.). The solution was brought to pH 9 with potassium carbonate and left overnight. The solution was acidified with 2N sulphuric acid and sodium metabisulphite added until the solution went completely colourless.

The solution was brought to pH 9 by addition of 10% sodium hydroxide solution, the tert-butanol removed under vacuum, and the solution reactified.

The solution of the acid products was extracted twice with ether, concentrated (1 ml.), and added to an ethereal solution of diazomethane (3 ml.) prepared from N-nitroso-methylurea (0.1g.) (Arndt, 1943). After half an hour the solution was concentrated and analysed by GLC on the SE-30 column by comparison with methyl nonoate and dimethyl nonandioate standards over a temperature programme of 110° - 215° at 10° per min. The oxidation was shown to have proceeded with 95% yield and gave two major peaks, one equivalent to methyl nonoate in retention time and the other eluting just after the dimethyl ester. These products would be expected from the oxidation at a $C_{(9)}$-$C_{(10)}$ double bond to two acid compounds which were then methylated.

5E.11 Lemieux Oxidation of the A.leucotreta Pheromone

One half combined active fractions (800 ♀ ME) mentioned in section 5E.9 was injected into the GLC, with the SE-30 column, and the pheromone peak collected (section 5E.3). A small area of mist was visible in the collection tube, the first occasion that the pheromone was present in sufficient quantities to be seen by the author. The tube was immediately washed with dichloromethane into test tube with a ground glass stopper (B 10) and the dichloromethane solution concentrated (10μl).

This solution and trans-dodec-7-en-1-yl acetate (0.2μl) were oxidised as described in section 5E.10 above, scaled down to give a total oxidation solution volume of 200μl. Unfortunately difficulty was found in obtaining a homogeneous solution, and so the solutions were shaken for twenty-four hours, and the products worked up as previously described. The final volumes of methylated products was 20μl each.

The optimum temperature programme was found to be 25° for 2 mins., followed by a linear gradient of 10° per min. to 215°. The injection port temperature set at 200°. With this programme methyl penanoate eluted with a retention time of 1.2 min. and trans-dodec-7-en-1-yl acetate at 15.3 min.(163°).

Injections of (10μl, each) of solution showed that most of the pheromone, natural and synthetic, was not oxidised.

Two small peaks, oxidation products from both natural and synthetic pheromones, were found to occur at retention times of 1.2 min. and 5.5 min.

This result lead the author to conclude that the double bond of the natural A.leucotreta sex pheromone was in the $C_{(7)}$-$C_{(8)}$ position and that the pheromone was trans-dodec-7-en-1-yl acetate.

The author feels that it is of interest to note that the latter purification procedure followed (sections 5E.8 and 5E.9) indicated that about one third of pheromone material was lost in obtaining the pure pheromone for mass spectrometry from the earlier purifications. The reasons for this are not immediately apparent as all extracts were sealed and kept cold in the fridge.

Chapter 6

THE SYNTHESES OF INSECT SEX PHEROMONES

6.1 The Nature of the Syntheses

The key step in the synthesis of the insect sex pheromone, found in the order Lepidoptera, is the introduction of unsaturation into an alkyl chain which has one or two alcohol groups which may be esterified. This has been accomplished often enough with unsaturated acids but in most cases these rely on elimination reactions and give mixtures of *cis* or *trans* isomers (Gowan and Wheeler, 1960). This is a wasteful process if only one is active, separation is difficult, and further more makes it useless for critical tests concerning structure and activity.

One method that has proved useful, in respect of the above criteria, is the synthesis of acetylenic compounds, which may then be selectively reduced to the *cis* or *trans* form of the olefinic compound. Since Ahmad and Strong (1948) introduced this method for the syntheses of unsaturated acids, other methods have now been developed. These are a stereospecific Wittig reaction and the cleavage of 2-alkyl-3-chlorotetrahydropyrans. Further points of synthesis are raised when more than one double bond, substituents such as secondary hydroxyls, or branched chains are required. These methods are reviewed below with special consideration towards the particular sex pheromones that have been isolated. These syntheses are summarised in Appendix 3.

6.2 Synthesis of Substituted Acetylenes

6.2.1 Synthesis yielding cis olefinic bond

This method was used by Ahmad and Strong who produced acetylenic chlorides from a reaction of monoalkyl acetylines with ω-chloro-α-iodoalkanes in the presence of sodamide in liquid ammonia. These, via the nitrile or Grignard reagent, gave the acetylenic acid which can be selectively reduced at the triple bond to the cis form of the unsaturated fatty acid. This reduction was done in the presence of "W6" Raney Nickel.

Berger (1966) used this method to synthesise cis-dodec-7-en-1-yl acetate, the T. ni sex pheromone. The only difference in his synthesis was the selective reduction to the cis-double bond using Lindlar catalyst (5%Pd-$CaCO_3$ with quinoline) instead of "W6" Raney Nickel. The acid was then reduced with lithium aluminium hydride and the alcohol acetylated with acetyl chloride. The yield of the 1-chloroalkylacetylene was fairly low (44%) and so was the total yield (28%).

Jacobson's group (Green et al., 1967) synthesised this pheromone with a slightly different approach. 2- (6-chlorohexyl)oxy tetrahydropyran was reacted with lithium acetylide to give the required 2-(7-Octynyloxy) tetrahydropyran in good yield (88%). Reaction with sodamide and n-butyl bromide in liquid ammonia gave the O-protected alkyne chain of correct length but only in moderate yields (57%).

This group have used this method in the syntheses of two other sex pheromones. When they synthesised the first insect sex pheromone, (dl)-10-acetoxy-cis-hexadec-7-en-1-ol, the gypsy moth (P. dispar) pheromone, they followed the basic method of Ahmad and Strong (1948) (Jacobson et al., 1961). In the synthesis of the sex pheromone from female pink bollworm moths (P. gossypiella) (Jones et al., 1966), 10-propyl-trans 5,9-tridecadien-1-yl acetate, they used 4-propyl-hex-3-en-1-ol to prepare a bromide (unstable) which they immediately reacted with the tetrahydropyranyl ether of hex-5-yn-1-ol using sodamide in liquid ammonia. This gave 10-propyl-(tetrahydro-2-pyranyloxy)-tridec-9-en-5-yne in low yield (23%).

Later Warthen and Jacobson (1967) synthesised congeners of this sex pheromone lacking the propyl group. The alkylnylating reaction was not done in liquid ammonia but in dioxane using lithium amide. The yields were no improvement on the liquid ammonia method (43%) (Fig. 23).

6.2.2 Synthesis yielding the trans-olefinic bond

In cases where the trans-olefinic bond is required it is possible at the next stage in the synthesis to reduce the acetylene bond to the trans-configuration with sodium in liquid ammonia (Henne and Greenlee, 1943). This reduction has been used by Jacobson's group in the synthesis of the pink bollworm moth pheromone, 10-propyl-trans-trideca-5,9-dien-1-yl acetate (Jones et al., 1966),

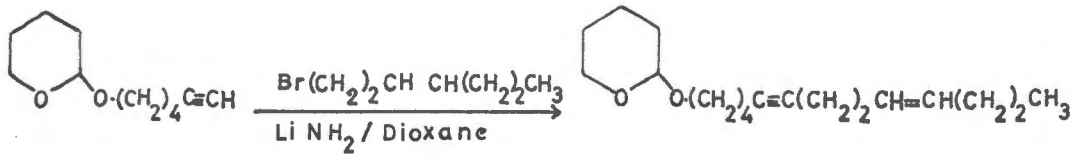


Fig. 23 A step in the synthesis of the isomers of tridec-5,9-dien-1-yl acetates, congeners of the pink bollworm moth female sex attractant. (Warthen and Jacobson 1967).

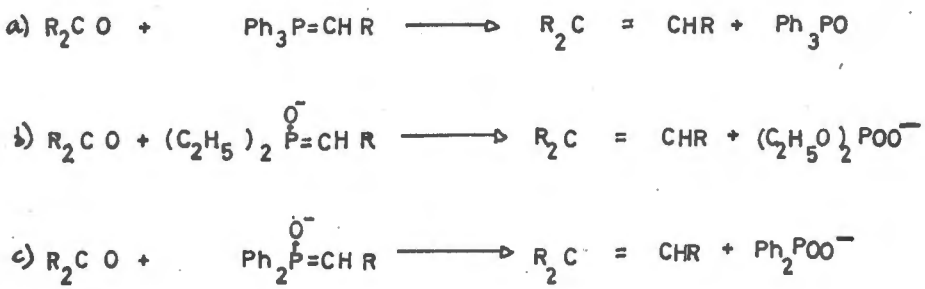


Fig. 24 Wittig condensation reactions undergone by carbonyl compounds and a) alkylidenetriphenylphosphoranes b) dialkyl phosphonate or c) diphenylphosphine oxide ions.

and its congeners, the 5, 9 tridecadien-1-yl acetates (Warthen and Jacobson, 1967).

6.3 Wittig Condensation (Carbonyl Olefination)

Until recently this method has not found much favour in the synthesis of stereospecific olefins, for it would give a mixture of cis and trans isomers, the trans form usually predominating. Bergelson and Shemyakin (1964) investigated the reactions between carbonyl compounds and alkylidenetriphenylphosphoranes, or dialkylphosphonate and diphenylphosphine oxide ions, to give olefins (Fig. 24).

These reactions, which once proved of little value in the synthesis of naturally occuring cis unsaturated fatty acids can now be utilised for this purpose by the choice of suitable reaction conditions and structural factors. These have been reviewed recently by Bergelson and Shemyakin (1964) and House (1965). The principles that Bergelson and Shemyakin give may be summarised briefly into four points and concern the proposed reaction mechanism given in Fig. 25.

6.3.1 Principles for stereospecifity in carbonyl olefination

6.3.1.1 The presence of a sufficiently neucleophilic Lewis base (e.g. I^-) promotes cis-olefin formation, in the reaction between aldehydes and alkylidenetriphenylphosphoranes.

In the absence of Lewis bases and in non-polar media, dipole-

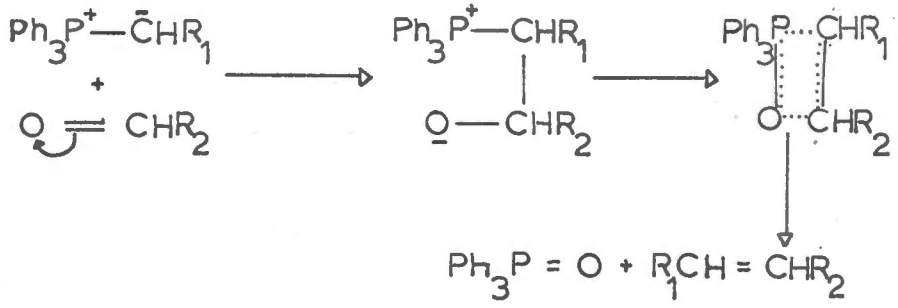


Fig. 25 Proposed mechanism of the Wittig condensation reaction.

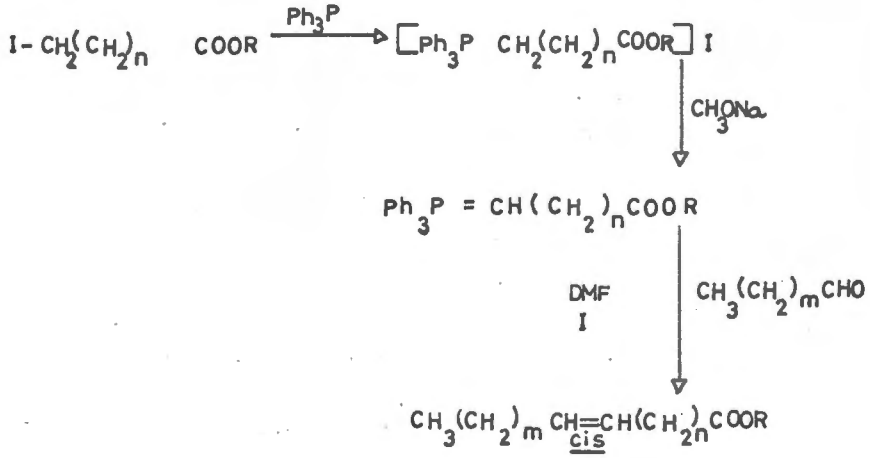


Fig. 26 Reaction sequence for synthesis of cis unsaturated fatty acids.

dipole interaction and mutual repulsion of the substituents (R) lead predominantly to trans-olefins. However the halogenide ion (I^-) for example interacts with the phosphorous atom tending to expand its electronic octet to a decet. This makes the phosphorous less electrophilic and sterically less accessible in the complex. This tends to cut down the dipole-dipole interaction between the phosphorane and aldehyde, causing the formation of an erythro intermediate and the cis-olefin.

6.3.1.2 Polar aprotic solvents (e.g. dimethylformamide (DMF)), increase the relative yield of the cis-isomer when aldehydes are treated with alkylidenetriphenylphospheranes. This effect is probably due to the solvation of the betaine intermediate which of then is in the preferential erythro form.

6.3.1.3 A carbonyl group on the α-carbon of the alkylidene triphenylphosphoranes tends to hinder the formation of the erythro compound due to the mutual repulsion of the oxygen from the aldehyde oxygen. This results in the predominant formation of the trans-olefins. This is true when α, β-unsaturated esters are synthesised by carbonyl olefination.

6.3.1.4 Alkylidenephosphonates with sufficiently low positive charge

on the phosphorous atom, give predominantly trans-olefins with aldehydes, independent of the reaction conditions. It is found that dialkylidenephosphonate and diphenylphosphine oxide anions, for example, cannot effectively form complexes with Lewis bases and react to give predominantly the trans olefin.

These principles have been applied to the synthesis of numerous unsaturated fatty acids by Bergelson and Shemyakin (1964) and an example is given in Fig. 26. The active groups can be carried in the reverse manner, and in fact it is sometimes necessary as the ω-alkoxycarbonylalkylidenephosphorane (especially when n = 3) can ring close to give the stable 2-oxycyclopentylidenetriphenyl phosphorane for example (Bergelson and Shemyakin, 1964; House and Babad, 1963).

6.4 Specific synthesis of trans-alk-4-en-1-ols

In the synthesis of trans-dodec-7-en-1-yl acetate, the stereoisomer of the T. ni pheromone (Green et al., 1967), a method was used based on a finding of Crombie and Harper (1950). They found that ring scission of 2-alkyl-3-chlorotetrahydropyrans produced only the trans form of alk-4-en-1-ol. The reaction is carried out with granulated sodium in ether. In this case trans-non-4-en-1-ol was prepared from 2-butyl-3-chlorotetrahydropyran in excellent yield (94%). The chain was extended by the use of diethyl malonate followed by a Grignard reaction with carbon dioxide.

6.5 Syntheses of Isomers of Hexadeca-10,12-dien-1-ol

Two major papers on these syntheses have been published (Butenandt et al., 1962; Truscheit and Eiter, 1962), and in both cases two or three syntheses per isomer were given.

When a specific geometrical isomer was not available, as for example trans-hex-2-en-1-al (Truscheit and Eiter, 1962), an acetylenic compound was used in the synthesis and reduced to the required isomer. In this way at least one double bond could be specifically synthesised in quantitative yields. The other double bond was usually synthesised by a Wittig condensation or by way of the Reformatsky synthesis of secondary hydroxy-esters. The Wittig condensation was usually non-stereospecific, and the elimination reactions on the Reformatsky products also gave a mixture of isomers of the conjugated product. Truscheit and Eiter were able to improve the yield of trans isomer following the Reformasky reaction, by the use of 1,5-diazabicyclo [4.3.0] non-5-ene in the dihydrohalogenation of 10-chloro-tridec-12-ynoate methyl ester to give trans-tridec-10-en-12-ynoate methyl ester (70%).

Both groups made use of the urea inclusion compound formed with the linear trans isomers, such as compound just mentioned, to accomplish separation and purification. The isomers could also be separated by distillation through a spinning band column.

APPENDIX

Appendix 1

INSECT OLFACTION AND ATTRACTANT PERCEPTION

A1.1 Site and Structure of the Olfactory Receptors

Little is known about what provides the stimulus for pheromone release. The only case investigated successfully shows that a plant odour is responsible. The next event in the communication system is the sensory perception of the attractant, the inlet mechanism for the release of a certain behaviour pattern.

The location of the olfactory organs has been shown to be on the antennae. In 1880 Hauser reviewed the subject and reported that males of Saturnia pavonia and P. dispar never mated when deprived of their antennae. Experiments by Mayer (1900), Fabre (1904), Kellog (1907), showed that chemoreception of the odour by the two antennae enabled the male moth to find the "calling" virgin female. Sight was not involved and other stray odours such as carbon disulphide and napthalene did not deter the male moths. Excising one antennae reduced the direction finding ability of the male. Kellog observed that B. mori males deprived of their right antennae circled to the left, and vice versa while seeking the female within a radius of a few inches.

Since then, antennae have been implicated many times as the bearers of the olfactory sense organs for most insects. Other minor areas that have been described are the maxillary and labial palpi, legs, and ovipositors (Detheir, 1963). The role of the antennae

is often well emphasised by the differences found between the antennae of male and female of the same species. Female antennae are usually small and thread-like, whereas the males' are well - developed, plumous structures. This increased surface area enhances the male olfactory sensitivity.

The olfactory receptors are in supporting structures of cuticular specialisation, the sensillae. These take various forms, of which the following are the most common; pegs (sensillae basiconica), hairs (sensillae trichodea), plates (sensillae placoidea) and pits (sensillae coeloconica). Depending on the insect, all of these have been demonstrated to contain olfactory receptors (Dethier, 1963). In the Lepidoptera, the pegs or hairs usually contain the sex attractant receptors (Schneider, 1964).

The invertebrate receptor cells in the sensillae differ basically from the vertebrate receptor cells, in that they are true "primary" sense cells (Wigglesworth, 1953). They are bipolar neurons with axons that terminate in the central nervous system (CNS) without an intermediate synapse. In contrast, the specialised receptor cells in the vertebrate synapse with the primary sensory neurons which in turn synapse with further neurons before the CNS is reached. This is a probable reason why the sex attractants act instantly as a behavioural release mechanism with few possibilities of inhibition. The insect receptor neurons are small fibres, relative to other insect receptors, of about 1μ in diameter, in fixed tissues. The dendrites of these neurons extend up into the pegs and hairs (Hodgson, 1964).

The sensillae basiconica of the grasshopper have been investigated by electron microscopy (Slifer, 1961; Slifer and Sekhon, 1964) and the following points noted. The dendrites of the olfactory receptors are complex but unlike the axons have no sheath. Initially, they branch into three or four, each terminating at holes in the cuticle. At the termination are found twenty-four microvilli structures which arise from the sides of the dendrite. Each microvillus is about 0.02 μ in diameter, and is considered to be an adaption to increase the surface area of the responsive surface membrane.

The ultrastructure of the receptor membrane is to a large extent unknown. However, the unit membrane has so far been investigated with x-ray diffraction, optical bifringence and electron microscopy using many tissue types, but especially nerve tissue. All these studies indicate a similar structure throughout (Fernandez-Moran, 1959; Robertson, 1960; Schmitt, 1959; Sjostrand, 1959; Vanderheuvel, 1963), and do not contradict the basic structure put forward by Finean (1958). This was a bimolecular layer arrangement of lipids, with the non-polar chains held together with strong van der Waal's interaction, orientated radially. Then concentric layers of protein, interacting with the polar ends of the lipid molecules on both sides of the bimolecular lipid layer. The lipids are phosphatides, cholestrol and cerebrosides. The protein structure is not well understood, though a so-called "neurokeratin" reticulate

structure has been identified, for it seems that the molecular configuration of this protein is similar to the keratins (Robertson, 1960).

A1.2 Electrical Events in Nervous Excitation

Nerve cells have been found to differ considerably in the way that electrical events are manifested. There are differences between events occuring in the dendrites and the axon of the same neuron, and between neurons of different anatomical position, and function. These various types of membrane potentials and their interrelationships are compared in Fig. 27 (Bullock, 1959).

The (Chemo) receptor potentials, like the initial synaptic potentials, are graded responses to external stimuli. These potentials occur in limited areas of the neuron. They are sub-threshold events and couple to give a graded local potential. When this reaches a threshold level, there follows an intergrated result of "all-or-none spike impulses" at a particular frequency.

On the "analog" principle it will be realised that the primitive graded response is more appropriate for the quantitative reception of fine stimuli. However, its amplitude decreases rapidly with distance. The all-or-none impulse, on the other hand, conducts over a considerable distance without decrement.

Therefore the chemoreceptor neuron is a transducing mechanism for converting intensity of chemical stimuli into frequency of

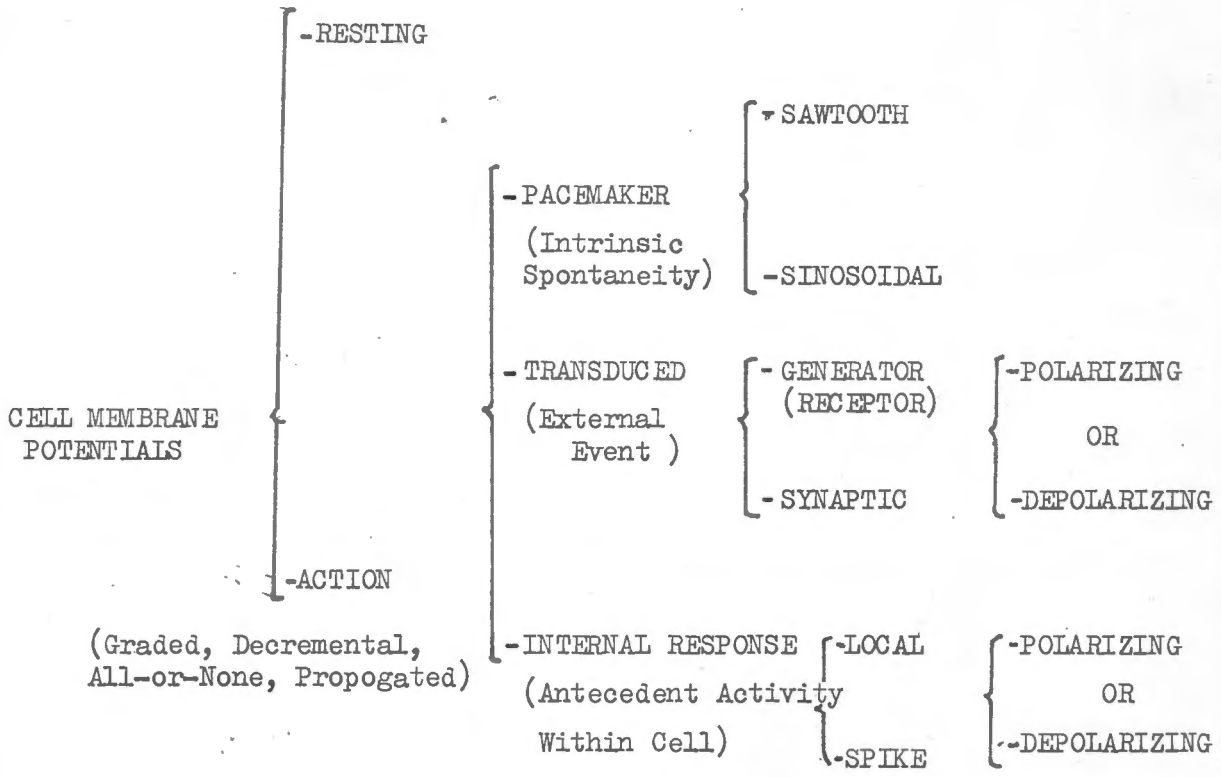


Fig. 27 The Systemization of Cell Membrane Potentials (Bullock, 1959).

nervous impulses, and a comparison can then be made between the action of acetylcholine crossing a synaptic gap of 200 Å to excite the synaptic membrane, and a sex attractant crossing a gap of 1-1000 metres to excite the males olfactory receptor membrane.

The biophysics of the neuron membrane potentials are not particularly well known. The theory based on differences in ionic concentrations across the membrane has received considerable support, but in this, as in every other, ignorance of the membrane structure and function prevents any conclusive proof. The neuron membrane, it seems, is able to maintain a difference in potential between the interior and exterior of the cell. The interior is about -70 mV with respect to the exterior. This is done by maintaining differences in ionic concenyration against electrochemical gradients (Eccles, 1965). The two ions of particular importance are the sodium and potassium ions. The role of the other metal ions, such as calcium is uncertain. The potassium ion concentration is about thirty times higher inside, and the sodium ion concentration ten times higher outside. The chloride ion concentrations are in equilibrium (Eccles, 1965).

At a resting potential there is an exchange of sodium ions and potassium ions, but the permeability is small (Keynes, 1951). The condition is maintained by the "sodium pump" in the membrane (Hodgkin and Keynes, 1955; Hokin and Hokin, 1963). The propagation of the impulse coincides with abrupt changes in the permeability of the membrane

and the flux of sodium ions and potassium ions increases greatly (Keynes, 1951). Using sodium-24 and potassium-42 and a "voltage clamp" (Marmont, 1949), Hodgkin and Keynes (1953) were able to show that the two phases of the impulse were due to an initial Na^+ influx followed by a K^+ efflux. The resulting change in ions is negligible when considering the overall difference in ionic composition in the cell, and the neuron can transmit thousands of consecutive impulses without ill effect. Immediately following the impulse however, there is a momentary refactory period when the membrane is inexcitable, this is a measure of the time for the normal permeability relations to return to normal (Katz, 1959). So far, most descriptions of the "all-or-none-spike impulse" seem to indicate that the membrane permeability is the crucial factor in the neuron potentials. This has often been argued against by theories that propose a binding hypothosis (Ungar, 1963), where the potential difference is seen to be due to preferential absorption by the membrane, or the cell as a whole.

These explanations generally concern the spike impulse, the initial changes in receptor potential have not been investigated as thoroughly. However, in determining the effect of the stimulus on the receptor dendrite, the graded receptor potentials are the best manifestations of receptor activity to measure. This has been done in a limited number of cases with insect chemoreceptors.

Morita and Yamashita (1959) have recorded receptor activity of the labellar hair of Lucilia caesar by cracking the side wall of the

hair between two microelectrodes, one of which was filled with a non-stimulating electrolyte. This forms an electrical contact with the inside of the sensory hair. On chemical stimulation, they recorded a sustained negative potential, which they concluded to be the receptor potential.

Schneider (1963) investigating the sex pheromone olfaction of the silkworm moth, B. mori, has shown that by placing a recording electrode in the sensory epithelium, a slow olfactory potential results on olfactory contact by the pheromone. This potential he called the electroantennogram (EAG). He has shown it to be the sum of many olfactory receptor potentials recorded more or less simultaneously, for he recorded an identical sustained negative potential in working with a single olfactory receptor. The recording is extracellular and the receptor site is temporarily negative in relation to the reference elctrode to the axon.

The graded receptor potentials are subject to membrane process known as defacilitation, and facilitation (Bullock, 1959). When the EAG of B. mori is recorded using successive puffs of sex pheromone the recorded potential shows a decreased amplitude with each successive stimulation. This is termed defacilitation and explains the behavioural pattern of loss of male response to continued pheromone exposure. Defacilitation indicates a possible way to use the pheromone in pest control.

A1.3 Theories of Olfaction

In the attempts to correlate molecular parameters and properties with olfaction, and gustation, many correlation experiments have been carried out. The many different theories usually have a physical property as a basis, for example solubility, vibrational and steric properties have been investigated. All have some experimental verification. Lipid solubility has been considered by Davies(1962) in his "dislocation theory". Davies also recognized that the dislocating power of an odourant would depend on its shape and size. Amoore (1964) has brought forward a steric theory of odour for human olfaction. He describes seven primary odours; ethereal, camphoraceous, musky, floral, minty, pungent and putrid all of which have corresponding receptor sites of specific shape, and possibly an electrophilic link. Amoore made certain predictions and was able to verify his theory.

Wright (1963, 1964) recognises the importance of primaries in olfaction (for comparison, the 'bits' required in information theory would be similar). However he correlates these primaries with the ability of the odourant molecules to undergo specific low frequency vibrations and rotations. These would be picked up in the far infra-red and Raman spectra of the molecules. He has discussed this theory in relation to insect olfaction (1963) and has recently shown correlation between electrophysiological measurements and far infra-red vibrations with the human and rabbit (1967).

The difference of Wright's theory from the steric theory is uncertain; these low frequency vibrations are in fact determined mainly by the conformation of the molecule. The properties of the molecule as a whole are reflected in the region below 600 cm^{-1} (Wright, 1964).

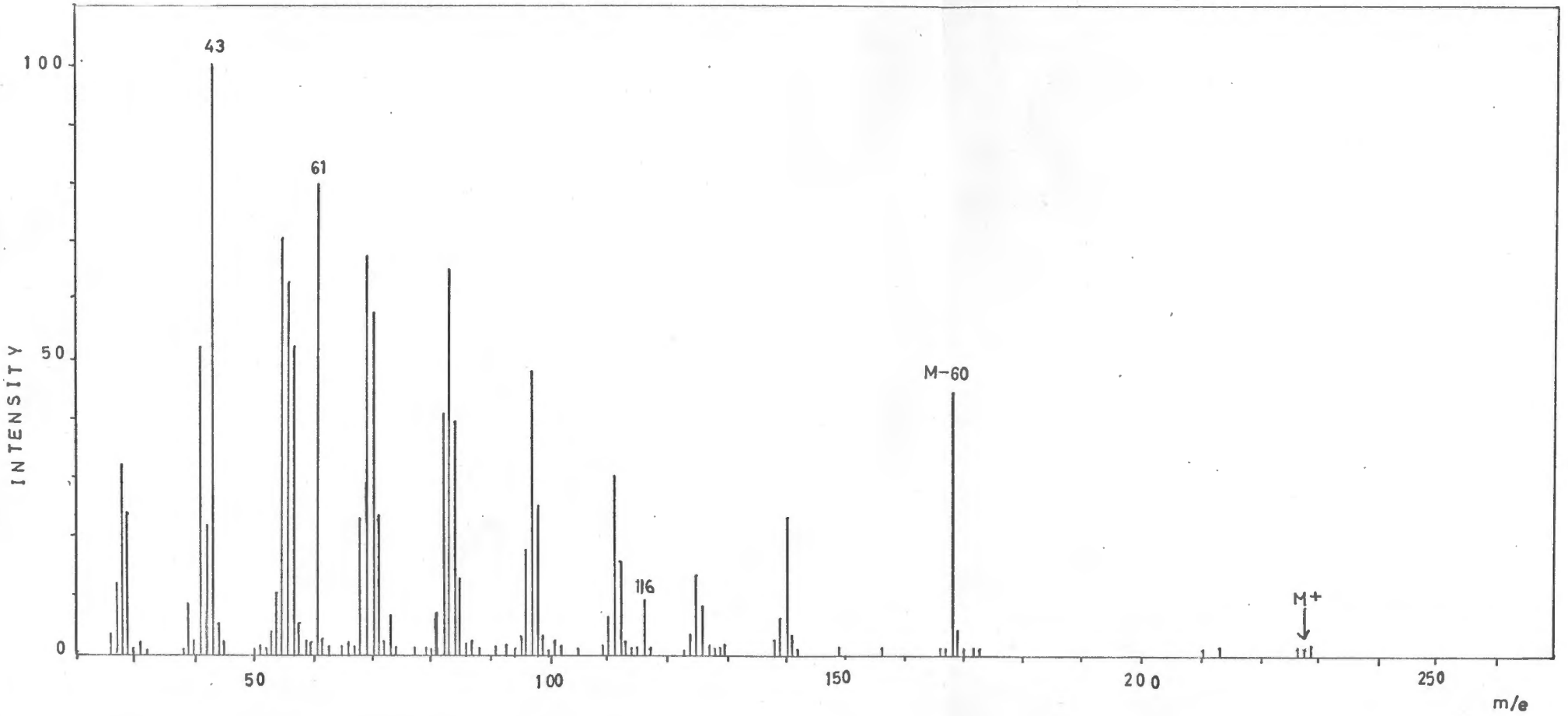
It is apparent however that the steric requirements are fairly rigid for pheromone activity. Only the 10-*trans*, 12-*cis* form of bombycol is highly active. The reverse isomer is only slightly active (Hecker, 1960) and the other two geometrical isomers are inactive. Apart from this, conditions for activity are a straight chain primary alcohol containing 14 - 18 carbon atoms with at least three carbons on either side of the conjugated system (Butenandt *et al.* 1962).

The sex pheromone activity for the male gypsy moth has been shown to require the *cis* configuration and 5 (natural) or 7 (synthetic) carbon atoms between the primary hydroxyl and double bond. The synthetic pheromone is called gyplure. The d-9-acetoxy-*cis*-octadec-12-en-1-ol isomer of gyplure was found to be inactive, Jacobson (1964) concludes that the acetoxy group must be in a position β-to a *cis* double bond for male response.

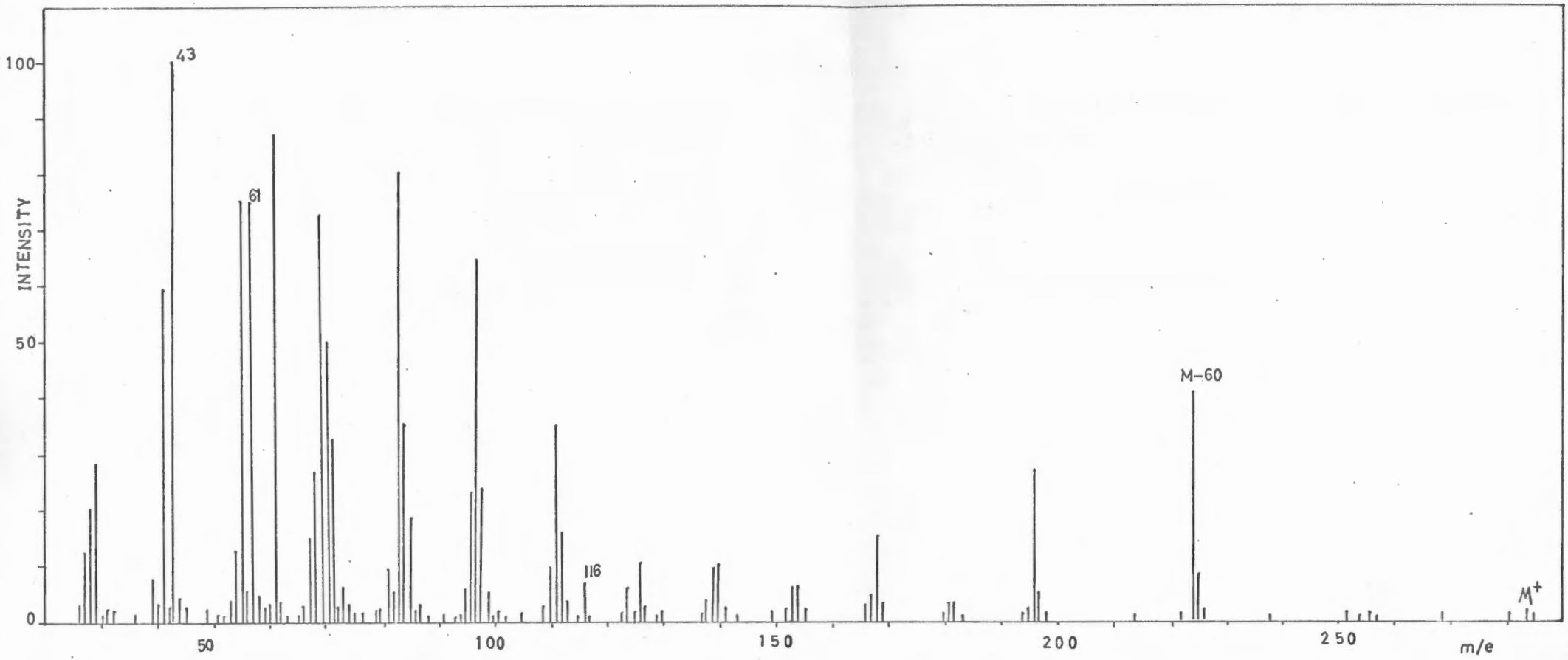
The pink bollworm moth pheromone is found to require the propyl branch chain for sex pheromone activity (Warthen and Jacobson, 1967). The two noctuid moth males require the *cis*-alkenyl acetates for activity (Green *et al.*, 1967, Sekul and Sparks, 1967).

Appendix 2

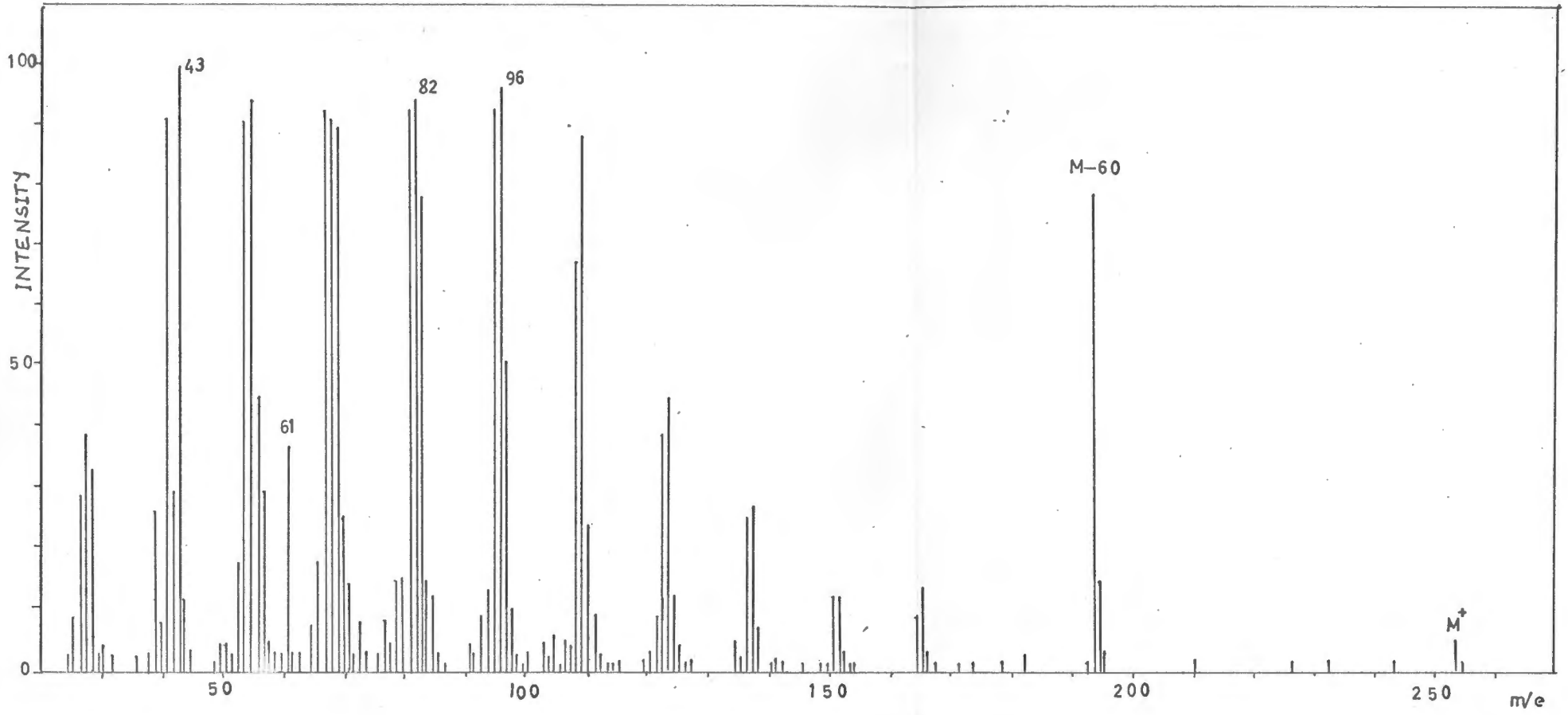
MASS SPECTRA



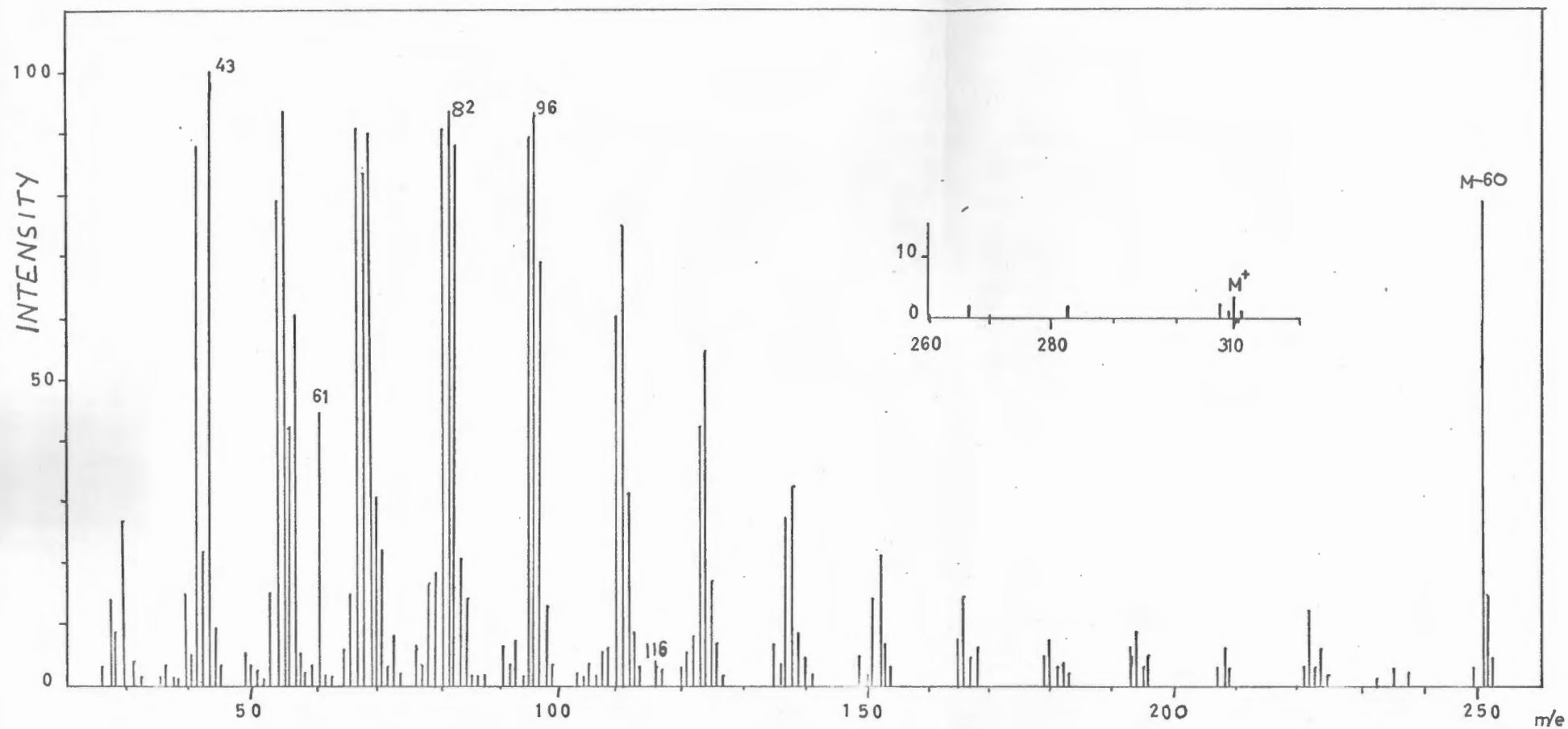
Mass spectrum of dodecanyl acetate.



Mass spectrum of hexadecanyl acetate.



Mass spectrum of cis-tetradec-9-en-1-yl acetate.



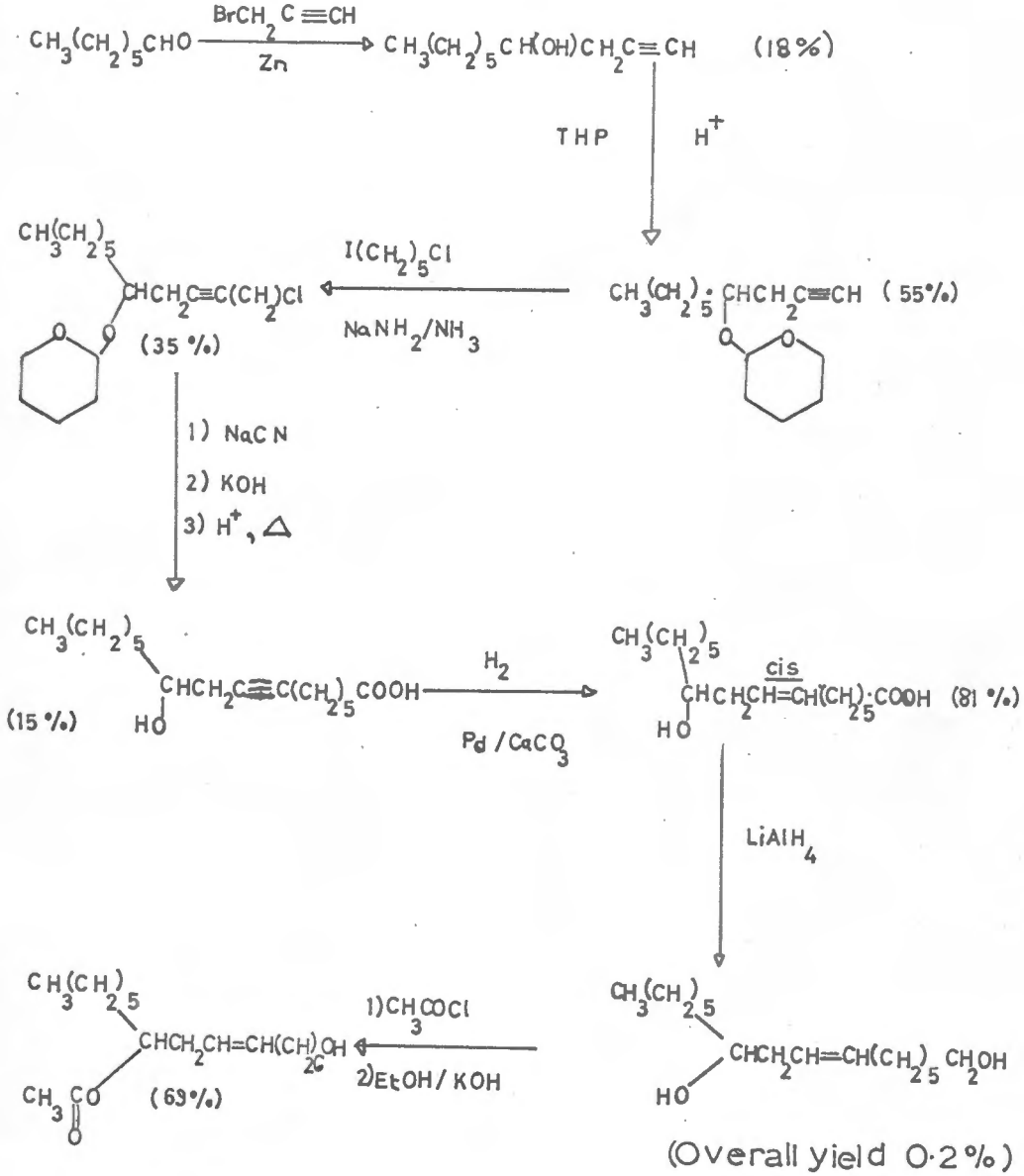
Mass spectrum of cis-octadec-9-en-1-yl acetate

Appendix 3

SYNTHESES OF THE SEX PHEROMONES IN THE ORDER LEPIDOPTERA

SYNTHESIS OF *P.dispar* PHEROMONE:

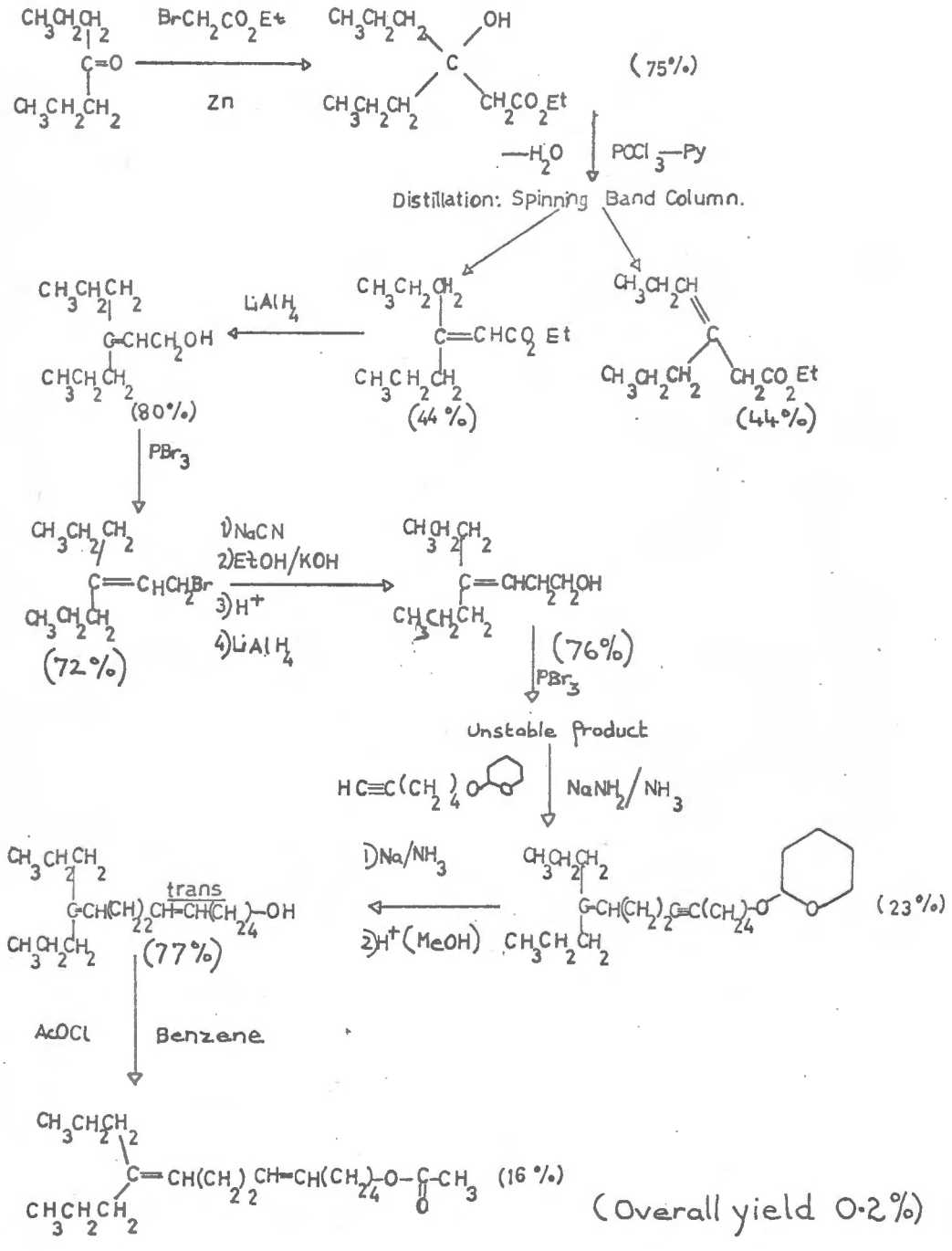
dl-10-Acetoxy-*cis*-hexadec-7-en-1-ol



Ref. Jacobson *et al.* (1961)

SYNTHESIS OF *P. gossypiella* PHEROMONE

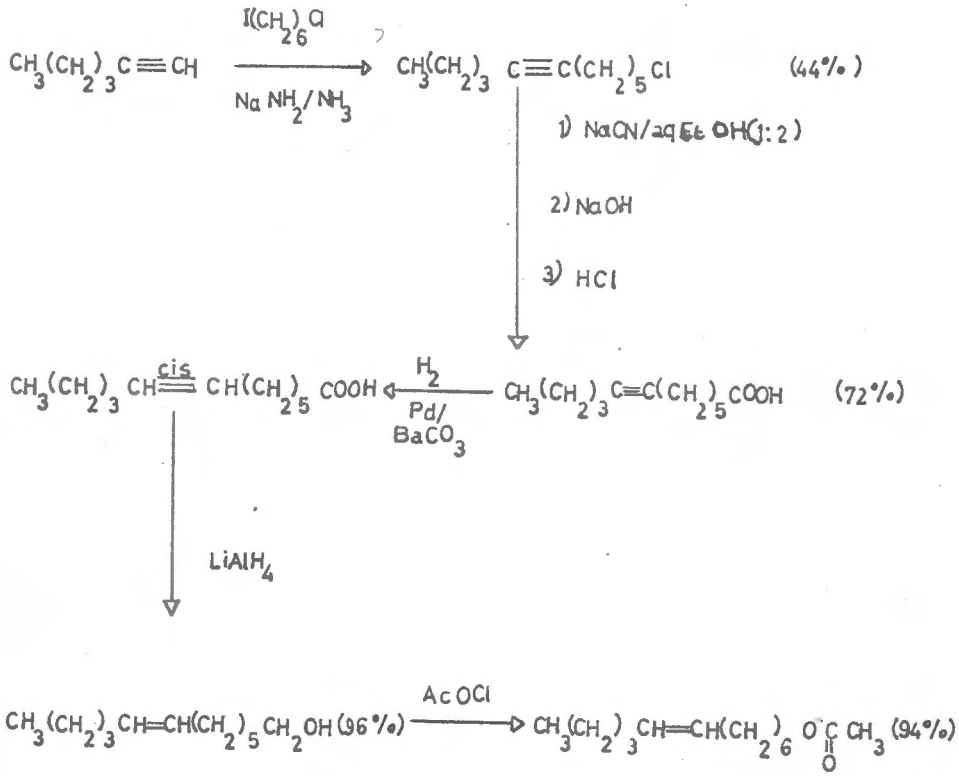
10-Propyl-*trans*-trideca-5,9-dien-1-yl acetate



Ref. Jones *et al.* (1966).

SYNTHESIS OF *T.ni* PHEROMONE I:

cis-Dodecen-7-en-1-yl acetate.

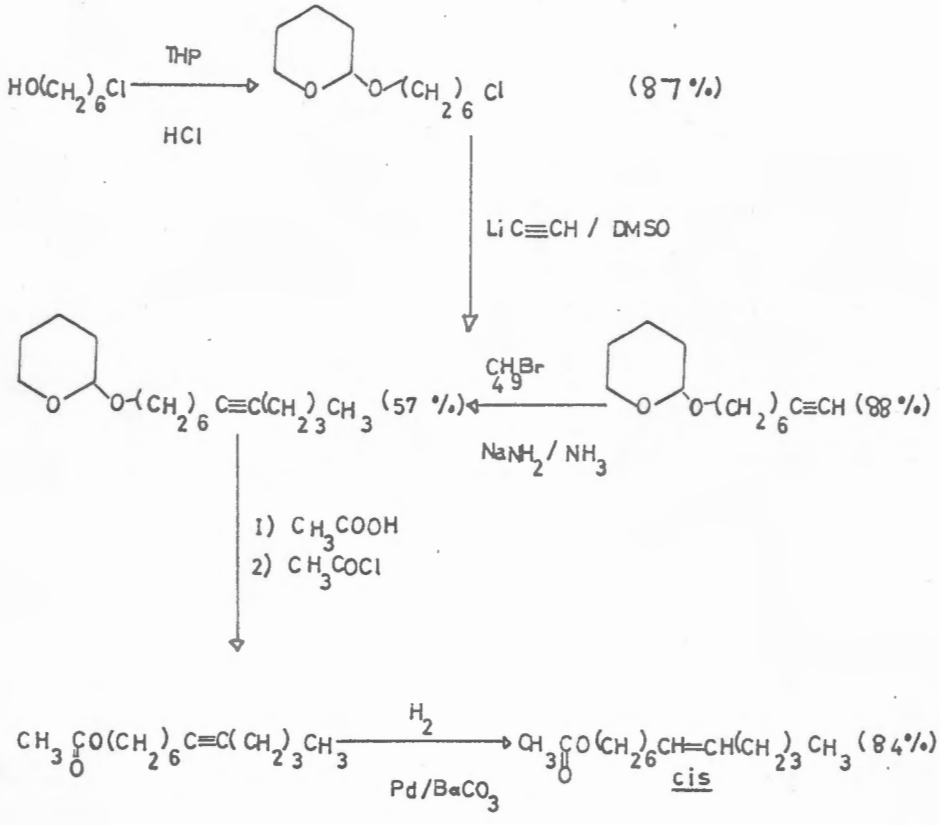


(Overall yield 28%)

Ref. Berger (1966)

SYNTHESIS OF *T.ni* PHEROMONE II:

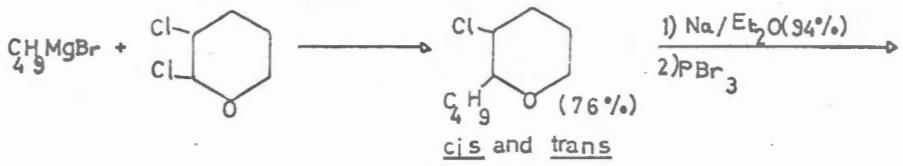
cis-Dodec-7-en-1-yl acetate.



(Overall yield 36 %)

Ref. Green *et al.* (1967)

SYNTHESIS OF A. leucotreta PHEROMONE: trans-Dodec-7-en-1-yl acetate.



cis and trans

$CH_3(CH_2)_3CH = CH(CH_2)_3Br$ (55%) $\xrightarrow{NaCH(COOEt)_2}$

$CH_3(CH_2)_3CH = CH(CH_2)_3CH(COOEt)_2$ $\xrightarrow[2)\ HCl\ \ 3)\ heat\ 150^\circ]{1)\ KOH}$

$CH_3(CH_2)_3CH = CH(CH_2)_4COOH$ (61%) $\xrightarrow[2)\ PBr_3\ (48\%)\ \ 3)\ Mg\ \ 4)\ CO_2]{1)\ LiAlH_4\ (95\%)}$

$CH_3(CH_2)_3CH = CH(CH_2)_5COOH$ (44%) $\xrightarrow[2)\ CH_3COCl\ pyridine]{1)\ LiAlH_4\ (88\%)}$

$CH_3(CH_2)_3CH = CH(CH_2)_6OCOCH_3$

trans (95%)

(Overall yield 4%)

Ref. Green et al. (1967)

REFERENCES

Acree, F. (1953 a) J. Econ. Entomol. 46, 313.

Acree, F. (1953 b) J. Econ. Entomol. 46, 900.

Acree, F. (1954) J. Econ. Entomol. 47, 321.

Ahmad, K. and Strong, F.M. (1948) J. Am. Chem. Soc. 70, 1699.

Amoore, J.E. (1964) Ann. N.Y. Acad. Sc. 116, 457.

Arndt, F. (1943) Org. Syn. Coll. Vol. 2 (Ed. Blatt, A.H.), Wiley, London, 165.

Barnes, M.M., Petersen, D.N. and O'Connor, J.J. (1966) Ann. Soc. Entomol. Soc. Am. 59, 732.

Baumann, F. and Gill, J.M. (1966) Aerograph. Res. Notes Spring'66.2.

Bergelson, L.D. and Shemyakin, M.M. (1964) Angew. Chem. (Intern. Ed.) 3, 250.

Berger, R.S. (1963) Science 141, 902.

Berger, R.S., McGough, J.M., Martin, D.F. and Ball, L.R. (1964) Ann. Entomol. Soc. Am. 57, 606.

Berger, R.S. (1966) Ann. Entomol. Soc. Am. 59, 767.

Beroza, M. and Sarmiento, R. (1963) Anal. Chem. 35, 1353.

Boeckh, J., Priesner, E., Schneider, D. and Jacobson, M. (1963) Science 141, 716.

Bullock, T.H. (1959) Biophys. Science Study Prog. (Ed. Oncley, J.L.) Wiley, New York, 515.

Butenandt, A. (1941) Angew. Chem. 54, 89.

Butenandt, A. (1955) Naturwissenschaft. 8, 457.

Butenandt, A., Beckmann, R., Stann, D. and Hecker, E. (1959) Z.Naturforsch. 14b, 203.

Butenandt, A. and Hecker, E. (1961) Angew. Chem. 73, 349.

Butenandt, A., Hecker, E., Hopp, M. and Koch, W. (1962) Ann. 658, 39.

Butenandt, A. (1963) J. Endocrinol. 27, 9.

Butler, C.G. and Fairey, E.N. (1964) J. Apicult. Research 3, 65.

Callow, R.K., Chapman, J.R. and Paton, P.N. (1964) J. Apicult. Res. 3, 77.

Corey, E.J. and Mock, W.L. (1962) J. Am. Chem. Soc. 84, 685.

Crombie, L. and Harper, S.H. (1950) J. Chem. Soc. 1707.

Davies, J.T. (1962) Symp. Soc. Exp. Biol. 16, 170.

Dethier, V.G. (1963) "Physiology of Insect Senses". Wiley,New York.

Eccles, J. (1965) Sc. American 212, 56.

Fabre, J.H. (1904) "Souvenirs Entomologiques" 8th Ed., Paris.

Farquhar, J.W., Insull, W., Rosen, P., Staffel, W. and Ahrends, E.H. (1959) Nutr. Rev., Suppl. 17, 1.

Fernandez-Moran, H. (1959) Biophys. Science Study Prog. (Ed. Oncley, J.L.) Wiley, New York. 319.

Finnean, J.B. (1958) Expt. Cell. Res. 5, 18.

Flaschentrager, B. and Amin, E.S. (1950) Nature. 165, 394.

Gowan, J.E. and Wheeler, T.S. (1960) "Name Index of Organic Reactions" Longmans, London.

Haller, H.L., Acree, F., Potts, S.F. (1944) J. Am. Chem. Soc. 66, 1659.

Harken, J.K. (1967) J. Chromatog. 26, 17.

Hauser, G. (1880) Z. Wiss. Zool. 34, 367.

Hecker, E. (1956) Proc. X th Intern. Cong. Entomol. 2, 293.

Hecker, E. (1960) Proc. XIth Intern. Cong. Entomol. 3B, 69.

Henne, A.L. and Greenly, K.W. (1943) J. Am. Chem. Soc. 65, 2020.

Hodgkin, A.L. and Keynes, R.D. (1953) J. Physiol. 121, 403.

Hodgkin, A.L. and Keynes, R.D. (1955) J. Physiol. 128, 28.

Hodgson, E.S. (1964) "The Physiology of Insecta" (Ed. Rockstein). 1, 363.

Hokin, L.F. and Hokin, M.R. (1963) Biochem. Biophys. 67, 470.

House, H.O. (1965) "Modern Synthetic Reaction" Benjamin, New York.

House, H.O. and Babad, H. (1963) J. Org. Chem. 28, 90.

Hungar, G. (1963) "Excitation", Thomas, Springfield. Ill.

Jacobson, M., Beroza, M. and Jones, W.A. (1960) Science 132, 1011.

Jacobson, M., Beroza, M. and Jones, W.A. (1961) J. Am. Chem. Soc. 83, 4819.

Jacobson, M. (1965) "Insect Sex Attractants" Interscience, (Wiley), New York.

James, A.T. and Morris, L.J. (1964) "New Biochemical Separations" van Nostrand, London.

Jamieson, G.R. and Reid, E.H. (1967) J. Chromatog. 26, 8.

Jones, W.A., Jacobson, M. and Martin, D.F. (1966) Science 152, 1516.

Karlson, P. and Lüscher, N. (1959) Nature. 183, 55.

Karlson, P. and Butenandt, A. (1959) Ann. Rev. Entomol. 4, 39.

Katz, B. (1959) Biophys. Science Study Prog. (Ed. Oncley, J.L.) Wiley, New York. 466.

Kellog, V.L. (1907) Biol. Bull. Marine Biol. Lab. 12, 152 - cited Jacobson, M. (1965).

Keynes, R.D. (1951) J. Physiol. 114, 119.

Kirschenblatt, J.D. (1962) Nature. 195, 916.

Kuemmel, D.F. (1964) Anal. Chem. 36, 426.

Makino, R., Satoh, K. and Inagami, K. (1956) Biochem. Biophys. Acta 19, 394.

Marmont, G. (1949) J. Cell. Comp. Physiol. 121, 403.

McCloskey, J.A. McClelland, M.J. (1965) J. Am. Chem. Soc. 87, 5090.

McLafferty, F.W. (1959) Anal. Chem. 31, 82.

Meyer, A.G. (1900) Psyche. 9, 15 - cited Jacobson, M. 1965.

Miwa, T.K., Mikolajczak, K.L., Earle, F.R. and Wolff, I.A. (1960) Anal. Chem. 32, 1739.

Morita, H. and Yamashita, S. (1959) Science 130, 922.

Novak, V.J.A. (1966) "Insect Hormones" 3rd Ed. Methuen, London.

Polacheck, J.W., Tropp, B.E., Law, J.H. and McCloskey, J.A. (1966) J. Biol. Chem. 241, 3362.

Riddiford, L.M. (1967) Science 158, 139.

Robertson, J.D. (1960) Prog. Biophys. and Biophys. Chem. 10, 343.

Ryhage, R. and Stenhagen, E. (1959) Arkiv. Kemi. 14, 483.

Ryhage, R. and Stenhagen, E. (1960) J. Lipid. Res. 1, 361.

Schmitt, F.O. (1959) Biophys. Science Study Prog. (Ed. Oncley, J.L.) Wiley, New York. 455.

Schneider, D. (1957) Z. Vergl. Physiol. 40, 8.

Schneider, D. (1963 a) Proc. Ist Intern. Symp. Olfaction Taste. 1, 85.

Schneider, D. (1963 b) Proc. XVIth Intern. Cong. Zoo. 3, 84.

Schneider, D. (1964) Ann. Rev. Entomol. 9, 103.

Sekul, A.A. and Sparks, A.N. (1967) J. Econ. Entomol. 60, 1270.

Shorey, H.H. and Gaston, L.K. (1964) J. Econ. Entomol. 57, 252.

Shorey, H.H. and Gaston, L.K. (1965) Ann. Entomol. Soc. Am. 58, 597.

Sjostrand, F.S. (1959) Biophys. Science Study Prog. (Ed. Oncley, J.L.) Wiley, New York. 301.

Slifer, E.H. (1961) Intern. Rev. Cytol. 11, 125.

Slifer, E.H. and Sekhon, S.S. (1964) J. Morphol. 109, 351.

Stein, G. (1963) Naturwissenschaft. 50, 305.

Steinbrecht, R.A. (1964 a) Z. Vergl. Physiol. 48, 341.

Steinbrecht, R.A. (1964 b) Z.Zellforsch. 64, 227.

Swart, P.L. (1966) Ann. Univ. Stellenbosch. 41A, 603.

Truscheit, E. and Eiter, K. (1962) Ann. 658, 65.

Vandenheuvel, F.A. (1963) J. Am. Oil Chem. Soc. 40, 455.

Warthen, D. and Jacobson, M. (1967) J. Med. Chem. 10, 1190.

Wigglesworth, V.B. (1953) "The Principles of Insect Physiology" Methuen, London.

Wright, R.H. (1963) Nature. 198, 455.

Wright, R.H. (1964) Nature. 204, 603.

Wright, R.H. (1967) Nature. 216, 404.

Zayed, S.M.A.D., Hussein, T.N., and Fakr, I.M.I. (1963) Z. Naturforsch. 18b, 265.