Cover picture : part of the antenna of *Periplaneta americana*
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DER
LANDBOUWHOGESCHOOL
WAGENINGEN

STRUCTURE ELUCIDATION OF SOME INSECT PHEROMONES:

A CONTRIBUTION TO THE DEVELOPMENT OF SELECTIVE PEST CONTROL AGENTS.

Dit proefschrift met stellingen van Cornelis Jacobus Persoons, landbouwkundig ingenieur, geboren te Diessen op 5 oktober 1935, is goedgekeurd door de promotoren, dr. J. de Wilde, hoogleraar in het dierkundig deel van de plantenziektenkunde en dr. H. O. Huisman, hoogleraar in de organische chemie aan de Universiteit van Amsterdam.

De rector magnificus van
de Landbouwhogeschool,

J. P. H. van der Want

Wageningen, 26 mei 1977

NN 8201 693

C. J. PERSOONS

STRUCTURE ELUCIDATION OF SOME INSECT PHEROMONES:

A CONTRIBUTION TO THE DEVELOPMENT OF SELECTIVE PEST CONTROL AGENTS.

PROEFSCHRIFT

ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. H. C. van der Plas,
hoogleraar in de organische scheikunde,
in het openbaar te verdedigen
op vrijdag 30 september 1977
des namiddags te vier uur
in de aula
van de Landbouwhogeschool te Wageningen

ISN = 107079-03

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NN08201, 693

STELLINGEN

I

Uit het door Yamada et al. verrichte onderzoek wordt door hen ten onrechte geconcludeerd tot het bestaan van "sex pheromone specialists" in de hersenen van de Amerikaanse kakkerlak.

Yamada et al., Nature 227, 855 (1970).

II

De conclusie van Nishino et al. betreffende de relatie chemische structuur en biologische activiteit bij feromonen, wordt door hun experimenten onvolledig ondersteund en is ook om andere redenen voorbarig.

Nishino et al., J. Insect Physiol. 23, 415 (1977).

III

De conclusie van Read et al., dat het sexferomoon van de "false codling moth", *Cryptophlebia leucotreta*, *trans*-7-dodecenyl acetaat zou zijn, is onjuist.

Read et al., Chem. Comm. 1968, 792 (1968).
Read et al., J. Insect Physiol. 20, 441 (1974).
Dit proefschrift hoofdstukken 4 en 5.

IV

In hun opsomming van de kwalitatieve eisen waaraan spoorvolgferomonen moeten voldoen, verwaarlozen Howard et al. ten onrechte een aantal kwantitatieve aspecten.

Howard et al., J. Chem. Ecol. 2, 147 (1976).

V

Voor een succesvolle toepassing van sexferomonen als bestrijdingsmiddel is een gedragsanalyse van het betreffende plaaginsect niet alleen in het laboratorium, maar vooral ook in het veld een stringente voorwaarde.

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VI

In het kader van de samenwerking overheid-industrie op het gebied van de ontwikkeling van selectieve pesticiden, zou ook de industriële ontwikkeling van de toepassing van feromonen dienen te worden opgenomen.

VII

De bedenkingen die worden gemaakt tegen het gebruik van insectenvirussen als rupsenbestrijdingsmiddelen, zijn ongegrond.

VIII

De sociale en landbouwkundige aspecten van een anti-trypanosomiasis campagne worden onvoldoende onderkend.

IX

De bewering dat de faraomier met de Bijlmer-expres vanuit Suriname naar Nederland zou zijn overgebracht, mist elke grond.

De Telegraaf dd. 7-2-1976.

X

De vraagstelling "kerkelijke gezindte" op diverse inlichtingenformulieren voor bijstandsaanvrage, is niet ter zake dienend.

C.J. Persoons.
Wageningen, 30-9-1977.

aan mijn ouders,
aan Jo,
Audrey en Bernine

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1. GENERAL INTRODUCTION

The increasing resistance of insects to insecticides, and the growing concern about contamination of the environment by insecticides, has induced investigators to search for novel methods of controlling insect pests. Such alternative methods should be as effective as conventional methods, and should also be acceptable to the public at large.

The search for such methods has been strongly accelerated by the appearance of Rachel Carson's book "Silent Spring" (Carson, 1962). But even before its appearance, scientists all over the world had begun to realize that insecticides were having adverse effects, and had started to look for alternative ways of controlling insect pests. One result of the search is the development of integrated pest control programmes (Marx, 1973; Brader, 1975), which combine several methods of insect control. Their aim is to keep the use of pesticides to a minimum, and to combine the best and most acceptable techniques, rather than to use a single technique. They do not completely exclude the use of insecticides, and the eradication of a pest is not expected to be achieved but rather its regulation below economic thresholds. Among the approaches being followed is the application of compounds which interfere with the communication between insects.

The chemicals serving the communication between organisms in general are called semiochemicals. More specific those that act between individuals of the same species are called pheromones. On the basis of the behavioural response in the receiving animal, the most important groups are:

- *sex pheromones:* a receptive female or male looking for a mate.
- *trail pheromones:* (in social insects) leading the way from the nest to a food source or the way back to the nest.
- *aggregation pheromones:* indicating a safe hiding place or a suitable place for mating and/or oviposition.

This work deals with the first group only, and the question arises how they can be applied in integrated pest control programmes. It is clear, in any case, that pheromones are of vital importance for the survival of insects. Methods using pheromones for pest control usually depend on utilizing the normal response of an insect to a pheromone for its own destruction. As each insect species has its own sex pheromone, they are highly specific. Moreover, they are active at very low concentrations. These properties favour their use as insect control agents.

If a pest is to be controlled effectively, its presence must be detected early on. Owing to their high specificity and high potency, pheromones were soon recognized as being very well suited to this purpose. The light traps so far used for the same purpose have the disadvantage of not being specific to a single insect species and of requiring much more work in the field.

Pheromones are nowadays mostly used in sex traps for *monitoring* the existance or expansion of an insect population. They are also used for detecting pests in goods held in quarantine and suspected of harbouring certain insects.

The simplest and most obvious way of controlling an insect pest seems to be *mass trapping*, resulting in the elimination of most or all of the insects of a

given population. However, the practical application of this technique has proved to be very difficult. The number of insects caught must be so large that a considerable reduction is achieved not only of the existing generation of insects, but also of the next. This can only be accomplished by the use of sex traps in large numbers. Theoretical calculations (Roelofs et al., 1970) indicate for example, that an initial trap : female ratio of at least 5 : 1 would be needed to obtain 95 % suppression of mating.

One of the first methods to be suggested for the direct control of insects by pheromones or other chemicals that modify or inhibit insect behaviour was *saturation of the air* with such chemicals. Nowadays this technique is often called the "confusion technique", because it results in, for instance, disorientation of males in search of females.

Many other techniques have been developed and tested, but their practical application still has to cope with many problems (concerning, e.g., release rate, formulation, purity, availability, etc.). The volume of literature on pheromones and their practical application is vast. For some general reviews see, e.g., Birch (1974), Roelofs (1975), Inscoe and Beroza (1976), and Ritter and Persoons (1976).

The practical application of pheromones in integrated pest control schemes requires that the compounds are available in sufficient amounts: synthesis of the compounds therefore is necessary. However, knowledge of their chemical structure is a first requisite. Accordingly, the main theme of this thesis is the elucidation of the structures of several pheromones, a minor theme being their evaluation in the field.

The next five chapters deal successively with the sex pheromones of the American cockroach (*Periplaneta americana*), the leafroller moth (*Archips podana*), the false codling moth (*Cryptophlebia leucotreta*), and the potato tuberworm moth (*Phthorimaea operculella*).

A summary is given in Chapter 8.

2. SEX PHEROMONES OF THE AMERICAN COCKROACH, *PERIPLANETA AMERICANA*

2.1 INTRODUCTION

The structure elucidation of the sex pheromone of the American cockroach, *Periplaneta americana*, is a problem of long standing. In 1963 Jacobson and co-workers (Jacobson et al., 1963) presented evidence to the effect that the pheromone is 2,2-dimethyl-3-isopropylidenecyclopropyl propionate (Fig. 1).

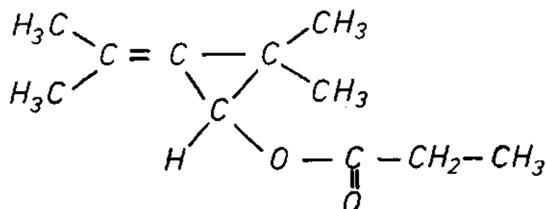


Fig. 1. Jacobson's structure of the sex pheromone of the American cockroach, *Periplaneta americana* (Jacobson et al., 1963).

At about the same time, Wharton and co-workers (Wharton et al., 1962, 1963) published data (mainly gas chromatographic retention times) which cast some doubt on Jacobson's structure, although this could not be disproved. The enormous difference in gas chromatographic retention times between Jacobson's and Wharton's material (6 and 145 min, respectively, under comparable conditions) suggests that the two groups were working on entirely different substances. At the time, however, this was not taken as an indication that the pheromone might consist of several different substances.

When Jacobson's compound was later synthesized and found to be biologically inactive (Day and Whiting, 1964) Jacobson withdrew the proposed structure (Jacobson and Beroza, 1965). Jacobson's compound was found to be entirely different from the natural product (Day and Whiting, 1966; Wakabayashi, 1967). Apparently, Jacobson's material mainly consisted of inactive compounds contaminated with minute amounts of active material. In order to explain the spectroscopic evidence published by Jacobson, Day and Whiting (1964) suggested the following structure (Fig. 2):

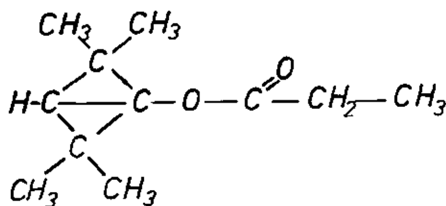


Fig. 2. Sex pheromone of *P. americana*: structure proposed by Day and Whiting (1964) to explain Jacobson's spectroscopic evidence.

No further structures have been proposed for the pheromone, although several attempts have been made for its isolation and identification (Chen, 1974; Chow et al., 1976; Takahashi and Kitamura, 1976[b] ; Kitamura and Takahashi, 1976). All such attempts have so far been unsuccessful. The presence of two biologically active compounds (called periplanone-A and periplanone-B) in extracts of faeces obtained from virgin females, and their isolation, purification and molecular formulae have been described before (Persoons et al., 1974; Ritter and Persoons, 1975) and a tentative structure of periplanone-B has been proposed (Persoons et al., 1976).

In addition to periplanone-A and periplanone-B, another four biologically active compounds have been isolated in minute amounts (PA_{22-I}, PA_{22-IV}, PA_{22-V} and PA_{22-VI}). The structure elucidation of periplanone-B will be described in detail. Being rather unstable (periplanone-A) or available in minute amounts only (PA_{22}-series) the other compounds could not be fully identified.

2.2 MATERIALS AND METHODS

2.2.1 Mass rearing of *Periplaneta americana*

The insects were reared at 30 °C (± 1 °C) and at a relative humidity of 50-60 % under a day/night regime of 17 hours light and 7 hours darkness. Water and food (Bonzo puppy) were supplied in abundance.

Once a month the egg capsules were collected from the stock cultures. Nymphs originating from egg capsules that hatched within a week, were collected in plastic containers measuring 70 × 30 × 40 cm, provided with an abundance of shelters. Ten to fifteen containers with 2000-2500 insects each were used simultaneously.

At the end of the larval stage the male and female nymphs were separated, because copulation decreases pheromone production considerably (Roth and Willis, 1952; Wharton and Wharton, 1957). Male nymphs were reared to adults for the required bioassays. The females were used for the production of the starting materials after reaching the adult stage. From 15,000 to 40,000 virgin females were reared at the same time.

2.2.2 Bioassays

Behavioural test

For monitoring the successive purification steps of the pheromone, we used tests developed by Wharton and co-workers (Wharton et al., 1954[a], 1954[b]) and by Takahashi and Kitamura (1972) based on the sexual excitation of the males.

A pasteur pipette was rinsed with a solution of the pheromone. The solvent was evaporated and air was blown for 1-2 seconds through the pipette into glass jars containing 15-25 male cockroaches. A sample was considered to be active when at least 50 % of the males showed the signs of sexual excitement (wing raising, copulatory attempts).

As a rule the test was not applied more than once a day to the same batch of males, except when large numbers of samples had to be tested. The test can be repeated more than once a day provided that the test jars are flushed with

air for at least half an hour immediately after a test, and provided that the insects are allowed sufficient time for recovery (Hawkins and Rust, 1976).

The tests were performed irrespective of the time of day (Lipton and Sutherland, 1970), although other investigators prefer to carry them out in the dark, when the males are more excitable (Butz and Aranoff, 1970; Takahashi and Kitamura, 1972; Hawkins and Rust, 1976; Chow et al., 1976). Compounds known to mimic the natural pheromone (borneol, borneol acetate, α- and β-santalol (Bowers and Bodenstein, 1971) and germacrene-D (Kitamura et al., 1976) were tested according to the same procedure (1 μg - 1 mg per test sample in hexane).

To test the attractive power of germacrene-D and of periplanone-B, a modified version of the olfactometers described by McCluskey et al. (1969) and Takahashi and Kitamura (1972) was used. A rearing bottle (5 l) was placed horizontally and two plastic tubes (4 cm Ø, 30 cm long), separated by a piece of plastic at the entrance, were fitted by means of a two-hole rubber stopper. The proximal ends of the tubes were provided with plastic funnels that acted as one-way doors, while the distal ends were closed with one-hole rubber stoppers. These holes permitted the insertion and removal of disposable pipettes, through which air and stimuli entered the tubes and the container. A glass tube through the stopper functioned as air outlet.

Males were tested in batches of about 20 individuals. An air speed of 500 ml/min/tube was most commonly used. Germacrene-D was applied on filter paper strips, whereas for periplanone-B the glass wall of the pipette was used. The exposure time was one minute and every 15 seconds the insects in both tubes and those at the entrance of the tubes were counted. Periplanone-B was tested in quantities ranging from 1 pg to 100 pg, whereas germacrene-D was tested in quantities of 10 μg and 100 μg. Tests conditions were as described above.

Electroantennographic test

The electroantennography (EAG) tests were used as a supplement to the behavioural tests. The technique was as described by Roelofs and Comeau (1971).

The signals were displayed on an oscilloscope (Philips, P.M. 3200)and an UV recorder (Honeywell, 906 T visicorder) was used to record the signals.

2.2.3 Collection of starting material from virgin females

Extraction of excrement-soiled filter paper

The technique was a modification of that used by Roth and Willis (1952) and by Wharton et al. (1962).

Although female cockroaches do not begin to produce pheromone before a week after their last moult (Wharton and Wharton, 1957; Hawkins and Rust, 1976), young virgin females were collected in plastic boxes measuring 70 × 30 × 40 cm immediately after the last moult (600-650 insects per box). About 20 rolls of Whatman no. 3 chromatographic paper (45 cm long, 3 cm Ø) were placed in each container. Every 3-4 weeks the excrement-soaked rolls were collected and replaced with new rolls until the insects died. Up to ten containers were used at the same time. The collected material was stored at -15 °C before being processed. The paper containing the excrements of one batch of 600 females was soaked in 10 litres of water to which lead nitrate was added. The paper was

ground and the resulting slurry centrifuged. This procedure was repeated three times*. 50 % of the aqueous extract was distilled, and the distillate extracted four times with hexane. The extracts were combined, dried for 24 hours over anhydrous magnesium sulphate, concentrated to a volume of 20-30 ml and stored at 2-3 °C.

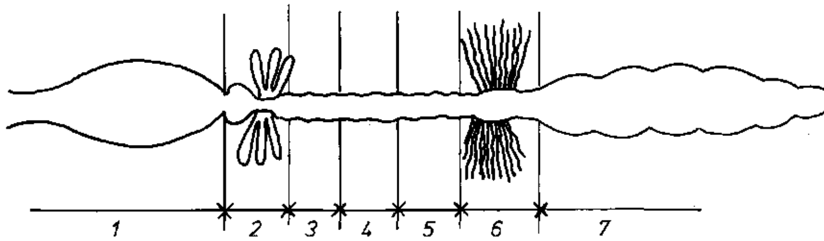
Extraction of faeces

The excrements produced by 6000-7000 virgin females during four weeks was scraped off the filter paper rolls, after which the filter paper and the excrements were processed separately. The method of collecting excrements was later on modified in such a way that the faeces of thousands of insects could be collected easily. To this end 15,000-20,000 virgin females were confined in a large cage (1 × 1 × 1 m), in which 15 pieces of nylon cloth (1 m^2 each) were stretched vertically in such a way that they could easily be removed. The bottom of the container consisted of a gauze screen, below which a drawer was placed to collect most of the excrements. The nylon sheets were replaced with new ones every 2-3 weeks. The soiled sheets were rinsed with water, to which were added the excrements that had been collected in the drawer. The resulting aqueous suspension was processed for extraction of the pheromone.

Extraction of alimentary tracts

In a pilot experiment the alimentary tracts of 20 virgin females (2-3 weeks old) were collected. To avoid contamination of the other parts of the gut with active material via the faeces, the insects were starved for ten days (Cornwell, 1968), during which they were kept individually in jars without access to water or food. The dissected tracts were each divided into seven parts, as shown in Fig. 3. The corresponding parts were pooled, homogenized in acetone, centrifuged and concentrated to 0.5 ml aliquots. Each fraction was bioassayed by the behavioural test.

*The large-scale processing of the starting material was carried out at the Pilot Plant of the Department of Chemical Technology of the Technical University, Delft.



1: crop + oesophagus
2: caeca + proventriculus
3: } ventriculus } midgut
4:
5:
6: ileum + malpighian tubules
7: colon + rectum

Fig. 3. Alimentary tract of *Periplaneta americana* (schematic). The numbered sections were investigated separately.

A total of 32,000 alimentary tracts were dissected and processed in batches of 2000 each. The tracts were collected without solvent, freeze-dried at -180 °C, ground and some water was added. The pheromone was co-distilled with water. During the distillation water was added dropwise.

2.2.4 Purification of crude extracts

Precipitation in acetone at -20 °C

The various hexane concentrates from the filter paper, faeces and alimentary tracts were concentrated, and the residue was dissolved in as little acetone as possible and transferred to a centrifuge tube. This solution was stored overnight at -20 °C, and the precipitate which had separated was removed by centrifugation (5-10 min, 3000 rpm). The supernatant was collected, the residue re-dissolved in acetone and again stored overnight at -20 °C. This procedure was repeated four times. The supernatants were combined, concentrated and subjected to gel permeation chromatography.

Gel permeation on Sephadex LH20

Samples of 0.5 ml were applied on a glass column (100 × 1.4 cm i.d.) loaded with Sephadex LH20 by means of a three-way valve. Freshly distilled acetone was used as the eluent. 5 ml fractions were collected and assayed as described above. Dilution series were made for location of maximum activity. Highly active fractions, and less active fractions were collected separately and the latter were re-chromatographed over the same system. The most active fractions from all runs were combined, concentrated, and stored at 2-3 °C.

2.2.5 Separation of periplanone-A, periplanone-B and some unidentified compounds

Column chromatography on silicagel

The active concentrate obtained by gel permeation chromatography was dissolved in hexane-2 % ethyl acetate, and subjected to column chromatography under the following conditions: column 60 × 0.8 cm i.d., particle size ≤ 50 μm, flow rate of eluent (hexane-2 % ethyl acetate) 25 ml per hour, pressure 8 atm. 15 minute fractions were collected and bioassayed. Samples of 250 μl were applied on the column by means of a sample injection valve. The most active fractions of the two distinctly separate peaks of activity (ascribed to periplanone-A and periplanone-B, respectively) were combined. Fractions containing less active material were combined and re-chromatographed under the same conditions.

High-pressure liquid chromatography (HPLC) on silicagel

The combined active fractions collected from the Sephadex column were taken up in hexane-2 % ethyl acetate and pre-purified on a preparative silicagel column : column stainless steel, 50 × 1 cm i.d., loaded with silicagel (Merck HR 60), particle size 5-20 μm, working pressure 90-100 atm, flow rate 20 ml per minute, eluent 2 % ethyl acetate in hexane (both freshly distilled), sample size 1 ml injected by means of a high pressure injection valve. Fractions of 7.5 ml were collected and bioassayed. Fractions containing periplanone-A were combined and concentrated; this was also done with the fractions containing periplanone-B. Less active fractions were combined and re-chromatographed over the same system.

The periplanone-A and periplanone-B concentrates were re-chromatographed with a HPLC system, incorporating analytical columns. The periplanone-A concentrate was re-chromatographed under the following conditions: two columns (25 × 0.5 cm i.d.) were loaded with Lichrosorb Si 60 (10 μm) and connected by capillary tubing; working pressure 85 atm, flow rate 4 ml per minute, eluent 1 % ethyl acetate in hexane (both freshly distilled), sample size 300 μl; 1-minute fractions were collected and bioassayed. The fractions of three distinct areas of activity were combined, viz. PA_{22-VI}, periplanone-A and PA_{22-V}. (Gas chromatography showed that the second mentioned contained another two biologically active compounds, viz. PA_{22-I} and PA_{22-IV}; see also Fig. 5.) Fractions containing less active material were combined, and re-chromatographed under the same conditions.

The fractions containing periplanone-B were also re-chromatographed on analytical columns, but under slightly different conditions: three columns (25 × 0.5 cm i.d.) were loaded with Lichrosorb Si 60 (particle size 5-8 μm) and connected as above. Working conditions: pressure 230 atm, flow rate 2.3 ml per minute, eluent 1 % ethyl acetate in hexane (freshly distilled), sample size 300 μl, detection with a DuPont UV spectrophotometer with flow cell, set at 254 nm; 2-minute fractions were collected (= 4.5 ml). The most active fractions were combined, and concentrated. Less active fractions were combined, and re-chromatographed under the same conditions.

Gas chromatography of purified extracts

Corresponding active fractions from various SiO_2 and HPLC runs were combined, and further analysed by gas chromatography. All gas chromatographic analyses were carried out with a Pye Unicam gas chromatograph, Model 104, both columns of which were fitted with end-flow splitters.

To locate the activity of the compounds on various columns, one-minute fractions were collected from the gas chromatograph in chilled capillaries (Burson and Kenner, 1969) and these capillaries assayed by the behavioural test or the EAG method, or by both. After location of the activity, 10-50 μl aliquots of the samples were injected on a polar column (5 % DEGS, diethyleneglycol succinate on Chromosorb W (AW), 80-100 mesh, 160 °C, 200 × 0.4 cm i.d., N_2 = 45 ml/min, or 10 % Carbowax 20 M on Chromosorb GAW-DMCS, 180 °C, 250 × 0.4 cm i.d., N_2 = 45 ml/min). The previously determined area of activity was collected and re-injected on a non-polar column (5 % OV 101 on Chromosorb G (AW-DMCS), 80-100 mesh, 200 × 0.4 cm i.d., 180 °C, N_2 = 45 ml/min). Those parts containing the activity were again collected and bioassayed.

2.2.6 Identification techniques

Mass spectrometry

The mass spectra of periplanone-A and periplanone-B were run on a combined gas chromatograph-mass spectrometer (LKB 9000) fitted with a 2 m, 5 % OV 101 column, operated at 180 °C. The mass spectra of PA_{22-I}, PA_{22-IV}, PA_{22-V} and PA_{22-VI} were run on a Mat 112 gas chromatograph-mass spectrometer, fitted with an SE 30 column (48 m × 0.3 mm i.d., 190 °C). Samples varying from 10 ng to 1 μg of the various components were injected into the GC/MS apparatus. Mass spectra were taken at different spots in the gas chromatographic peaks as a check of the purity of the compounds. A compound was considered to be pure when its mass spectra taken from different spots were identical.

Mass spectrometry of hydrogenated compounds

The active compounds were hydrogenated and subjected to mass spectrometry in the GC/MS apparatus (LKB 9000) with an OV 225 column (periplanone-B) and an OV 101 column (periplanone-A). The tops of both columns (7 cm) were loaded with palladium chloride as the hydrogenation catalyst. The columns were heated from 150 to 210 °C (temperature programmed), with hydrogen as the carrier gas and mass spectra were taken at different spots of the chromatographic peaks.

The efficiency of the system was checked by hydrogenation of a number of unsaturated straight-chain and cyclic compounds in amounts of 1-6 μg. Of the various compounds isolated from *P. americana*, only periplanone-A and periplanone-B were available in sufficient amounts for the hydrogenation experiments.

Infrared analysis

Samples containing about 10 μg of periplanone-A and about 7 μg of periplanone-B, respectively, were subjected to infrared analysis, using a special micro-technique. Spectra were recorded in KBr on a double-beam grating spectrophotometer (Perkin-Elmer, Model 421).

Ultraviolet analysis

The sample of periplanone-A used for infrared analysis was worked up for UV analysis as follows. The KBr pellet was dissolved in 1 ml of water, the solution extracted three times with hexane, the hexane extracts were combined and dried over anhydrous $MgSO_4$. Periplanone-A was recovered from the extract by gas chromatography. A second sample (1 μg) was purified for UV analysis as described before. For the UV analysis of periplanone-B, 1.5-2.0 μg of the substance was isolated as described before. The substances were dissolved in 150 μl of hexane, and the spectra recorded with a double-beam grating UV spectrophotometer (Cary, Model 4).

NMR analysis

A first NMR spectrum was run with a sample containing about 100 μg of periplanone-B in 10 μl $CDCl_3$ on a Varian 220 MHz apparatus, with and without $Eu(dpm)_3$. As the substance appeared to decompose rather rapidly in $CDCl_3$, a second sample of about 70 μg was collected, and its spectrum taken in 10 μl of CS_2, $CS_2 + C_6D_6$ (10:1), and in C_6D_6. These spectra were run on a Varian HR 300 and a Varian SC 300 spectrometer, the latter with a digital resolution better than 0.2 Hz over a spectral width of 3000 Hz (Fourier transformation of a 32 K data table).

The other compounds extracted from *P. americana* were not available in sufficient amounts for the same series of NMR analysis.

2.3 RESULTS

2.3.1 Mass rearing of *P. americana*

The amount of active material extractable from one female proved to be extremely small. The total amount of periplanone-B collected in the course of the experimental period was about 200 μg, and that of periplanone-A about ten times less. For this reason up to 40,000 insects had to be reared at the same

time.

2.3.2 Bioassays

Behavioural test

In tests of a series of fractions containing different amounts of active compounds, quantitative differences in response were difficult to observe. Preparation of dilution series allowed the number of fractions per series to be reduced from about 20 to 5 or less.

Germacrene-D proved to be the only compound active as a mimic of the natural pheromone, although several compounds have been reported to do so. This compound was active at a minimum dose of 10 µg. At this level its action is indistinguishable from that of the natural pheromone. The latter, however, has a threshold of activity of less than 10^{-6}µg (or even 10^{-10}µg according to Takahashi and Kitamura, 1976[b]).

The experiments with periplanone-B and germacrene-D in the olfactometer experiments indicated that both compounds are attractive to males of *P. americana*.

Periplanone-B applied in the amount of 1 pg on glass attracted only a few insects, about 10 % of the insects entering the tubes or assembling at the entrance of the tubes. A dose of 10 pg increased this percentage, whereas with a dose of 100 pg up to 50 % of the insects were trapped. A dose of 1 ng completely confused the insects and once they had been exposed to such a high dose, they needed a recovery time of at least one day or more before they could be used as test insects again. Best results were obtained when rearing bottles were used, in which the insects had been kept for some time already before the experiments were carried out.

Germacrene-D could also attract males of *P. americana*, but much higher quantities were needed. The most effective dose was found to be 100 µg, attracting between 10-30 % of the insects (no higher amounts could be tested, due to shortage of material). Lower amounts gave hardly any attraction in these experiments.

Applying the attractant on glass was found to lower the effective concentration limit for periplanone-B by a factor of about 1000. For germacrene-D filter paper seemed to be the best dispenser.

It seems well established now that both periplanone-B and germacrene-D have attractive qualities (McCluskey, 1969; Takahashi and Kitamura, 1972; Chow et al., 1976; Rust, 1976; and Rust et al., 1976). However, the experiments described by these investigators, were all carried out with partly purified extracts, possibly containing more than one single active component. Although nothing has been reported about synergistic effects of the unknown compounds, the possibility that they act as such may not be neglected. Chow et al. (1976) working with crude extracts in a heavily infested rice barn, trapped significantly more males with the crude extract than with any of the other chemicals tested. He also caught large numbers of females and nymphs, possibly indicating the presence of an aggregation pheromone. No indications have been found for the presence of a trail following pheromone as reported by Brousse-Gaury (1975).

Electroantennography (EAG)

This technique was used as a supplement to the behavioural test for monitoring the successive purification steps. It provides a crude quantitative measure of the distribution of activity within a series of samples, and renders the preparation of dilution series unnecessary. Figure 4 gives an example.

Fig. 4. Sex pheromone of the American cockroach, *P. americana*. Distribution of activity in a series of samples collected from a Sephadex column, and measured by the EAG technique. Sequence of testing the samples was at random. C = standard test sample (<< 1 μg).

In the example, the activity was confined to fractions 31, 32 and 33, which were also found to be active in the behavioural test. All isolated compounds elicit distinct EAG responses. Compounds known to mimic the natural pheromone were also tested by EAG. Their potencies are compared with that of periplanone-B in Table 1. This shows that all "mimics" are active only at very high concentrations, and that their potency is much less than that of the natural pheromone, less than 1 μg of which suffices to evoke a strong response.

Table 1 Activity in EAG tests of various compounds, including the natural pheromone periplanone-B. The tests were conducted in fivefold using five different antennae

compound	amount tested (μg)	relative EAG response
natural pheromone	<< 1	100
borneol[1]	1	0
	10	25
	100	27
	1000	38
borneol acetate[1]	1	0
	10	20
	100	35
	1000	43
L-borneol acetate[2]	1	6
	10	13
	100	27
	1000	46
α-santalol[3]	1	6
	10	4
	100	9
	1000	10
β-santalol[3]	1	13
	10	7
	100	12
	1000	12
germacrene-D[4]	1	7
	10	19
	100	30

1) racemic mixture; purity ⩾ 95 %

2) purity ⩾ 98.5 %

3) α- and β-santalol were separated with a silver-impregnated nucleosil column; purity ⩾ 95 %

4) kindly provided by S. Takahashi, Kyoto University, Kyoto, Japan; purity ⩾ 95 %

2.3.3 Collection of starting material

Extraction of filter paper

Several methods to collect the active material that have been tested could not be used at the scale required. Although rather time-consuming, the "filter paper technique" proved to be very successful. By this technique we collected about 100 µg of periplanone-B and about 10 µg of periplanone-A.

Extraction of faecal material

Work by Bodenstein (1970) and our own experience has shown that the pheromone is produced in the alimentary tract and is excreted with the faeces. In a pilot experiment we obtained about 10 µg of periplanone-B and about 1 µg of periplanone-A from the excrements produced by 6000 - 7000 virgin females in four weeks.

Extraction of alimentary tracts

The pheromone could only be recovered from that section of the alimentary tract which comprises the proventriculus and the caeca. Other parts of the gut were found to be inactive. For practical reasons, however, complete intestinal tracts were excised from insects which had not been starved. In this way, a total of about 32,000 intestines were collected.

2.3.4 Purification of crude extracts

Precipitation of impurities with acetone at -20 °C

Treatment of the hexane extract with a small amount of acetone and overnight storage of the mixture at -20 °C resulted in the formation of a precipitate, which was removed by centrifugation and discarded. The supernatant thus purified was found to be less likely to cause saturation of the column in gel permeation chromatography.

Gel permeation on Sephadex LH 20

Separation on a Sephadex column usually afforded 3-4 active fractions [nos (30), 31, 32, 33 and (34)]. (See also Fig. 4.) The most active fractions could usually be diluted by a factor of 10,000 without loss of activity. When these active fractions were combined and the solvent removed, a minute amount of a clear yellow oil (50 µl) was left behind.

2.3.5 Isolation of periplanone-A, periplanone-B and some unidentified compounds

Silicagel column chromatography

Column chromatography on silicagel revealed that there were two active compounds. The first active peak (periplanone-A) emerged from the column after 3-4 hours, and the second (periplanone-B) after 11-13 hours. Dilution series of the two regions of activity indicated that most of the periplanone-A was concentrated in two fractions, and most of the periplanone-B in 4-5 fractions. The fractions containing periplanone-A could be diluted by a factor of 100,

and those containing periplanone-B by a factor of about 1000 without loss of activity.

High pressure liquid chromatography on SiO_2 (HPLC)

Prepurification on a preparative column of the combined active fractions from the Sephadex column resulted in complete separation of periplanone-A and periplanone-B within 20 minutes. Periplanone-A was eluted after 3-5 minutes and periplanone-B after 13-18 minutes. Upon re-chromatography of the fractions containing periplanone-A over analytical columns, three distinct regions of activity emerged: fractions 12-14 (= PA_{22}-$_{VI}$), fractions 26-34 (= periplanone-A) and fractions 35-44 (= PA_{22}-$_{V}$). Upon re-chromatography over analytical columns, periplanone-B was eluted after 64-68 minutes. Unlike periplanone-A, the periplanone-B thus obtained is not contaminated with other biologically active compounds.

Gas chromatography

Gas chromatography of the fractions containing periplanone-A after HPLC on SiO_2, revealed another two active components. The other active fractions obtained by HPLC each contained a single active compound (see Fig. 5).

From the gas chromatographic results it appeared that periplanone-B was the most abundant compound. Extracts of faeces were found to contain about 10 times less of periplanone-A than of periplanone-B, and the alimentary tracts contained hardly any periplanone-A at all. The other compounds occurred in even smaller amounts. We therefore limited ourselves to the isolation and identification of periplanone-A and periplanone-B.

For the two major active compounds, viz. periplanone-A and periplanone-B, the concurrence of the chromatographic peaks with biological activity was checked on five different stationary phases; on all of these, the chromatographic peaks coincided with the biological activity. The Kovàts Retention Indices of the two compounds on the various stationary phases are given in Table 2.

Table 2 Kovàts Retention Indices of periplanone-A and periplanone-B on five different gas chromatographic stationary phases

compounds	stationary phases				
	DEGS	OV 101	OV 17	OV 210	DC 200
periplanone-A	2250	1790	1890	2280	1800
periplanone-B	2450	1790	1890	2290	1800

Of all column materials tested, DEGS is the only one capable of separating the two compounds into well-resolved peaks. Re-chromatography of a pure sample

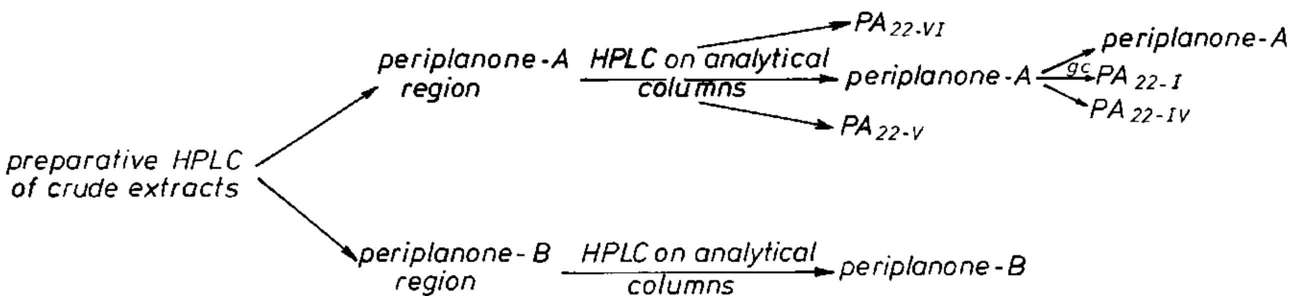


Fig. 5. Separation of active components in a crude extract of females of *P. americana* by means of high pressure liquid chromatography and gas chromatography.

of periplanone-A that had been kept for some time at a low temperature, gave an inactive product in addition to the original active compound. This finding indicates that the compound is unstable. The same inactive compound is found with several different gas chromatographic systems. Since both periplanone-A and its decomposition product give two sharp and well-resolved peaks, it seems unlikely that the inactive compound results from thermal decomposition. It will be seen later that the inactive compound is formed by isomerization rather than by decomposition. Due to the extreme small amounts available, accurate determination of the Kovàts Retention Indices of the minor compounds proved to be impossible. The measured values are listed in Table 3.

Table 3 Approximate values of Kovàts Retention Indices of the minor components of the sex pheromone of *P. americana* on different gas chromatographic systems

components	stationary phases		
	Carbowax 20 M	DEGS	OV 101
PA_{22}-I	± 2760	–	1650
PA_{22}-IV	2060–2160	2165–2230	1570
PA_{22}-V	2370	2460	1740
PA_{22}-VI	2090	2145	± 1600

2.3.6 Mass spectrometric analysis

Mass spectra of periplanone-A and its isomerization product

These spectra are shown in Figs 6 and 7. The parent peak of periplanone-A was found at m/e = 232. The spectrum showed weak peaks at m/e = 172 and m/e = 189. Peakmatching gave m/e = 172 = $C_{13}H_{16}$ (found 172.1239, calculated 172.1252) and m/e = 189 = $C_{12}H_{13}O_2$ (found 189.0931, calculated 189.0915). The active compound therefore contains at least 13 C-atoms, 16 H-atoms and 2 O-atoms. Since $C_{13}H_{16}O_2$ has a molecular weight of 204, the parent peak at m/e = 232 can only be attributed to $C_{15}H_{20}O_2$ or to $C_{14}H_{16}O_3$. The latter can be ruled out, because its presence would imply the rather unlikely fragment M-CO_3 at m/e = 172. In accordance with this formula are the fragments m/e = 105 = C_8H_9 = 105.0696 (calculated 105.0704) and m/e = 127 = $C_7H_{11}O_2$= 127.0768 (calculated 127.0759) which are complementary parts of the molecule.

The composition $C_{15}H_{20}O_2$ suggests that the compound might be a sesquiterpenoid.

The mass spectra of periplanone-A and its isomerization product are very similar, but they also show some marked differences. Both spectra have m/e = 232 as the parent peak, but their fragmentation patterns are very different.

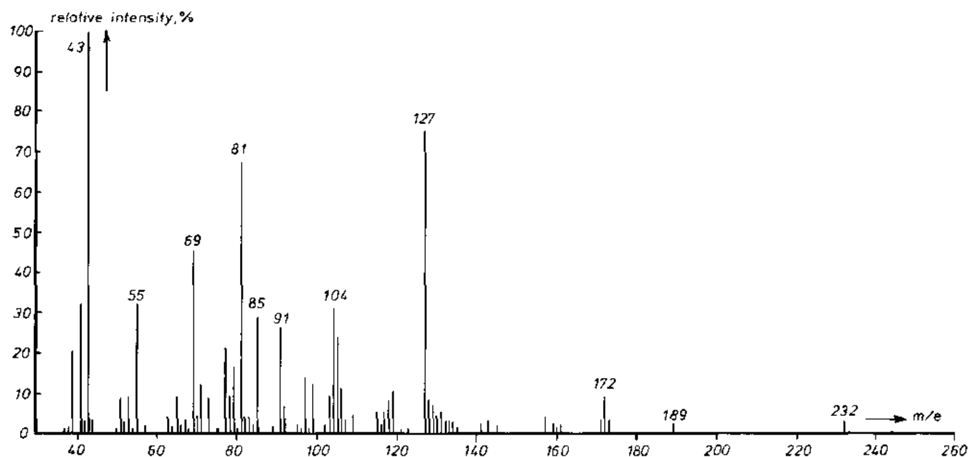


Fig. 6. The mass spectrum of periplanone-A.

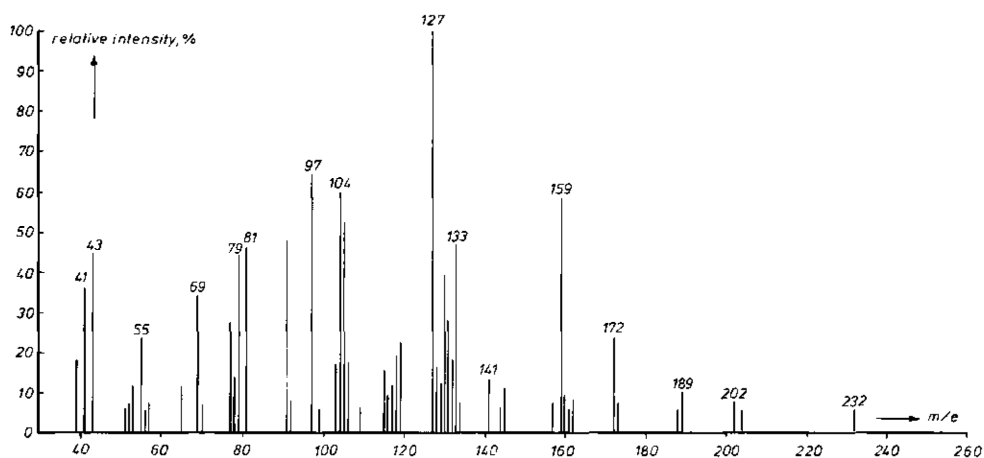


Fig. 7. The mass spectrum of the isomerization product of periplanone-A.

By whatever method the isomerization product is isolated, its mass spectrum invariably has the highest peak at m/e = 127, which is much weaker in periplanone-A. The fragment m/e = 159 appears as a low peak in the spectrum of periplanone-A. However, it is one of the most prominent fragments in the spectrum of the isomerization product. This also holds for the peak at m/e = 133. Apart from these marked differences, there are some minor differences as well.

Mass spectrum of periplanone-B

This is illustrated in Fig. 8. Peakmatching of the parent peak shows that

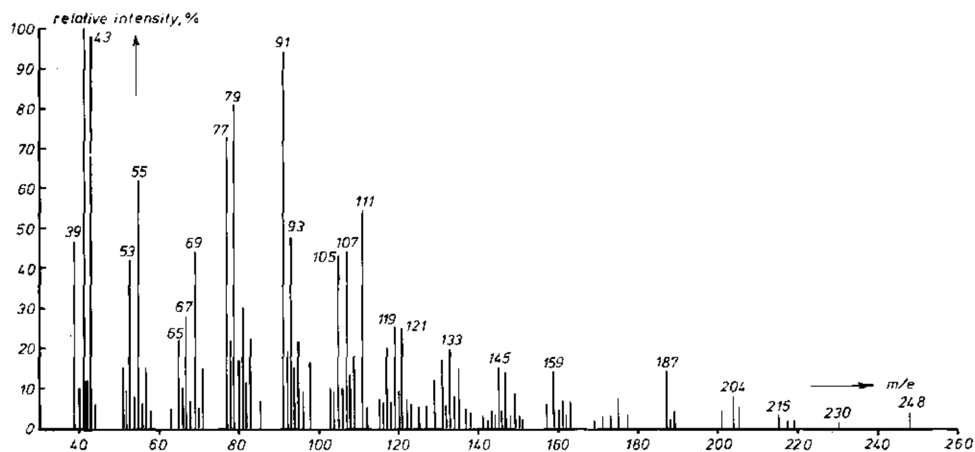


Fig. 8. The mass spectrum of periplanone-B.

it corresponds to $C_{15}H_{20}O_3$ (calculated 248.1412; found 248.1386 and 248.1343). The spectrum agrees very well with that of a compound isolated by Chen (1974) (Nakanishi, personal communication), and with that published by Takahashi and Kitamura (1976[b]), and Kitamura and Takahashi (1976). The molecular formula of periplanone-B suggests that it is a sesquiterpenoid.

Mass spectra of PA_{22-I}, PA_{22-IV}, PA_{22-V}, and PA_{22-VI}

The mass spectra of these compounds are illustrated in Figs 9, 10, 11 and

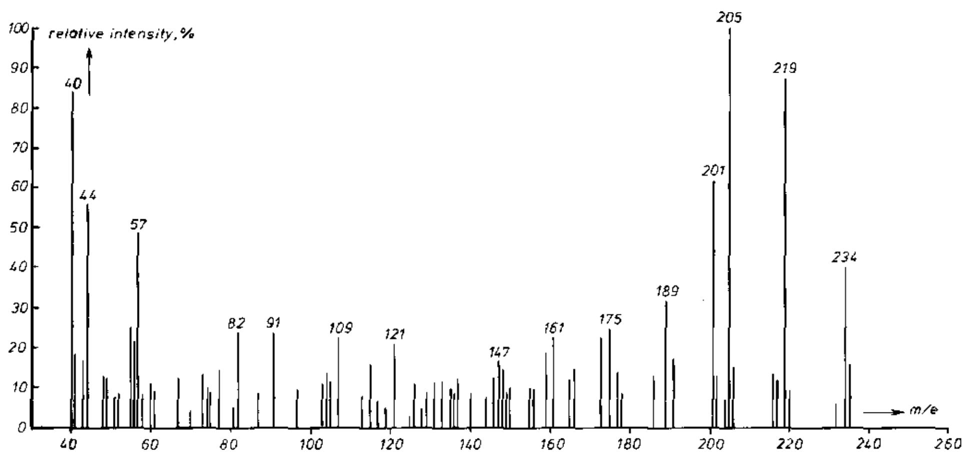


Fig. 9. The mass spectrum of PA_{22}-I.

12. The fragmentation patterns and their parent peaks (PA_{22-I} = 234; PA_{22-IV} = 161; PA_{22-V} = 189 and PA_{22-VI} = 175) differ markedly from those of periplanone-A and -B (Jacobson and Beroza (1965) found for their compound M = 182). The PA-compounds could only be obtained in nanogram amounts, and therefore they could not be investigated further.

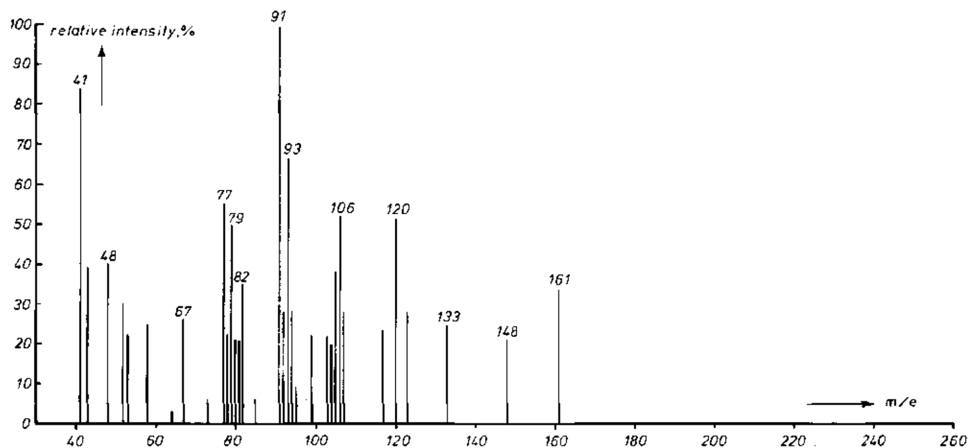


Fig. 10. The mass spectrum of PA_{22}-IV.

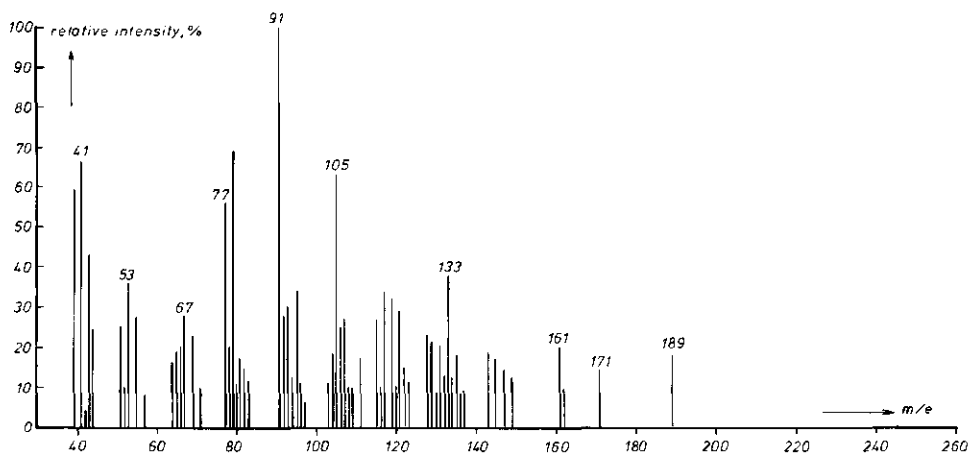


Fig. 11. The mass spectrum of PA_{22}-V.

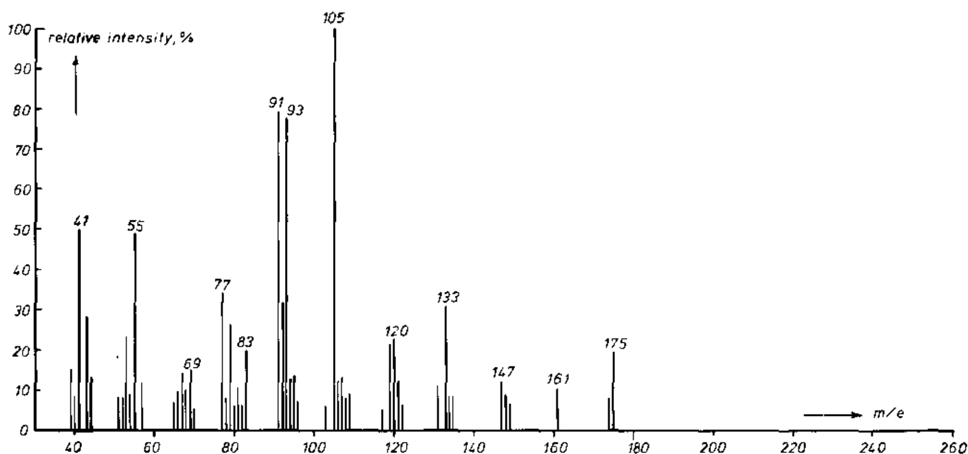


Fig. 12. The mass spectrum of PA_{22}-VI.

Determination of the number of double bonds in some reference compounds by combined hydrogenation-GC/MS

The efficiency of the system was checked by hydrogenation of several reference compounds. The results are given in Table 4.

Although the percentage hydrogenation varied rather widely, the number of double bonds in all compounds tested could be derived from the differences between the mass spectra of the hydrogenated and the non-hydrogenated compounds. The spectra that were obtained from the hydrogenated compounds were all mixed spectra, but the percentage hydrogenation of most compounds could be calculated from the intensities of certain peaks in the spectra. This could not be done for both periplanones.

Table 4 Percentage hydrogenation of various unsaturated straight-chain and cyclic compounds as determined by gas chromatography-mass spectrometry

compound	sample (μg)	% hydrogenation
(Z)-7-tetradecenyl acetate	2	± 90
(E)-2-tetradecenyl acetate	1	± 50
3,6,8-dodecatrienol[1)]	3	± 100
α-santalol	6	± 30
α-santalol	2	± 30
α-santalol	2	± 30
nerolidol	7	± 10
nerolidol	1	± 70
cedrene	1.5	± 20
citral	1	± 30
decanal	1	± 0
farnesol	1	not determined
compound P ($C_{15}H_{24}O$)[2)] (unknown structure)	1	not determined
periplanone-A	1	not determined
periplanone-B	1	not determined

1) (Z)/(E) mixture
2) Ritter et al., 1973

Mass spectrum of hydrogenated periplanone-A

The parent peak of hydrogenated periplanone-A (Fig. 13) is found at m/e = 236. That of periplanone-A is found at m/e = 232, and the increment indicates that the compound contains at least two double bonds. Although in many respects similar, the fragmentation pattern of hydrogenated periplanone-A differs from that of the non-hydrogenated compound in showing a peak at m/e = 137 as well as several groups of peaks which have moved up the m/e scale by 2-4 mass units. It therefore seems likely that periplanone-A contains two double bonds.

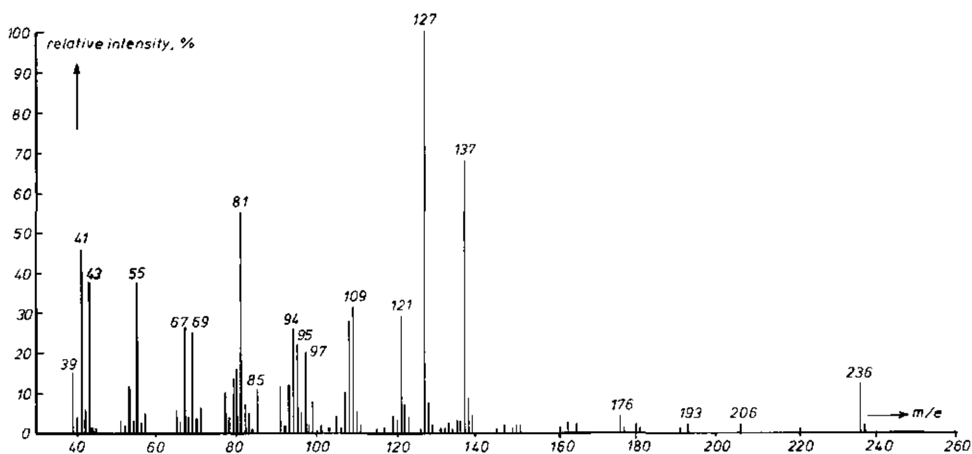


Fig. 13. The mass spectrum of hydrogenated periplanone-A.

Mass spectrum of hydrogenated periplanone-B

The highest m/e value found for hydrogenated periplanone-B was 250 (Fig. 14) (originally 248), indicating that periplanone-B contains at least one double bond. The peak at m/e = 234 (= M − 18) suggests the presence of m/e = 252 as a parent peak, but no such peak was found. The absence of a peak at m/e = 254, which would indicate tri-unsaturation, in addition to the fact that some groups of peaks in the mass spectrum of hydrogenated periplanone-B are moved 2-4 mass

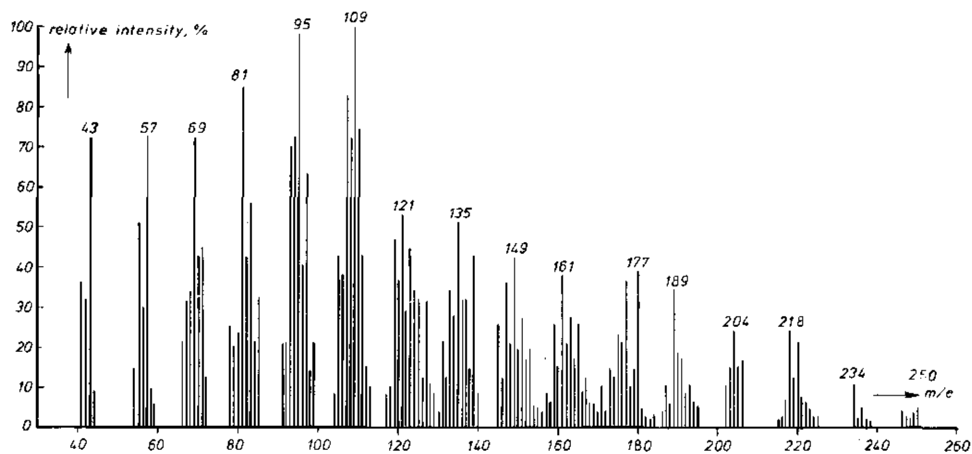


Fig. 14. The mass spectrum of hydrogenated periplanone-B.

units up the m/e scale with respect to the spectrum of periplanone-B, constitutes strong evidence that the molecule of periplanone-B contains two double bonds.

2.3.7 Infrared analysis

Infrared spectrum of periplanone-A

The spectrum is shown in Fig. 15, and the results of a functional group analysis are given in Table 5.

Table 5 Functional groups found in periplanone-A, with the corresponding theoretical and empirical wave numbers of the absorption bands[1]

functional group	absorption band (cm^{-1})		remarks
	theoretical	empirical	
$>C=O$	1725-1705 1325-1215	1710 1235 or 1280	strong medium not conjugated
$(CH_3)_2-C<$	1385-1380 1370-1365	1380 1360	doublet; bands of about the same intensity
CH_3-	2960 ± 10 2870 ± 10	2960 2870	two strong bands
$-CH_2-$	2920 ± 10 2850 ± 10	2925 2850	strong weak
$CH_2=C<$	3095-3075 895-885	3085 875	weak strong

The wave number of the C=O band (1710 cm^{-1}) indicates that this groups is non-conjugated and incorporated either in an open chain or in a ring of six or more carbon atoms. The broad band at 3430 cm^{-1} in the IR spectrum of periplanone-A has about the same intensity as that in the IR spectra of curdione[2] and germacrone-diepoxide[3] (due to the water present in the KBr pellet). The presence of an OH-group therefore can be ruled out.

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1) Infrared spectra of various germacrane derivatives were kindly supplied by Dr Morikawa, The Institute of Food Chemistry, Osaka, Japan; Dr Takeda, Shionogi Research Laboratory, Osaka, Japan, and Dr Yamamura, Meijo University, Nagoya, Japan.

2) Curdione was provided by Dr Hikino, Sendai, Japan.

3) Germacrone-diepoxide was provided by Dr Herout, Prague, Czechoslovakia.

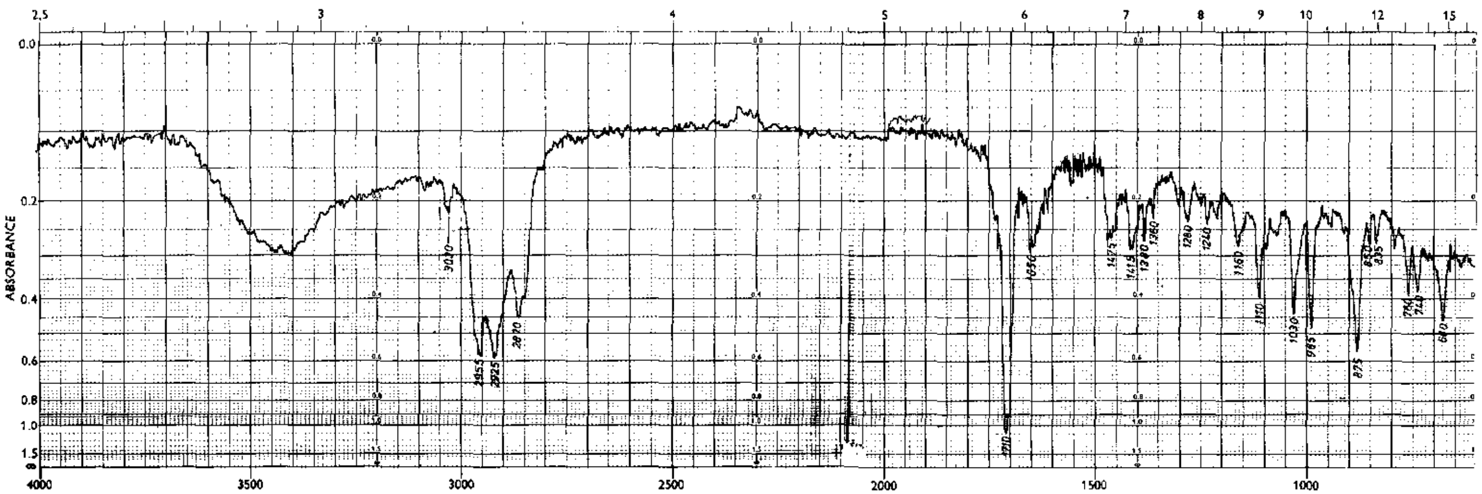


Fig. 15. The infrared spectrum of periplanone-A.

The intensity of the ketone absorption band is about the same as that of the C=O band in the spectrum of germacrone[1)] and germacrone-diepoxide. Therefore the possibility that the second oxygen atom also forms part of a carbonyl group cannot be excluded completely, although this seems less probable.

The spectrum provides no evidence for the presence of hydroxyl groups (band at about 3400 cm^{-1}), ether groups (open chain or a ring containing four or more C-atoms) (strong band near 1150-1060 cm^{-1}), ester groups (a strong band near 1300-1200 cm^{-1}) or carboxyl groups (broad band between 3200-2600 cm^{-1}). The only remaining possibility is that the second oxygen atom is incorporated in an epoxide ring. The absorption band at 3030 cm^{-1} (CH stretch of a CH_2–O–C< group) and bands between 1280-1230 cm^{-1} (-C-O- stretch) support this (Borrow and Searles, 1953; Jones and Sandorfy, 1956).

Since the spectrum does not show the usual bands for (Z)- and (E)-epoxides at 830 cm^{-1} and 900 cm^{-1}, respectively (Shreve et al., 1951), the epoxide (if present) probably occupies an exo position.

The absorption band at 3030 cm^{-1} may be attributed to a (Z)-disubstituted double bond. The mass spectrum of hydrogenated periplanone-A shows that the compound contains two double bonds. If, therefore, the 3030 cm^{-1} absorption band is attributed to an exo-epoxide, there must be other absorption bands for the missing double bond. Since there are no absorption bands in the range 850-800 cm^{-1}, a trisubstituted double bond probably is absent. In germacrone and curdione such bands are found at 850 cm^{-1} and 800 cm^{-1}, respectively. The presence of a (Z)-disubstituted double bond is confirmed by the absorption band at 680 cm^{-1}. The absence of an absorption band at 1600 cm^{-1} indicates that neither double bond is conjugated.

Infrared spectrum of periplanone-B

Figure 16 shows the infrared spectrum of periplanone-B, and Table 6 lists the main functional groups derived from the spectrum.

The mass spectrum of periplanone-B shows that the compound contains three oxygen atoms. One of these is accounted for by the absorption band at 1705 cm^{-1} which can be attributed to a non-conjugated keto group , either in an open chain or in a ring of six or more carbon atoms. Comparison of the IR spectrum of periplanone-B with those of germacrone, germacrone-diepoxide and curdione leads to the conclusion that the presence of another keto group in periplanone-B can be ruled out. Since, in addition, the IR spectrum of the latter shows no bands assignable to hydroxyl, ether, ester or carboxyl groups (see also the IR spectrum of periplanone-A), the two remaining oxygen atoms probably belong to epoxide groups. It is likely that one of these is an exo-epoxide responsible for the absorption at 3030 cm^{-1}, and for the -C-O-stretch absorption near 1250 cm^{-1} (Bellamy, 1958; Jones and Sandorfy, 1956). Since the IR spectrum of periplanone-B does not show the C-H stretch absorptions at 3000 cm^{-1} of the trisubstituted epoxide groups of germacrone-diepoxide, the remaining oxygen atom probably forms part of a (Z)- or an (E)-epoxide group (bands at 890 cm^{-1} and 830 cm^{-1}). The band at 1605 cm^{-1} can be attributed to

1) Germacrone was provided by Dr Sutherland, University of Manchester, England.

the two double bonds, whose presence could also be derived from the mass spectrum of hydrogenated periplanone-B.

Table 6 Functional group analysis of the infrared spectrum of periplanone-B. Theoretical and empirical values for the various absorption bands are given[1)]

functional group	absorption band (cm^{-1})		remarks
	theoretical	empirical	
$>C=O$	1725-1705 1325-1215	1705 1325-1245	strong medium not conjugated
$(CH_3)_2-C<$	1385-1380 1370-1365	1380 1360	medium; doublet
CH_3-	2960 ± 10 2870 ± 10	2950 2870	two strong bands
$-CH_2-$	2920 ± 10 2850 ± 10	2925 2850	medium very weak
$CH_2=C<$	3095-3075 895-885	3075 905	weak strong
C=C-C=C	1600	1605	medium
-C(H)=C(H)-	970-960	970	strong

1) See note Table 5.

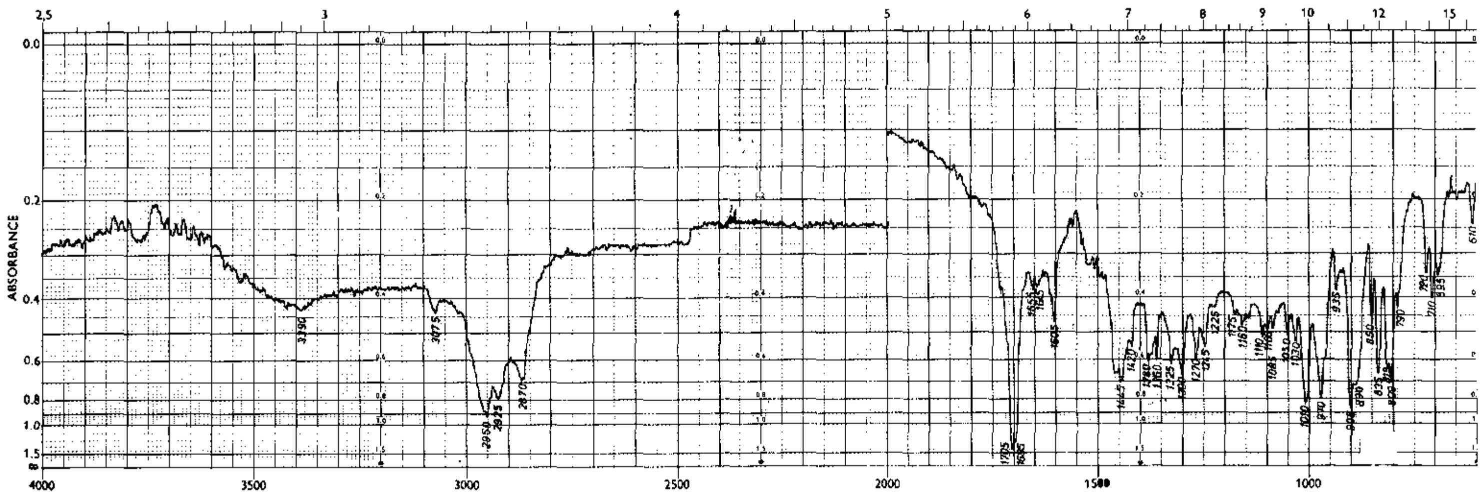


Fig. 16. The infrared spectrum of periplanone-B.

2.3.8 Ultraviolet analysis

Ultraviolet spectrum of periplanone-A

This compound does not absorb in the range 200-400 nm. This agrees with the infrared data, which gave no indication of the presence of a conjugated system (absence of an absorption band at 1600 cm^{-1}).

Ultraviolet spectrum of periplanone-B

This shows a maximum absorption at 226 nm (see Fig. 17), confirming the presence of a conjugated diene system and agrees with that calculated (229 nm) for a conjugated diene system with two alkyl substituents or two ring residues (Woodward, 1942; Scott, 1964).

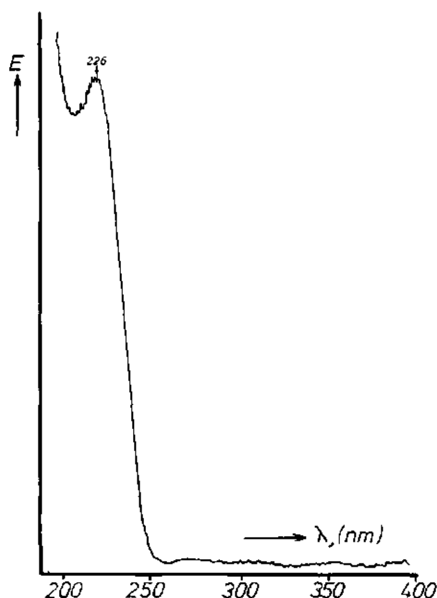


Fig. 17. The ultraviolet spectrum of periplanone-B.

2.3.9 NMR analysis

NMR spectrum of periplanone-B

The spectrum (in CS_2) is shown in Fig. 18. The chemical shifts of the various protons in different solvents are listed in Table 7.

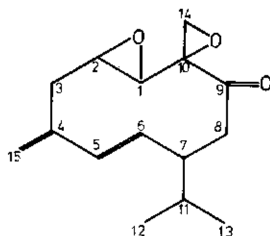
On the basis of mass spectrometry and peak matching the molecular formula was concluded to be $C_{15}H_{20}O_3$ (see MS of periplanone-B). In satisfactory agreement with this, the signals in the NMR spectrum integrate to a maximum of 21 hydrogen atoms.

The mass spectrum of hydrogenated periplanone-B, as well as its infrared spectrum (band at 1600 cm^{-1}) and UV spectrum (absorption at λ_{max} = 226 nm) in-

dicate the presence of a system of conjugated double bonds. That this is a conjugated diene $H_2C = \overset{|}{C} - \underset{H}{C} = \overset{H}{C} - \overset{|}{C} - H$ (E) is borne out by the NMR signals of four olefinic hydrogen atoms (Fig. 18) at δ = 4.87 (s, broadened), δ = 5.02 (s, broadened), δ = 5.91 (d. 16 Hz, broadened) and δ = 5.78 (dd. 16 and 10 Hz). Saturation of the proton at δ = 5.78 showed that it is coupled to a multiplet at δ = 2.06, which is part of an AB-type system (δ = 2.04 dd. 5.5 Hz and (-) . 11.5 Hz ; δ = 2.06 ddt. 5.5 Hz, 7.5 Hz and 10 Hz).

Table 7 Proton chemical shifts of periplanone-B in different solvents (top), and numbering of C-atoms in structure I (below)

solvent / protons	$CDCl_3$ (Varian HR 220, 20 °C)	CS_2 (Varian HR 300, 30 °C)
1-H	3.81	3.52
2-H	2.94	2.68
$3-H_2$	2.69	2.58
	2.76	2.58
$15-H_2$	4.98	4.87
	5.11	5.02
5-H	6.05	5.91
6-H	5.90	5.78
7-H	2.20	2.06
11-H	1.63	1.56
$12-H_3$	0.88[5]	0.87
$13-H_3$	0.91[5]	0.89
$8-H_2$	2.68	2.55
	2.20	2.04
$14-H_2$	2.83	2.63
	3.04	2.84



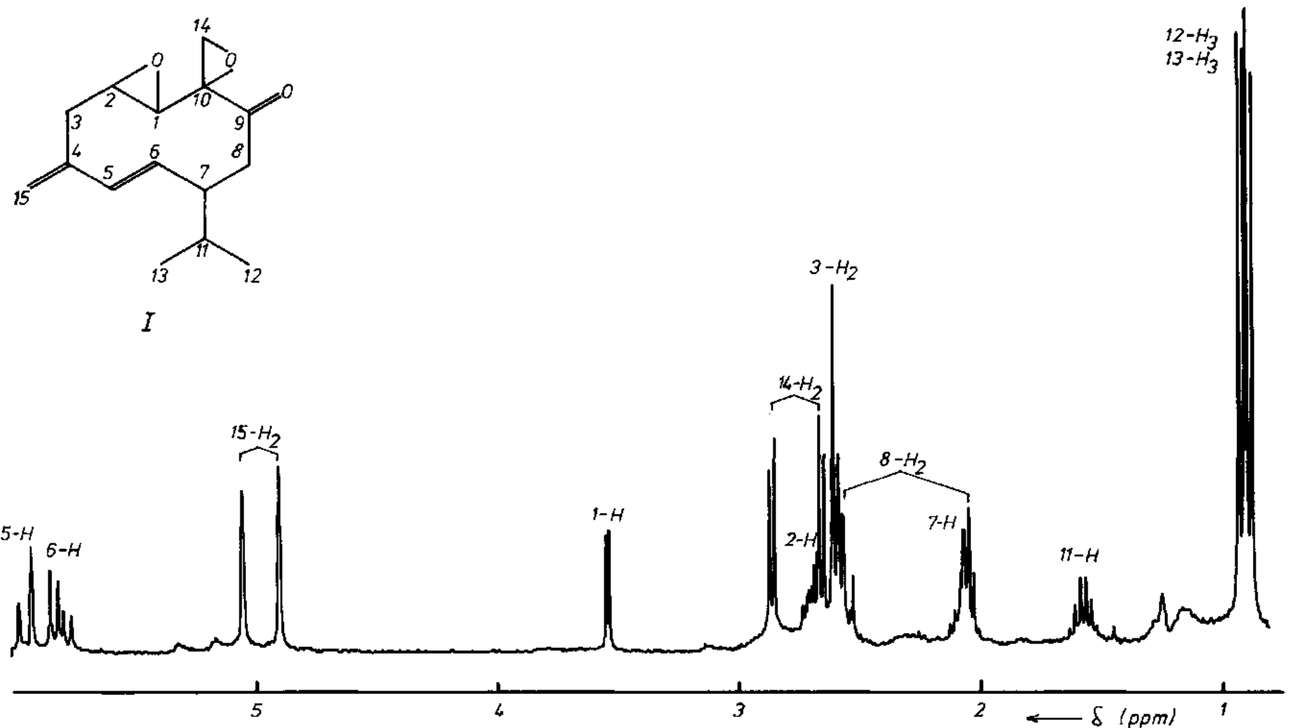
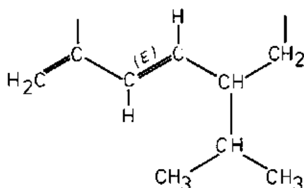


Fig. 18. 300 MHz proton NMR spectrum of periplanone-B in CS_2; assignments of protons in structure I.

Saturation of this two-proton AB-multiplet in turn causes the triplet-like signal at δ = 2.55 (with J = 10 Hz and (-) 11.5 Hz) to collapse into a singlet. Therefore, a CH_2 (δ = 2.04 and δ = 2.55) must be attached to the allylic CH (δ = 2.06).

Saturation of a one-proton multiplet at δ = 1.56 (octuplet ∿ 6.5 Hz) showed that it is coupled to two methyl groups (δ = 0.87 d. 6.5 Hz; δ = 0.89 d.6.5 Hz) as well as to the allylic CH (δ = 2.06 Hz). These data are in accordance with the following partial structure:



Computer simulations of the NMR pattern of this system are in good agreement with the observed spectrum.

The AB pattern at δ = 2.63 and δ = 2.84 (J = 6 Hz) is attributed to an exo--epoxide group >C–O–CH_2, because the other plausible explanation, viz. a CH-CH group without further vicinal couplings is impossible in view of the other structural elements present (see below).

The strong IR absorption at 1705 cm^{-1} constitutes evidence for a non-conjugated ketone. The one-proton doublet at δ = 3.52 (d. 4 Hz) accounts for the third oxygen atom, which forms part of the epoxide group in - CH_2 - CH–O–CH -. Evidence for the proton sequence in this partial structure was obtained by saturation of the one-proton doublet at δ = 3.52, which resulted in simplification of the one-proton multiplet at δ = 2.68 (ddd. 8 Hz, 6 Hz, and 4 Hz). The signal at δ = 2.68 is strongly coupled to a CH_2 signal at δ = 2.58 (J_{gem} = (-) 12 Hz). Spectrum simulation of this ABCX system (for the CS_2 as well as for the $CDCl_3$ solution of the compound, the spectra of which have quite different patterns) confirmed the CH-CH_2 couplings. The vicinal coupling constant of 4 Hz indicates that the epoxide has the (Z) configuration.

Re-running of the spectrum on the SC 300 spectrometer revealed several small couplings that had not been observed before. These provided important additional information on the structure. One result of saturation of the protons around δ = 2.5-2.6 was that the broadened singlet at δ = 5.02 was changed into a narrow triplet (Fig. 19, trace b.). Saturation of the olefinic proton at δ = 5.02 in turn sharpened the signal at δ = 2.58 (Fig. 19, trace c.).

The demonstrated coupling constitutes evidence that the methylene group in - CH_2 - CH–O–CH - must be allylic to the vinylidene double bond, and the following partial structure emerges:

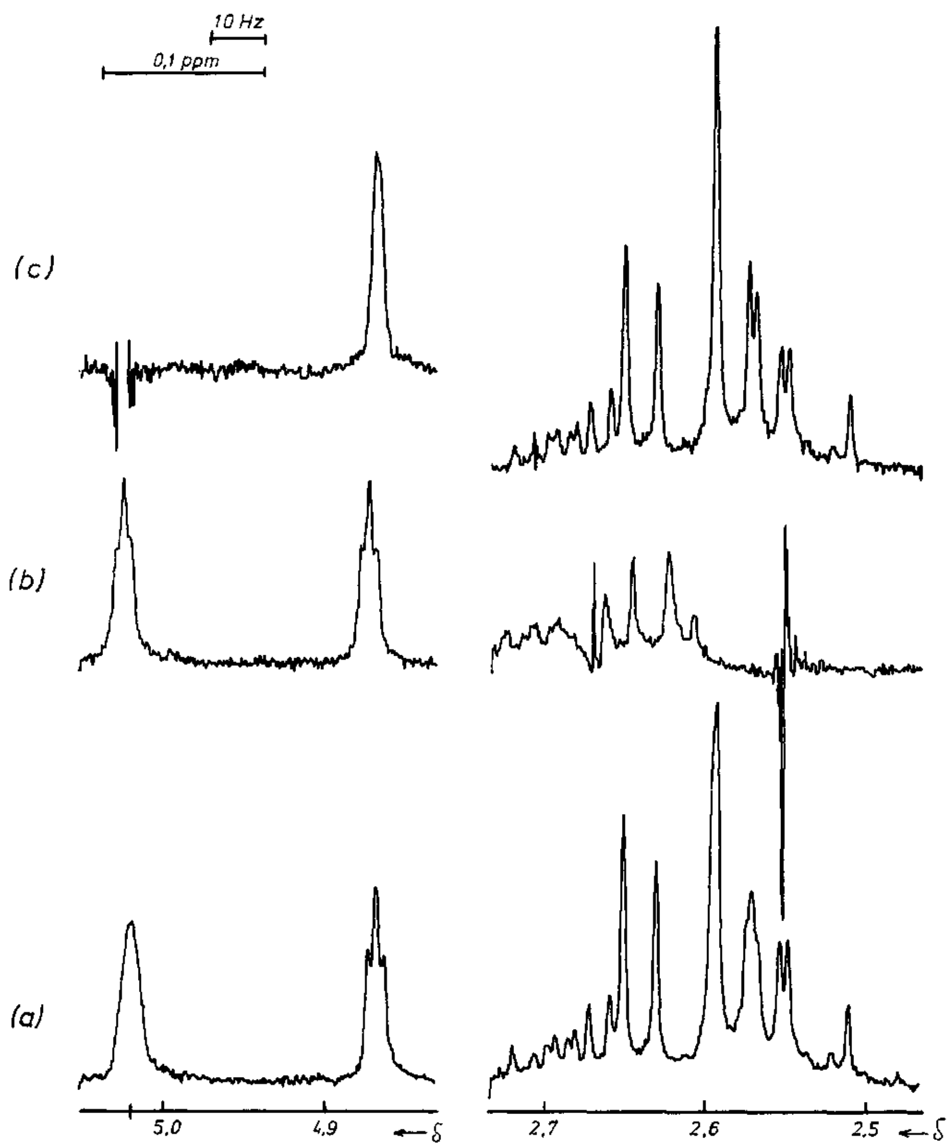
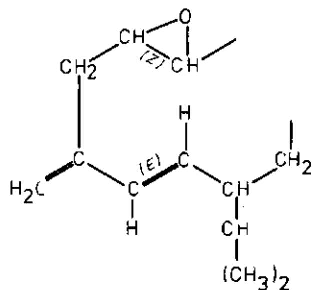
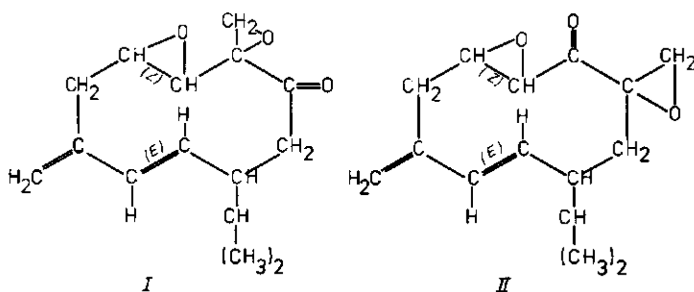


Fig. 19. Partial proton NMR spectra of periplanone-B in CS_2:
(a) without saturation
(b) saturation around δ = 2.5 - 2.6
(c) saturation of δ = 5.02



Combination of this partial structure with the remaining ketone and epoxide groups leads to two possible structures, I and II (apart from stereochemical differences), both possessing a ten-membered alicyclic ring:



Only structure I has the germacrane-type skeleton, which obeys the head-to--tail isoprene rule.

Structure II is also composed of three isoprene units, but in addition to a head-to-tail coupling, it also contains a tail-to-tail arrangement.

The chemical shifts expected for - $C\underline{H}_2$ - CH - $CH(CH_3)_2$ in structure II are about 0.5 ppm lower than those found for periplanone-B, whereas the expected values in structure I are in good agreement with those found (see Fig. 20).

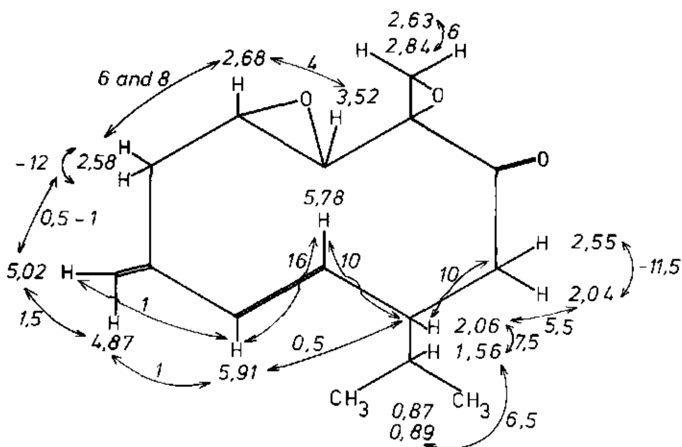


Fig. 20. Proton chemical shifts and coupling constants of periplanone-B in CS_2, as assigned to structure I.
(1 Z, 5 E)-1, 10(14)-diepoxy-4(15), 5-germacradiene-9-one).

2.4 DISCUSSION

The scientific dispute that arose when Jacobson et al. (1963) published the structure they assigned to the sex pheromone of the American cockroach, *Periplaneta americana*, could have been avoided, if they had realized that the pheromone was not a single compound (Jacobson et al., 1963; Wharton et al., 1963; Jacobson and Beroza, 1965). The discussion came to an end when the proposed compound was synthesized and shown to be completely inactive (Day and Whiting, 1964 and 1966; Wakabayashi, 1967). None of the compounds described in this paper have any spectroscopic resemblance to the compound isolated by Jacobson et al. (1963). The phenomenon that a pheromone consists of more than one component seems to be the rule rahter than the exception. The fact that the pheromone of *P. americana* contains more than one component is now firmly established (Chen, 1974; Persoons et al., 1974; Chow et al., 1976, and Takahashi and Kitamura, 1976[b]; Kitamura and Takahashi, 1976). Although it is difficult to compare the experimental data, it seems certain that periplanone-B has been isolated by Chen (1974) (Nakanishi, personal communication) and also by Takahashi and Kitamura (1976[b]) and Kitamura and Takahashi (1976). The mass spectra of the compounds they isolated are in good agreement with the mass spectrum of peripla-

none-B. The amounts they were able to isolate so far have been insufficient for a complete structure elucidation. No spectral data have yet been published for the other compounds mentioned in the literature. Chow et al. (1976) obtained one active fraction by column chromatography on SiO_2 of a crude extract of whole insects. Gas chromatography of this fraction on DEGS and OV 17 yielded one and two active fractions, respectively. Because Chow et al. give retention times instead of Kovât's Retention Indices, it is impossible to say whether the compounds isolated are the same as ours. The question whether all compounds isolated are true pheromones or (biologically active) degradation products remains unsolved. The main degradation (= isomerization) product of periplanone-A is biologically inactive. The other compounds (PA_{22}-series) could be isolated only in minute amounts, so that it was impossible to establish whether they are structurally related to one another or to periplanone-A or periplanone-B. The IR spectra of periplanone-A and periplanone-B suggest that the two are structurally related.

The molecular formulae of periplanone-A and periplanone-B might suggest that the latter is an oxidation product of the former. However, attempts to oxidize periplanone-A to periplanone-B, were unsuccessful. If periplanone-B is a simple oxidation product of periplanone-A, the latter would probably be present in the insect's gut in much larger amounts. However, the faecal extracts contain periplanone-A and periplanone-B in a ratio of about 1:10, and extracts of alimentary tracts mainly contain periplanone-B with very little periplanone-A.

The finding that the pheromone is produced in the midgut conflicts with a statement by Stürckow and Bodenstein (1966) that the pheromone is synthesized in the head, although they were unable to find a pheromone gland there. Their experiments are not convincing, because biologically active material can be obtained from all parts of the insect by extraction with an organic solvent. This finding may be explained by diffusion of the pheromone into the wax layer of the insect's cuticle as the pheromone is excreted. Our finding that the pheromone of *P. americana* is produced in the midgut rather than in the head, has found confirmation in work by Bodenstein (1970) and by Takahashi and Kitamura (1976[a]). Our own attempts to find a gland or a gland-like organ in the region where the pheromone is produced were unsuccessful, but Takahashi and Kitamura (1976[a]) found marked differences between the midgut epithelium of males and that of pheromone producing females. For the cockroach, *Byrsotria fumigata*, Moore and Barth (1976) proposed the genital atrium as the main point of pheromone release and, possibly, production.

Interspecific responses exist between various *Periplaneta sp.*, *P. americana*, *P. brunnea*, *P. autralasiae* and *P. fuliginosa* (Frazier, 1970), and also between *P. americana* and *P. japonica*. Moreover, *P. americana* and *P. japonica* also respond manifestly to germacrene-D (Tahara et al., 1975; Takahashi and Kitamura, 1976[b]). Since it has been found that the major component of the sex pheromone of *P. americana* is a germacrane derivative, these interspecific responses suggest that the sex pheromones of *Periplaneta* cockroaches are all germacrane derivatives. If this is indeed the case, the situation would be the same as for several families of *Lepidoptera*, the sex pheromones of which are also closely related.

The responses of cockroaches to pheromone mimics, are rather confusing. Several compounds have been reported to be sexual excitants for *P. americana*: D/L-borneol acetate, α-santalol, β-santalol (Bowers and Bodenstein, 1971), α-cadinol and T-cadinol (Nishino and Tsuzuki, 1975) and germacrene-D (Tahara et al., 1975; Kitamura et al., 1976). In our behavioural tests, the only compound which elicited a complete display of sexual excitation was germacrene-D. This is in agreement with the work of Kitamura et al. (1976). Takahashi and Kitamura (1976[b]) also found a slight response (a 10 mg sample was used) to D/L-borneol acetate. Not being available, α-cadinol and T-cadinol could not be tested. In EAG experiments the compounds mentioned above also elicit responses, but only in very high concentrations. This weakness of EAG responses throws some doubt upon the value of experiments conducted by Washio and Nishino (1976) and by Washio et al. (1976), who tested a variety of terpenoids, including germacrene-D, in EAG tests, not supplemented by behavioural experiments.

Isomerization of germacrane derivatives has been reported before (Herout et al., 1959). Germacrone isomerizes at temperatures above 150 °C to elemenone by ring-closure of the cyclodecane ring (Fig. 21). This finding was confirmed in the present investigation by comparison of the MS, IR and NMR spectra of germacrone and its isomerization product. Periplanone-A seems to be less stable and isomerizes even at low temperatures.

It has also been reported that, under slightly more forcing conditions, several cyclodecane oxides undergo thermal rearrangements involving closure of the cyclodecane ring (Brown et al., 1969; Wada et al., 1969).

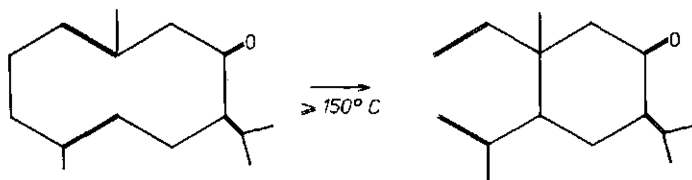


Fig. 21. Isomerization of germacrone to elemenone (Herout et al., 1959).

Compounds with a methylene group adjacent to a ketone group are characterized by an absorption band in the infrared spectrum at 1410-1415 cm^{-1}, as has been established for a variety of compounds (Francis, 1951; Jones et al.,1952; Nolin and Jones, 1953; Tsankova and Ognyanov, 1976). This absorption band is very intense in some compounds, but very weak in others. In periplanone-A it is of moderate intensity, whereas in periplanone-B it is very weak. Comparison of the infrared spectra of several germacrane derivatives (see note to Table 5), with and without a CH_2-group adjacent to a ketone group, allows of no definite conclusions.

The presence of the group $-\overset{O}{\overset{\|}{C}}-CH_2-$ in periplanone-B is borne out by the occurrence of a fragment M-44 (= $M-CH_3C{\lt}^{O}_{H}$)(carbon atoms nos 8 and 9 in structure I) in the MS of periplanone-B. Formation of this fragment from structure I

may well take place via several types of rearrangement that seem less plausible for structure II. The reported biological activity of another germacrane derivative, viz. germacrene -D (Tahara et al., 1975) also supports structure I.

These data, together with those from the NMR spectrum of periplanone-B, lead to the conclusion that the ketone group in periplanone-B has a CH_2 group adjacent to it, even though this conclusion is not convincingly supported by the mass and IR spectra of the compound.

Attempts to find a possible Nuclear Overhauser Effect between the proton at δ = 5.78 (in CS_2) and several other hydrogen atoms were unsuccessful. Since the negative results may well have been due to instrumental factors, or the size or condition of the sample, no conclusions were drawn from these experiments.

Addition of $Eu(dpm)_3$ to a solution of periplanone-B in $CDCl_3$ caused the proton signals to shift slightly. After addition of a second amount of $Eu(dpm)_3$ the signals appeared to be no longer related to those in the original spectrum, probably owing to decomposition of the sample. The results obtained after the first addition of the shift reagent show the larger shifts for the protons in the - CH_2 - CH - CH - and >C - CH_2 groups (epoxide O bridging), all other protons exhibiting smaller shifts. These can be regarded as agreeing with both structure I and II.

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3. SEX PHEROMONES OF THE MOTH, *ARCHIPS PODANA*: ISOLATION, IDENTIFICATION AND FIELD EVALUATION OF TWO SYNERGISTIC GEOMETRICAL ISOMERS*

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ABSTRACT

By means of analytical and electroantennogram methods, *cis*-11- and *trans*-11-tetradecenyl acetate have been isolated and identified as the sex pheromones of the fruit tree tortrix moth, *Archips podana*. In contrast to the single compounds, mixtures in a ratio of about 1 : 1 are highly attractive to male moths in field experiments.

INTRODUCTION

The fruit tree tortrix moth *Archips podana* (Scop.) (= *Cacoecia podana*) is found in the greater part of Europe, from the Mediterranean area to as far north as Scandinavia. In many places this moth is a serious pest in apple and pear orchards, e.g. in the north of the Netherlands and in Kent, England. In preliminary experiments in which males of this insect were caught in traps baited with virgin females, we were able to demonstrate the excretion of a sex pheromone by the latter.

As for practical application of pheromones, e.g. in monitoring or direct control, these compounds have to be available in sufficient quantities, we decided to undertake its identification. This paper describes in detail the structural elucidation of the two sex pheromones isolated from *A. podana* and their evaluation in the field.

MATERIALS, METHODS AND RESULTS

Rearing and extraction of the female moths

A. podana moths were reared on a semi-synthetic diet of wheat germs, previously used for rearing *Adoxophyes orana* (Ankersmit, 1968). *A. podana* can be reared on the same diet only if apple leaves are added to it (100 g/l of diet).

From 1500 laboratory-reared virgin females 2 to 3 days after emergence, the last three abdominal segments were clipped and homogenized in methylene chloride. The resulting slurry was filtered and the residue re-extracted for 7 hrs with freshly distilled methylene chloride in a Soxhlet apparatus. The extracts were combined and the solvent was removed by distillation at atmospheric pressure. The residue was a small amount of a yellow oil, which elicited a weak response in the electroantennogram (EAG) assay.

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Electroantennography

Synthetic compounds. At a first screening, we tested the antennal responses elicited by *cis*- and *trans*-dodecenyl, tetradecenyl, and hexadecenyl acetates and the corresponding alcohols (Table 8). Distinct responses were produced only by the tetradecenyl acetates (tda) and to a lesser extent by the corre-

Table 8 Various C_{12}, C_{14}, and C_{16} acetates and alcohols tested by the EAG method on *Archips podana**

Position of double bond	C_{12}				C_{14}				C_{16}			
	Acetate		Alcohol		Acetate		Alcohol		Acetate		Alcohol	
	cis	*trans*	*cis*	*trans*	*cis*	*trans*	*cis*	*trans*	*cis*	*trans*	*cis*	*trans*
2	+	+	+		+	+						
3	+	+	+	+								
4	+	+	+	+	+	+						
5	+	+	+	+	+				+	+		
6	+	+	+		+	+	+	+	+	+		
7	+	+	+	+	+	+	+	+				
8	+	+	+	+	+	+	+	+				
9	+	+	+	+	+	+	+	+	+			
10	+		+	+	+	+	+	+	+			
11					+	+	+	+	+	+		
12					+	+	+					
13									+	+		
14									+	+		

*Only compounds marked with + were tested.

sponding alcohols. *Cis*-11- and *trans*-11-tda elicit much stronger responses than any of the other positional isomers (Fig. 22). Moreover, the recovery

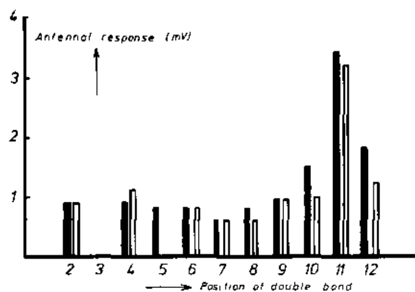

Fig. 22. Antennal responses of *A. podana* males to various mono-unsaturated *cis*- and *trans*-tetradecenyl acetates. Black bars: *cis*-isomers. White bars: *trans*-isomers.

Roelofs, W.L. and Tette, J.P. (1970) Sex pheromone of the oblique-banded leaf-roller moth. Nature, Lond. 226, 1172.

Tamaki, Y., Noguchi, H., Yushima, T., Hirano, C., Honma, K., and Sugawara, H. (1971) Sex pheromones of the summerfruit tortrix: isolation and identification. Kotyu 39, 338-340.

4. SEX PHEROMONE OF THE FALSE CODLING MOTH *CRYPTOPHLEBIA* (= *ARGYROPLOCE*) *LEUCOTRETA* (LEPIDOPTERA: TORTRICIDAE) TRANS-8-DODECENYL ACETATE, A CORRECTED STRUCTURE[1]

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ABSTRACT

The sex pheromone of the false codling moth, *Cryptophlebia leucotreta*, has been identified by means of electroantennographic and analytical techniques as *trans*-8-dodecenyl acetate possibly containing a small percentage of its *cis*-isomer. Traps baited with mixtures of *cis*-8- and *trans*-8-dodecenyl acetate are highly attractive in field experiments. This finding contradicts earlier reports, which state that the pheromone is *trans*-7-dodecenyl acetate.

INTRODUCTION

Isolation and identification of the sex pheromone of the false codling moth, *Cryptophlebia* (= *Argyroploce*) *leucotreta* (Meyr)(Tortricidae) has been reported by Read et al., (1968, 1974). They concluded that the sex pheromone is *trans*--7-dodecenyl acetate (*trans*-7-dda). However, field experiments by another research team indicated that *trans*-7-dda is not attractive to false codling moth males (Rauch, personal communication). These data seemed to justify a re-investigation of the structure of the sex pheromone of this moth.

METHODS AND MATERIALS

Electroantennography with model compounds

A first screening was carried out using the available series of dodecenyl, tetradecenyl and hexadecenyl acetates and the corresponding alcohols (Persoons et al. 1974).

Preparation of the pheromone extract

C. leucotreta moths were reared on an artificial medium at Procida Laboratories, Saint-Marcel, Marseille, France. The abdominal tips of about 1500 laboratory-reared virgin females, 2-3 days old had been collected in aqueous ethanol during the past year. It was used as the starting material. As an aqueous ethanol mixture is not suitable for pheromone extraction, both the abdominal tips and the aqueous ethanol had to be extracted with another solvent. The ethanol was extracted with methylene chloride and hexane respectively; the abdominal tips were homogenized and also extracted with methylene chloride. All solvents had

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1) Published in Med. Fac. Landbouww. Rijksuniv. Gent, 41/2, 1976, pp. 937-943.

been freshly distilled. However, no activity could be detected by electroantennography neither in the hexane extract, nor in the methylene chloride extract. As the starting material supplied by Procida had been kept for a long time in aqueous ethanol, hydrolysis was thought to be a possible cause of the loss of activity. Acetylation in a mixture of pyridine and acetic anhydride (10:1)restored the activity, as was shown by the electroantennogram (EAG) assay.

Purification of the pheromone extract by column chromatography

The re-acetylated product was subjected to gel permeation chromatography in acetone on a Sephadex LH 20 column (0.7 × 150 cm, flow rate 20 ml/h) and fractions of 5 ml were collected. The active fractions were determined by the EAG method, and the combined active fractions were subjected to column chromatography on silicagel (0.8 × 60 cm, particle size ⩽ 50 μm, flow rate 16 ml/h) using hexane-2.5 % ethylacetate as eluent. The activity was located by the EAG method.

Gaschromatography of pre-purified pheromone extract

To locate the activity, 1 μl samples of an active fraction collected from the silicagel column were injected on a DEGS column (diethyleneglycol succinate, 5 % on Chromosorb W(AW), 2 m, 165 °C, 20 ml N_2/min) and on an OV 101 column (5 % on Chromosorb G, 195 °C, 30 ml N_2/min), respectively. One minute fractions were collected and assayed by the EAG method. On the DEGS-column the activity was confined to fractions Nos 10 and 11 (retention time of the pheromone = 9.8 minutes; *cis*-8-dda = 10.6 min); on the OV 101 column the activity was recovered in fractions Nos 11 and 12 (retention time of the pheromone = 11.2 minutes; *cis*-8-dda = 11.0 min). After location of the activity samples (10 μl) of the pre-purified extract were injected on the DEGS-column and the active parts were collected and re-injected on an OV 101 column. The effluent from the OV 101 column was collected and used for further chemical analysis.

In order to determine more precisely the retention time of the pheromone a PDEAS column was used (3 % phenyldiethanolamine succinate on Chromosorb W(AW), 6.5 m × 2.3 mm, 170 °C, 17 ml N_2/min). Unlike the columns mentioned before, this column is capable of separating some of the *cis-trans* isomers of C_{12}acetates. The sample used was purified as described above.

Mass spectrometry

A sample containing about 1 μg of the pheromone was purified as described above and was re-injected on a GC/MS apparatus (LKB 9000) fitted with a 2 m 5 % OV 101 column at 190 °C.

Infrared analyses

An infrared spectrum (in KBr) was made of a sample containing about 15 μg of the purified pheromone, using a Perkin Elmer double beam spectrophotometer (model 421).

Ozonolysis

Two samples, each containing 2-3 μg pure pheromone were subjected to ozonolysis according to the method described by Beroza and Bierl (1967), using

either heptane or glycerol triacetate as a solvent.

Synthesis

The two geometric isomers of 8-dodecenyl acetate have been synthesized as shown in the diagram of Fig. 25. Upon treatment with hydrochloric acid in toluene at 90 °C, the diol (1), yields the alcohol (2), which is converted into its pyranyl ether (3). This compound (b.p. 100 °C/0.5 mm) reacts with lithium acetylide/ethylenediamine complex in dimethyl sulphoxide (4 hrs at 25 °C) to give the acetylenic compound (4). To a tetrahydrofuran solution of this derivative, butyllithium is added at 0 °C, followed by a solution of propyl bromide in hexamethylphosphoric triamide at 20 °C, after which the mixture is allowed to react for 3 hrs, yielding the C_{12} compound (5).

Upon being heated at 100 °C for 4 hrs in an acetic acid/acetyl chloride mixture, compound (5) is transformed into its acetate (6), which is purified by chromatography over silica (developing solvent: petroleum ether, G grade, containing 7 % of diethyl ether) and then submitted to hydrogenation over palladium (5 % on a barium sulphate carrier) in an ethanol solution containing a little quinoline. The resulting *cis*-8-dodecenyl acetate (*cis*-8-dda) (7) is isolated by chromatography on silica impregnated with silver nitrate (developing solvent: benzene) and purified by distillation (b.p. 80 °C/0.05 mm; n_D^{25} = 1.4425). Purity ⩾ 99 %.

Reduction of the acetylene (5) by lithium in liquid ammonia (7 hour reflux) yields the *trans*-ethylenic derivative (8), which is acetylated upon being heated at 100 °C for 5 hrs in an acetic acid/acetyl chloride mixture. The resulting *trans*-8-dodecenyl acetate (*trans*-8-dda) (9) is isolated by chromatography on silica (developing solvent: petroleum ether, G grade) and purified by distillation (b.p. 76 °C/0.05 mm; n_D^{25} = 1.442; IR maximum (in $CHCl_3$) at 960 cm^{-1}, corresponding to *trans*-CH=CH-). Purity > 99 %.

The *cis*- and *trans*-isomers of 8-dodecenyl acetate are clearly distinguishable by thin layer chromatography on silica impregnated with silver nitrate.

Field experiments

Field experiments were carried out in Ivory Coast in cotton fields during the 1975 season. Traps were baited with either the individual compounds (*cis*--8-dda or *trans*-8-dda) or with various mixtures of them (20 mg in polyethylene caps). The traps baited with virgin females contained two individuals each.

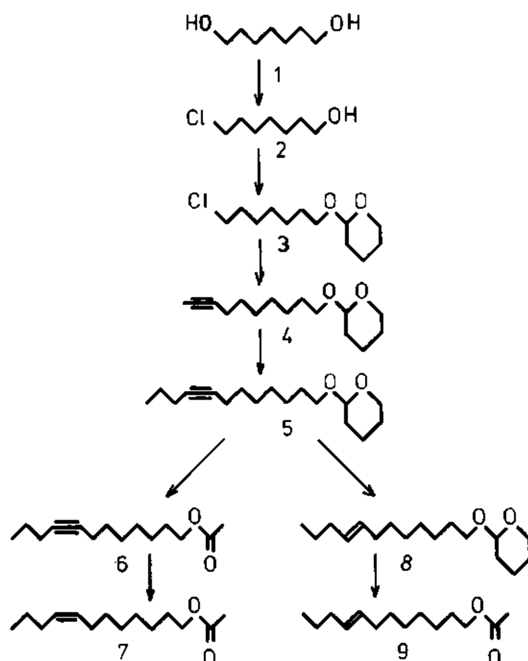

Fig. 25. Scheme for the synthesis of *cis*-8-dodecenyl acetate and *trans*-8-dodecenyl acetate.

RESULTS

In the electroantennographic experiments distinct responses were elicted by *cis*-8- and *trans*-8-dda (purities $\geqslant$ 99 %). The response to *trans*-7-dda (which, according to Read et al., 1968, 1974, is the pheromone) is much weaker. Figure 26 gives the responses of male *C. leucotreta* moths to a series of mono-unsaturated *cis*- and *trans*-dodecenyl acetates. These data suggest that the pheromone consists of either *cis*-8-dda, or *trans*-8-dda or a mixture of these compounds.

The gas chromatographic retention times on a PDEAS-column (see Table 11) do not allow definite conclusions to be drawn about the position of the double bond. The retention time of the pheromone is almost the same as that of *trans*--8-dda. Table 11 gives the retention times of several reference compounds and of the pheromone on a PDEAS column.

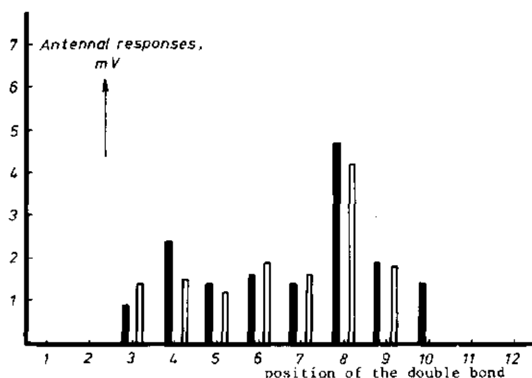

Fig. 26. Antennal responses of *C. leucotreta* male moths to various mono--unsaturated *cis*- and *trans*-dodecenyl acetates. Black bars: *cis*--isomers. White bars: *trans*-isomers. 5 duplicates, 10 µg samples.

Table 11 Retention times of the pheromone of *C. leucotreta* and of some reference compounds on a PDEAS column

C_{12}acetate	retention time (min)
saturated	13.6
trans-7-	14.9
trans-8-	15.1
cis-8-	15.6
pheromone	15.2

The mass spectrum confirmed the pheromone to be a mono-unsaturated C_{12}acetate (m/e = 166 = M $-CH_3COOH$). As acetates show no parent peaks or only very weak ones in the mass spectrum, the molecular weight can be calculated from these data as m/e = 226. Neither the position of the double bond nor its geometry can be derived from the mass spectrum. A band at 960 cm^{-1} in the infrared spectrum indicated the pheromone to be a *trans*-unsaturated compound. Apart from the intense band at 960 cm^{-1}, *trans*-compounds also have a weak band at 3010 cm^{-1}, where *cis*-compounds have an intense band. Therefore, the presence of a low percentage of the *cis*-isomer in the pheromone cannot be excluded.

By comparison of its retention time with those of synthetic aldehydes, butanal was identified as the only cleavage product of ozonolysis. This finding constitutes conclusive evidence for the 8-position of the double bond.

The results of the field experiments are summarized in Table 12. A more de-

tailed description of the field experiments will be given by Angelini et al. (1976, in press).

Table 12 Field trapping results of *C. leucotreta* males. The traps baited with pheromone were exposed for 104 days, and those with virgin females for 94 days. Three duplicates; purities of the compounds ⩾ 99 %; 20 mg per trap.

ratio *cis*-8/*trans*-8-dda	total numbers caught
100/0	115
70/30	2893
50/50	3009
30/70	3042
0/100	41
traps baited with two virgin females each	2341

DISCUSSION

The analytical data mentioned so far are only consistent with the structure *trans*-8-dda. They contradict previous publications (Read et al., 1968, 1974) not only on the basis of chemical analysis but also with regard to field data.

As far as we know it has not yet been proven, for any insect, that *trans*-8--dda is a natural pheromone or component of such a pheromone. On the other hand synthetic *trans*-8-dda has been shown to attract several tortricid species(Roelofs and Cardé, 1974[b]; Ritter and Persoons, 1975, 1976, in press). The sex pheromone of the oriental fruit moth, *Grapholita molesta*, has been identified as *cis*-8-dda (Roelofs et al., 1969). It is synergized by its geometrical isomer, *trans*-8-dda, but the presence of the latter in the insect is uncertain (Beroza et al., 1973; Roelofs and Cardé, 1974[a]). This can also be stated for the lesser appleworm, *Grapholita prunivora* (Roelofs and Cardé, 1974[a]).

The importance of investigation of the possible role of the geometrical isomer of an unsaturated pheromone has been stressed before (Persoons and Ritter, 1975). In the EAG experiments both *cis*-8-dda and *trans*-8-dda elicited responses of about equal intensities. From these results it was impossible to conclude which of these two compounds constitutes the pheromone. For the moth *Rhyacionia buoliana*, Smith et al. (1974) found in their EAG experiments also a marked response (of about equal intensity as for the pheromone) to the geometrical isomer of the pheromone *trans*-9-dda. In this case the *cis*-isomer proved to be a strong inhibitor. On the basis of EAG studies the *cis*-isomer was included in the field experiments with *C. leucotreta* although no *cis*-isomer could be detected in the extracts, neither by gaschromatographic analysis nor

in the infrared spectrum. Its synergistic role has clearly been demonstrated by the field experiments. For the ratios *cis*-8/*trans*-8-dda tested so far, no clear cut optimum in attractancy has been found, although a decrease in the amount of *cis*-8-dda produces a slight increase in numbers of moths caught. The optimum attraction ratio will have to be decided by experiments with traps baited with mixtures containing less than 30 % of *cis*-8-dda. It may also be necessary to use smaller quantities of the pheromone mixture than 20 mg. Too large amounts might give rise to a "plateau" in the curve relating isomer ratio and attractancy in sex traps.

The fact that the false codling moth pheromone is a 12-carbon chain compounds, agrees with the rule that members of the subfamily of the Olethreutinae have indeed 12-carbon chain compounds as their pheromones. No exceptions to this rule have been found so far. However, the large bud moth *Zeiraphera diniana*, also belonging to the subfamily of the Olethreutinae is attracted by *trans*-11-tetradecenyl acetate, but it has not been proven to be the natural pheromone of this moth (Roelofs et al., 1971).

ACKNOWLEDGMENTS

We thank P.J.F. Nooijen for the IR analysis, Dr E. Talman for the mass spectrometry, and A. Angelini for carrying out the preliminary field experiments. Model compounds for EAG were kindly supplied by Dr W.L. Roelofs and Drs S. Voerman. This research was supported by a grant from Procida, Saint-Marcel, Marseille, France.

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5. SEX PHEROMONE OF THE FALSE CODLING MOTH, *CRYPTOPHLEBIA LEUCOTRETA* (LEPIDOPTERA: TORTRICIDAE): EVIDENCE FOR A TWO COMPONENT SYSTEM*

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ABSTRACT

The sex pheromone of *Cryptophlebia leucotreta* females is a mixture of (E)-8- and (Z)-8-dodecenyl acetate (dda). The compounds have been isolated from extracts of *C. leucotreta* females and were identified by means of chromatography on a silver-impregnated column, and by combined GC-MS analysis and ozonolysis. Optimal catches of males were obtained with mixtures of (E)-8- and (Z)-8-dda in the range of 70 : 30 to 30 : 70.

Keywords

Sex pheromone, false codling moth, Tortricidae, *Cryptophlebia (Argyroploce)*, (Z)-8-dodecenyl acetate, (E)-8-dodecenyl acetate.

INTRODUCTION

In reports by Read et al. (1968, 1974), it is suggested that the sex pheromone of the false codling moth, *Cryptophlebia leucotreta*, is (E)-7-dodecenyl acetate ((E)-7-dda).

In field experiments, however, Rauch** found this compound to be inactive. A re-investigation of the problem (Persoons et al., 1976) yielded evidence that the true pheromone is a positional isomer of (E)-7-dda, namely (E)-8-dda. When the latter compound was tested in field experiments, it was found to be strongly synergized by (Z)-8-dda.

Conclusive evidence that the two compounds make up the sex pheromone of the insect was lacking at the time. Neither compound could be isolated from an extract in aqueous ethanol of virgin females, which had been standing for a long time before it could be analysed. However, upon acetylation the extract regained its biological activity, and the E-isomer could be isolated from it.

Fresh extracts of virgin females of *C. leucotreta* have now been shown to contain both isomers, (E)-8-dda and (Z)-8-dda.

METHODS AND MATERIALS

Extraction of the pheromone

A sample of 2-3-days-old laboratory-reared virgin females of *C. leucotreta* immersed in methylene chloride was supplied by Procida Laboratories, Saint Marcel, Marseille, France. The insects were homogenized three times in the methylene chloride suspension and the solid residue was extracted with freshly distilled methylene chloride for 8 hours in a Soxhlet extractor. The extracts were combined and dried over anhydrous $MgSO_4$, and the residue remaining after

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**Dr F. Rauch; Procida, Marseille, France.

removal of the solvent at reduced pressure was taken up in 0.5 ml of acetone.

Isolation

The acetone extract was subjected to preliminary purification by gel permeation chromatography on a Sephadex LH 20 column (150 × 0.7 cm, flow rate 20 ml acetone/h), 5 ml fractions being collected.

The geometric isomers were separated on a silver loaded nucleosil 10-SA column* (25 × 0.46 cm i.d., eluent hexane/ethyl acetate 9 : 1, flow rate 0.5 ml/min). Those fractions that had elution times corresponding to those of synthetic (Z)-8- and (E)-8-dda were collected for further purification by gas chromatography with PDEAS as the stationary phase (3 % phenyldiethanolamine succinate on Chromosorb W(AW), 650 × 0.23 cm, 175 °C, 11.5 ml N_2/min), followed by chromatography on a column of OV 101 (5 % on Chromosorb G, 200 × 0.4 cm, 194 °C, 40 ml N_2/min). The products of ozonolysis were separated on an OV 101 column at 175 °C.

The retention times of the isolated pheromones were compared with those of synthetic (Z)-8-dda and (E)-8-dda on the PDEAS column described above, and also with the retention times on a SP 2340 column (15 % cyanosilicone on Chromosorb P AW-DMCS, 600 × 0.23 cm, 210 °C, 15 ml N_2/min).

Mass Spectrometry

Mass spectra of the compounds were run with a Mat 112 GC-MS combination fitted with a 35 m SE 30 SCOT column maintained at 180 °C.

Ozonolysis

This was carried out according to the method of Beroza and Bierl (1967) with carbon disulphide as the solvent.

RESULTS

The fractions (nos 23-28) obtained by gel permeation chromatography of the extract and having elution times similar to those of synthetic (Z)-8-dda and (E)-8-dda were each concentrated to a residue of about 100 µl, and subjected to chromatography on the silver-impregnated column. Those fractions which had the same elution times as the synthetic compounds (11.7 and 9.5 min for (Z)- and (E)-8-dda, respectively) were again collected and subjected to gas chromatography on SP 2340 and PDEAS. The results are summarized in Table 13. Compounds with retention times identical to those of (E)-8-dda and (Z)-8-dda were isolated from fractions 3 and 5, respectively, fraction 4 containing a mixture of the isomers. Both compounds occur in the extract in about equal amounts.

*Custom-packed with silver-loaded Nucleosil 10 SA (Mackerey, Nagel & Co., Düren, G.F.R.) by Chrompack Nederland, Middelburg, The Netherlands.

Table 13 Comparison of the retention times of synthetic (E)-8-dda and (Z)-8-dda on columns of SP 2340[1] and PDEAS[2] with those of compounds isolated from a series of purified[3] fractions of an extract of virgin females

Fraction	Retention times (minutes)	
	on SP 2340	on PDEAS
2	-	-
3	12.4	8.4
4	12.4 + 13.7	8.4 + 8.8
5	13.7	8.8
6	-	-
Synthetic compounds:		
(E)-8-dda	12.4	8.4
(Z)-8-dda	13.7	8.8

1) 15 % cyanosilicone on Chromosorb PAW-DMCS, 6 m × 2.3 mm, 210 °C, N_2 = 15 ml/min.

2) 3 % phenyldiethanolamine succinate on Chromosorb W(AW), 6.5 m × 2.3 mm, 175 °C, N_2 = 11.5 ml/min.

3) By chromatography on a silver-impregnated column.

The mass spectra of the compounds isolated from fractions 3, 4 and 5 were identical with those of synthetic (Z)-8-dda and (E)-8-dda. Since the mass spectra of compounds of this type are very similar, the only conclusion that can be drawn from them is that the isolated compounds are mono-unsaturated dodecenyl acetates (m/e = 166 = M - CH_3COOH).

From mass spectra of the type in question, the positions of double bonds cannot be deduced. To this end the compounds isolated from fractions 3, 4 and 5, and purified over columns of PDEAS and OV 101, were subjected to ozonolysis. The reaction products were separated by chromatography on an OV 101 column, and had retention times identical to that of acetoxy octadecanal (7.1 min), which can also be obtained by ozonolysis of synthetic (Z)-8-dda or (E)-8-dda.

The ozonolysis products of (E)-7-dda and (E)-9-dda, on the other hand, have retention times on an OV 101 column of 4.2 min and 10.2 min, respectively. As (E)-7-dda is not separated from (E)-8-dda under the chromatographic conditions employed, the results of the ozonolysis demonstrate the absence of (E)-7-dda in the extracts.

The results of the electroantennographic experiments with model compounds, including (E)-7-dda, confirm the conclusion that the latter compound is not

the sex pheromone of *C. leucotreta* (Fig. 27).

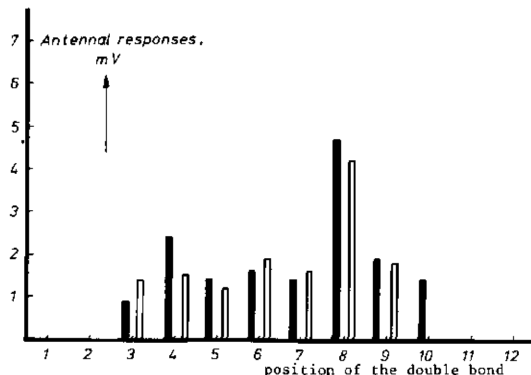

Fig. 27. Antennal responses of *C. leucotreta* male moths to various mono-unsaturated *cis*- and *trans*-dodecenyl acetates. Black bars: *cis*-isomers. White bars: *trans*-isomers. 5 duplicates, 10 µg samples. (Persoons et al. 1976)

These data, together with the earlier finding (Persoons et al., 1976), that butanal is a second product of ozonolysis, complete the evidence that the compounds isolated from the insect are (Z)-8-dda and (E)-8-dda. The fact that they are active as sex pheromones is demonstrated by the field data given in Table 14.

Table 14 Field trapping results of *C. leucotreta* males. The traps baited with pheromone were exposed for 104 days, and those with virgin females for 94 days. Three duplicates; purities of the compounds ⩾ 99 %; 20 mg per trap (Persoons et al., 1976)

ratio *cis*-8 / *trans*-8	total numbers caught
100 / 0	115
70 / 30	2893
50 / 50	3009
30 / 70	3042
0 / 100	41
traps baited with two virgin females each	2341

DISCUSSION

The experimental data presented above show unambiguously that the double bond in the sex pheromone of *C. leucotreta* is not at the 7-position, as reported by Read et al. (1968, 1974), but at the 8-position. In addition, the data show that the insect contains the (E)- as well as the (Z)-isomer, and that these two compounds are present in fresh extracts of virgin females. The conclusion that it is not (E)-7-dda, but a mixture of (E)-8-dda and (Z)-8-dda which constitutes the sex pheromone of *C. leucotreta* is also supported by the negative results of field experiments with (E)-7-dda, and by the positive results of similar experiments with (E)-8-dda, (Z)-8-dda, and particularly, mixtures of the two.

As far as we are aware, the false codling moth is the only insect whose sex pheromone contains (E)-8-dda, whereas (Z)-8-dda has been found in the pheromones of several other insects. For example, the oriental fruit moth, *Grapholita molesta*, has (Z)-8-dda as the main component of its pheromone (Roelofs et al., 1969). The activity of the (Z)-isomer in this insect is strongly synergized by the (E)-isomer, although the presence of the latter in the insect has not been established (Beroza et al., 1973[a] and 1973[b], Roelofs and Cardé, 1974).

A number of other tortricid moths are also attracted by mixtures of (Z)-8-dda and (E)-8-dda, or by the individual compounds, but the presence of these compounds in the insects has not been established (Inscoe and Beroza, 1976; Ritter and Persoons, 1976).

Although mixtures of geometrical isomers have been found in several members of the Tortricinae subfamily (Inscoe and Beroza, 1976; Ritter and Persoons, 1976), the false codling moth is the only member of the Olethreutinae subfamily which has been proved to produce a binary sex pheromone composed of about equal amounts of geometrical isomers.

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6. SEX PHEROMONE OF THE POTATO TUBERWORM MOTH, *PHTHORIMAEA OPERCULELLA*: ISOLATION, IDENTIFICATION AND FIELD EVALUATION*)

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The sex pheromone of the potato tuberworm moth, *Phthorimaea operculella* (Zell.) has been identified as a mixture of *trans*-4, *cis*-7-tridecadienyl acetate and *trans*-4, *cis*-7, *cis*-10-tridecatrienyl acetate. The identifications have been carried out by means of electroantennography, mass, infrared and NMR spectroscopy. The identifications have been confirmed by synthesis. Both compounds occur in the insects in about equal amounts.

The individual compounds are attractive to male moths in pheromone traps. The triolefinic ester was found to be much more attractive than the diolefinic ester.

However, mixtures of both compounds in the ranges of 4:1 to 1:4 are much more attractive than the individual compounds or traps baited with two virgin female moths.

The potato tuberworm moth, *Phthorimaea operculella*, is a serious pest in potato, and some other solanaceous crops in the warmer regions of all continents. It damages potato crops in the field as well as in storage. Although not occurring in the Netherlands, it has been imported on several occasions by potato shipments originating from infested areas. To detect possible field infestations in an early stage, it was found to be necessary to have a sensitive detection method for the insect available. As sex pheromones can be very suitable for this purpose, a program for the isolation and identification of the sex pheromone of this moth was set up.

In 1974 we reported that the sex pheromone of the potato tuberworm moth was a two-component system (Persoons & Ritter, 1974). Both compounds were C_{13} acetates, one being a diolefinic acetate (I) and the other a triolefinic acetate (II). This was the first example of an odd-carbon-chain sex pheromone.

During our investigations Roelofs et al. (1975[b]) published a paper on the structure elucidation and field evaluation of one of the constituents of the sex pheromone of this moth, namely *trans*-4, *cis*-7-tridecadienyl acetate. In an independent investigation we had reached the same conclusion.

Roelofs et al. (1975[b]) stated that a second pheromone component was present, but they did not give further information to support this statement. After the presentation of a preliminary communication on the identification of that second component (Persoons et al., 1976), we now wish to report on the structure

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elucidation and field evaluation of both compounds in more detail.

MATERIALS AND METHODS

P. operculella moths were reared in plastic containers (70 × 30 × 40 cm) on potato tubers at 20 °C and a relative humidity of 60 % under a day/night regime of 17 hours light/7 hours dark. Water was supplied in excess as a 3 % sucrose solution on cotton wicks. At 2-3 days intervals the adult moths were collected and sexed. Male moths, except those needed for the bioassay, were discarded. About 70,000 female moths were collected in methylene chloride.

Extracts of complete moths were made by grinding the insects in an electric blender in methylene chloride. Depending on the numbers of insects available, batches of different size were processed at the same time. The slurry was filtered and the homogenate re-extracted with fresh methylene chloride for 8 hours in a Soxhlet apparatus. The methylene chloride extracts were combined and dried overnight over anhydrous $MgSO_4$. The solvent was then removed by distillation and the residue dissolved in acetone.

For the bioassay ten male moths were confined in glass jars of one liter, covered with nylon gauze. A small cotton wick soaked in a 3 % sucrose solution was offered as food. The samples to be tested were applied on filter paper strips that were inserted in a pasteur pipette. A slow air stream was blown over the test sample into the jars and the reactions (wing fluttering, copulation attempts) scored. Initially these tests were performed in the first few hours of the photophase; later they were carried out at the end of the scotophase as the insects were found to be more sensitive during this period.

The technique of electroantennography (EAG) was essentially the same as that described by Roelofs & Comeau (1971). It was used for screening the available C_{12}, C_{13}, C_{14} and C_{16} acetates, alcohols and aldehydes as well as for monitoring the various purification steps.

Column chromatography on Sephadex was carried out by loading a glass column with Sephadex LH 20 in acetone (150 × 1.4 cm i.d., flow rate 20 ml/h, sample size 0.5 ml) and collecting 5 ml fractions. The activity was located by the EAG method. Larger amounts of extracts were purified via a much wider column (150 × 3 cm i.d., flow rate 150 ml/h, sample size 4 ml). Column chromatography on SiO_2 was carried out as high pressure liquid chromatography (HPLC). Three columns (25 × 0.5 cm i.d.) loaded with Lichrosorb Si 60 (5-10 μm) were connected. The working conditions were: eluent 1 % ethyl acetate in hexane, pressure 90 atm, flow rate 1.4 ml/min and one-minute fractions were collected. The fractions were bioassyed as described above.

A Pye Unicam, model 104 gas chromatograph was used. Analyses were carried out on the following columns: 5 % diethyleneglycol succinate (DEGS) on Chromosorb W(AW), 2 m, 165 °C, 30 ml N_2/min; 3 % phenyldiethanolamine succinate (PDEAS) on Chromosorb W(AW), 6.5 m × 1.65 mm i.d., 175 °C, 15 ml N_2/min, and 5 % OV 101 on Chromosorb G, 2 m, 190 °C, 30 ml N_2/min. Gas chromatographic fractions were collected according to the method of Burson & Kenner (1969).

Mass spectra were run on a GC/MS combination (LKB 9000) fitted with an OV 101 column at 190 °C. Infrared spectra (in KBr), were made on a Perkin Elmer double beam spectrophotometer (model 421). Sample size 5 μg and 25 μg for compound I and II, respectively. NMR spectra were run on a Varian HR 300 spectrom-

eter operating at 300 MHz. Sample size 30 μg for each compound in CCl_4.

Field experiments were carried out in Cyprus during the spring of 1976 in three different potato fields (one concentration series per field). Traps were water-filled open dishes (containing a detergent) placed on the ground between the potato plants. The pheromones (200 μg in CH_2Cl_2) were applied on rubber septa, placed in the centre just above the traps. Purities of both compounds were 98 %. Apart from the individual components, mixtures of them in the ratios of 4:1, 1:1 and 1:4 were also tested.

Female baited traps contained two virgin females per trap. To avoid the possible effect of site preference, the traps were rotated every day.

RESULTS

Because of the large numbers of insects necessary, complete insects were extracted instead of extracting abdominal tips only, although it is known that female potato tuberworm moths possess a sex pheromone producing gland (Adeesan et al., 1969). The relative large amounts of extracts that were obtained in this way could easily be purified by the preparative Sephadex column. The activity from both columns was recovered in the fractions that eluted 7-8 hours after the sample application. HPLC of the combined (EAG) active fractions from the Sephadex column demonstrated the presence of two active compounds eluting after 4-6 minutes (compound I) and 8-10 minutes (compound II),respectively.

When parts of these prepurified extracts were injected on a DEGS column, two well-resolved peaks appeared with retention times of 13.2 min (compound I) and 17.1 min (compound II),respectively. On an OV 101 column both (EAG) active peaks coincided (retention time = 12.0 min). This is demonstrated in Fig. 28.

As the activity coincided with neither an unsaturated dodecenyl acetate, nor with an unsaturated tetradecenyl acetate (or the corresponding alcohols) the impression was obtained that the compounds were different from the usual sex pheromones (mono- and di-unsaturated C_{12}, C_{14}, C_{16} acetates or alcohols). This confirmed our findings from the EAG experiments, where only weak responses were obtained from the C_{12}, C_{14}, C_{16} acetates or alcohols.

For further investigations both compounds were injected on a PDEAS column, the active peaks collected and reinjected on an OV 101 column. In all instances the activity coincided with the gas chromatographic peaks. These active peaks, however, did not coincide with the retention times of one of the compounds mentioned before. Since the samples collected from the HPLC contained only minor amounts of impurities, only one gas-chromatographic system (OV 101) sufficed for final purification (purity > 99 %).

Mass spectrometry suggested that both compounds were unsaturated tridecenyl acetates. Compound I had the parent peak at m/e = 238, and a peak at m/e = 178 (= M-60, loss of one molecule acetic acid). Compound II had the parent peak at m/e = 236 and a peak at m/e = 176 (= M-60). These data strongly suggested that compound I indeed was a di-unsaturated C_{13}-acetate and compound II a tri-unsaturated C_{13}-acetate. From these spectra no information was obtained regarding the position of the double bonds nor about their geometry. Infrared analyses confirmed the compounds to be unsaturated acetates, both having absorption bands characteristic for *cis*- and *trans*-unsaturation: 3010 cm^{-1} and 970 cm^{-1}, respectively.

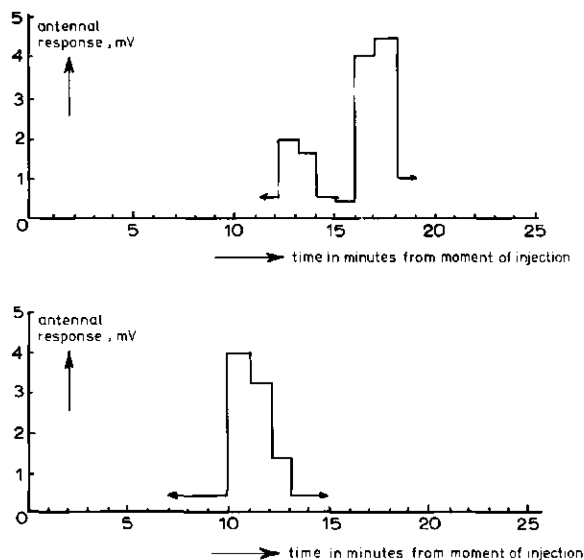

Fig. 28. Antennal responses of *Phthorimaea operculella* males to 1 minute gas chromatographic fractions collected from a polar column (DEGS,top) and a non-polar column (OV 101,bottom) after injection of a prepurified extract. The intensities of the fractions that are not indicated varied between 0.1 and 0.5 mV. Under the same experimental conditions *cis*-7-tetradecenol had retention times of 19.0 min and 10.6 min on the DEGS and OV 101 columns, respectively. The retention times of *cis*-7-tetradecenyl acetate on the same columns were 16.4 and 15.8 min, respectively.

The diolefinic ester (I) therefore must have one *cis* and one *trans* double bond. To establish the number of *cis* and *trans* double bonds in the triolefinic ester (II), infrared spectra were made of a number of di-, and tri-unsaturated acetates (Table 15). The relative intensities of the *trans* and *cis* double bonds of the various compounds were calculated by dividing the absolute absorbances at 970 cm^{-1}(*trans*) and 3010 cm^{-1} (*cis*) by the absolute absorbances at 1740 cm^{-1} (ester carbonyl) and 1040 cm^{-1} (ester C-O) × 100. The values of these relative intensities are given in Table 15. Comparison of the relative absorbances of the *cis* and *trans* double bonds of the various compounds, with those of the nat-

ural triolefinic ester (II), indicated a *trans*:*cis* ratio of 1:2 in the latter.

Table 15 Comparison of the relative intensities of the infrared absorbances of the *cis*- and *trans*- double bonds in the natural pheromones (I and II), with those of various di- and triolefinic esters

Compound	Relative intensities			
	trans		*cis*	
	$\frac{970\ cm^{-1}}{1740\ cm^{-1}}$	$\frac{970\ cm^{-1}}{1040\ cm^{-1}}$	$\frac{3010\ cm^{-1}}{1740\ cm^{-1}}$	$\frac{3010\ cm^{-1}}{1040\ cm^{-1}}$
natural I	16	78	7	37
natural II	17	81	17	82
c4,t7,tridda[1]	17	63	10	41
t4,c7,tridda	16	80	8	40
c4,t7,c10 tridta[2]	15	68	16	73
t4,c7,c10 tridta	16	73	22	95

1) tridecadienyl acetate
2) tridecatrienyl acetate

The available mono-unsaturated C_{12}, C_{14} and C_{16} acetates, alcohols and aldehydes were only weakly EAG active. A number of mono-unsaturated C_{13} acetates therefore was synthesized and tested by the EAG method. Much stronger responses were obtained with this series than with the others. The results are given in Fig. 29.

Although all responses evoked by the various mono-unsaturated C_{13} acetates are smaller than those elicited by the natural pheromone (compound I), one might conclude that at least two of the double bonds are in the 4- and the 7--position. The position of the 3rd double bond could not be derived from these results, although some preference for the 10-position could be derived from the EAG pattern. Based on the foregoing data, compound I therefore might be *cis*-4, *trans*-7-tridecadienyl acetate or *trans*-4, *cis* -7-tridecadienyl acetate. The EAG experiments indicated a preference for the latter.

We therefore ran an NMR spectrum of compound I and both isomers were synthesized (the *trans*-4, *cis*-7 isomer was synthesized according to Roelofs et al., 1975[b], while the synthesis of the *cis*-4, *trans*-7 isomer will be published elsewhere (Voerman, in prep). The NMR data constitute conclusive evidence that compound I is indeed a $\Delta^{4,7}$ tridecadienyl acetate. In Table 16 the calculated proton assignments are given for the four geometrical isomers, and the measured values of synthetic *cis*-4, *trans*-7-tridecadienyl acetate, *trans*-4, *cis*-7-tridecadienyl acetate and the natural pheromone (compound I).

The δ values of compound I are only in good agreement with the calculated and measured values of the *trans*-4, *cis*-7 isomer. Compound I therefore is *trans*-4 , *cis*-7-tridecadienyl acetate. Moreover, only the *trans*-4, *cis*-7 isomer

Table 16 Assignment of the various proton signals (δ values in CCl_4) of the natural pheromone (compound I) and the corresponding calculated (Frost, 1974)* and measured values for $\Delta^{4,7}$ tridecadienyl acetates

Structure :	AcO -	CH_2 -	CH_2 -	CH_2 -	CH = CH -	CH_2 -	CH = CH -	CH_2 -	CH_2 -	CH_2 -	CH_2 -	CH_3
Carbon atom no :		1	2	3	4 5	6	7 8	9	10	11	12	13
isolated compound I		3.98	1.65	2.01	(t)	2.68^5	(c)	2.01	(1.26-1.36)			0.89^5
calculated for t4,c7		3.97^5	1.64^5	2.04^5	t	2.67	c	2.01	1.32^5	1.28	1.30	0.89
found for synthetic t4,c7		3.98	1.65	2.00	t	2.68	c	2.00	(1.26-1.36)			0.89
calculated for c4,t7		3.99^5	1.65^5	2.10	c	2.67	t	1.95^5	1.31^5	1.26	1.28^5	0.88^5
found for synthetic c4,t7		3.98	1.65	2.09	c	2.68^5	t	1.94^5	(1.25-1.34)			0.89
calculated for c4,c7 (not synthesized)		3.99^5	1.66	2.11^5	c	2.71^5	c	2.02^5	1.33	1.28	1.31	0.89^5
calculated for t4,t7 (not synthesized)		3.97^5	1.64	2.03^5	t	2.62^5	t	1.94^5	1.31	1.26	1.28^5	0.88^5

*The influence of the -OAc group on the δ values of the chain methylene groups, which was not included in Frost's publication, was evaluated for 5 model compounds.
The calculated influence of the -OAc group is: α + 2.715; β + 0.330; γ + 0.09; δ + 0.03^5 and ε + 0.01 (α refers to the $-CH_2-$ group in position 1, etc.).

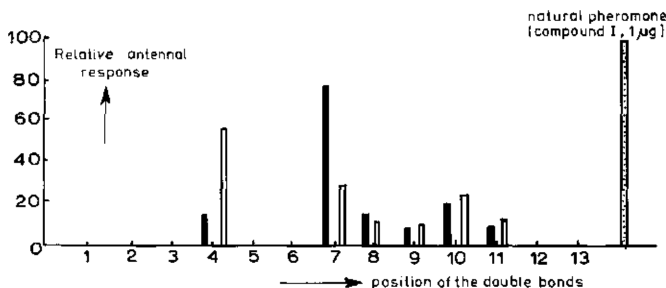

Fig. 29. Comparison of the antennal responses of *Phthorimaea operculella* males to the natural pheromone and to various mono-unsaturated *cis*- and *trans*-tridecenyl acetates. Black bars: *cis*-isomers. White bars: *trans*-isomers (five replicates; 10 µg samples, blank spaces not tested).

proved to be active in EAG, behavioural and field experiments. Other positional isomers were excluded on the basis of the chemical shift (δ 1.65) found for the methylene in CH_3-COO-CH_2- $\underline{CH_2}$- which only fits the $\Delta^{4,7}$ isomer.

For the triolefinic ester (II) also an NMR spectrum was run. The proton assignments are given in Table 17 (top). The NMR data indicate that the compound is a $\Delta^{4,7,10}$-tridecatrienyl acetate. This evidence, together with the infrared data (*trans*:*cis* = 1:2) leaves three possible isomers, namely t4, c7, c10; c4, t7, c10 and c4, c7, t10. For all possible isomers the proton signals were calculated (Frost, 1974) and compared with those of the natural pheromone. Of only two of these isomers the calculated values could be compared with their measured values (the c4, t7, c10 and the t4, c7, c10 isomer), the other isomers not having been synthesized (Table 17, bottom). (The synthesis of the *cis*-4, *trans*-7,*cis*-10 and the *trans*-4,*cis*-7,*cis*-10 isomer will be published elsewhere; Voerman, in prep.).

Only the proton signal positions calculated for the t4, c7, c10 isomer closely agree with those of the natural pheromone, while the calculated values for the other isomers differ much more. Compound II must therefore be *trans*-4, *cis*-7,*cis*-10-tridecatrienyl acetate. Natural as well as synthetic II elicited pronounced EAG responses. Moreover, it was also active in behavioural and field tests.

The results of the field experiments are given in Table 18. Both the diolefinic ester and the triolefinic ester proved to be attractive on their own (purities > 98 %).The triolefinic ester was even more attractive than traps containing live virgin females, while the diolefinic ester showed only weak attraction compared with live females. Only a limited number of ratios of the two compounds was tested, but all the ratios tested so far are much more attractive than the individual compounds or female baited traps. Males are effectively trapped by a rather wide range of concentrations, without a clear cut optimum.

Table 17. Assignment of the various proton signals (δ values in CCl_4) in the natural pheromone (compound II) and the corresponding calculated (Frost, 1974) and measured values for $\Delta^{4,7,10}$tridecatrienyl acetates

structure :	AcO - CH_2 -	CH_2 -	CH_2 -	CH =	CH -	CH_2 -	CH =	CH -	CH_2 -	CH =	CH -	CH_2 -	CH_3
carbon atom no :	1	2	3	4	5	6	7	8	9	10	11	12	13
isolated compound II :	3.97^5	1.65	2.05	(t)		2.73	(c)		2.73	(c)		2.05	0.97^5
calculated for t4, c7, c10 :	3.97^5	1.64^5	2.04^5	t		2.71	c		2,74	c		2.05^5	0.96
found for synthetic t4,c7,c10:	3.98	1.65	2.05	t		2.73	c		2.73	c		2.05	0.97^5
calculated for c4,t7,c10	3.99^5	1.65^5	2.10	c		2.68^5	t		2.68^5	c		2.04	0.95^5
found for synthetic c4,t7,c10	3.98	1.65	2.10	c		2.69^5	t		2.69^5	c		2.03	0.96
calculated for (not synthesized) c 4, c7, t10	3.99^5	1.66	2.11^5	c		2.74	c		2.71	t		1.98^5	0.94^5
c4, t7, t10	3.99^5	1.65^5	2.10	c		2.67^5	t		2.64	t		1.97^5	0.94
t4, c7, t10	3.97^5	1.64^5	2.04^5	t		2.69^5	c		2.69^5	t		1.98^5	0.94^5
t4, t7, c10	3.97^5	1.64	2.03^5	t		2.64	t		2.67^5	c		2.04	0.95^5
c4, c7, c10	3.99^5	1.66	2.11^5	c		2.75^5	c		2.75^5	c		2.05^5	0.96
t4, t7, t10	3.97^5	1.64	2.03^5	t		2.63	t		2.63	t		1.97^5	0.94

The fact that only a limited number of mixtures could be tested, may have been the cause that no clear cut optimum in attractancy has been found. However, in all three sites the ratio diolefinic ester/triolefinic ester = 1:4 showed the highest attraction. *Cis*-4,*trans*-7,*cis*-10-tridecatrienyl acetate has been tested in the field as well, either alone or in combination with *trans*-4,*cis*-7-tridecadienyl acetate. Increasing amounts of this compound in the mixtures tested considerably decreased catches. The compound itself showed only weak attraction.

Table 18 Total number of moths caught between 25 May and 7 June in traps containing different ratios of *trans*-4,*cis*-7-tridecadienyl acetate/*trans*-4,*cis*-7,*cis*-10-tridecatrienyl acetate* and traps baited with live virgin females**

Compounds tested (μg)		Number of moths caught in site no			Totals	Moths captured/trap; % of maximal catch
t4,c7	t4,c7,c10	1	2	3		
200	-	123	332	307	762	21
160	40	606	1264	931	2801	80
100	100	618	1321	1060	2999	85
40	160	674	1402	1435	3511	100
-	200	275	1065	614	1954	55
female baited traps (2 females/trap)		-	840	463	1303	37

* One series of traps per site; rubber dispensers.

** Blank traps caught no moths.

DISCUSSION

Apart from the compound, *trans*-4,*cis*-7-tridecadienyl acetate, isolated and identified by Roelofs et al. (1975[b]) as part of the pheromone of the potato tuberworm moth, the sex pheromones of only three other Gelechiidae have been isolated and identified, namely the sex pheromone of the pink bollworm, *Pectinophora gossypiella* (Hummel et al., 1973), that of the Angoumois grain moth, *Sitotroga cerealella* (Vick et al., 1974) and that of the peach twig borer, *Anarsia lineatella* (Roelofs et al., 1975[a]). The pheromone of the pink bollworm is a two-component system, namely an isomeric mixture of *cis*,*cis*-, and *cis*-,*trans*-7,11-hexadecadienyl acetate. The sex pheromone of the Angoumois grain moth is a single compound, namely *cis*-7,*trans*-11-hexadecadienyl acetate and that of the peach twig borer again is a two-component system, namely a mixture of *trans*-5-decenyl acetate and *trans*-5-decen-1-ol. Unlike the sex pheromones of the Tortricidae, which are C_{12} or C_{14} compounds (depending upon the subfamily to which the insect belongs), the sex pheromones of gelechiid moths show a much wider diversity.

At the time Hindenlang et al. (1975) started their work on the potato tuberworm moth, only C_{16}-carbon chain acetates were known as pheromones for gelechiid

moths. Therefore they focussed on C_{16}carbon chain acetates, but they soon had to return to the classical approach. Fouda et al. (1975) in their work on the potato tuberworm moth, isolated an acetate of a di-unsaturated C_{13}-alcohol. Several of the isomers of 7,9- 7,10-, 7,11-, and 7,12-tridecadienyl acetates were synthesized, of which the *cis*-7,*cis*-11 isomer appeared to be moderately attractive. The mass spectra of the natural and the synthetic compounds, however, were different. It therefore may be concluded that the natural pheromone is not the *cis*-7,*cis*-11 isomer. From the work of Roelofs et al. (1975[b]) it appeared that the di-unsaturated pheromone is in fact the *trans*-4,*cis*-7 isomer. This has now been confirmed by our work.

The potato tuberworm moth is the first example of a lepidopterous insect having a mixture of a di- and a tri-unsaturated acetate as its sex pheromone. Moreover, it is also the first example of an odd-carbon chain sex pheromone, although synthetic odd-carbon-chain compounds are known that are active as sex attractants (Herrebout,pers. comm., Ritter & Persoons, 1975; Voerman et al., 1975).

Binary mixtures as sex pheromones are gradually becoming well-known phenomena. This has repeatedly been established for the Tortricidae (Roelofs & Cardé, 1974; Persoons & Ritter, 1975; Arn, 1975; Ritter & Persoons, 1976), where it seems to be more a rule rather than the exception. However, binary mixtures are also found in other moth families (Mayer & McLaughlin, 1975).

Synergistic effects of binary pheromone mixtures have often been observed, but in these cases the individual compounds are hardly (and very often not at all) active in behavioural or field tests (Persoons & Ritter, 1975; Ritter & Persoons, 1976). However, in the case of the potato tuberworm moth, both compounds are individually active in behavioural tests as well as in field tests, although they strongly synergize each other in the latter.

Although the compounds were isolated from the insects in about equal amounts, the ratio does not seem to be very critical for attraction, as has been found for *Adoxophyes orana* (Minks & Voerman, 1973) and *Archips podana* (Persoons et al., 1974). This is also the case for another gelechiid moth, the peach twig borer, *Anarsia lineatella*, which is also attracted to wide range of ratios (Roelofs et al., 1975[a]).

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ZUSAMMENFASSUNG

SEXUALPHEROMONE DER KARTOFFELMOTTE, *PHTHORIMAEA OPERCULELLA*, ISOLIERUNG, IDENTIFIKATION UND FREILANDBEWERTUNG

Das Sexualpheromon von *Phthorimaea operculella* (Gelechiidae) wurde identifiziert als ein Gemisch von *trans*-4,*cis*-7-Tridecadienylacetat und *trans*-4,*cis*-7,*cis*-10-Tridecatrienylacetat. Die beiden Identifizierungen wurden durchgeführt mit Hilfe von Elektroantennografie, Massenspektroskopie, Ultrarotspektroskopie und Kernspinnresonanzspektroskopie.

Die beiden Identifizierungen wurden durch Synthese bestätigt. Die Pheromonkomponenten wurden in etwa gleiche Mengen von den Faltern isoliert. Beide Substanzen erwiesen sich sowohl in natürlicher wie in synthetischer Form als aktiv in der Elektroantennografie sowie im biologischen Test und in Sexfallen.

Ein Mischungsverhältnis der beiden Komponenten von 4:1 bis 1:4 weist eine viel höhere Lockstoffwirkung auf als die beiden Einzelkomponenten oder als zwei lebende Weibchen.

Ein Isomer des natürlichen Tridecatrienylacetat, nämlich *cis*-4,*trans*-7,*cis*-10-Tridecatrienylacetate, war nur schwach aktiv in den verschiedenen Testen. In Sexfallen wurde die Aktivität dieses Isomers durch Zusatz von *trans*-4,*cis*-7-Tridecadienylacetat nicht erhöht.

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7. EVALUATION AND PROSPECTS OF THE IDENTIFIED PHEROMONES

During the past few decades, it has become increasingly clear that the use of commercial insecticides is subject to severe limitations. This has led to much research to find alternative methods to control insect pests. As it was assumed that the development of resistance against physiologically active substances, playing an indispensable role in the life processes of insects was less probable, research in this area was strongly promoted.

Among this group of compounds as candidate control agents, the insect growth regulators (the juvenile hormones and their analogs) and the insect pheromones are currently intensively being investigated (e.g., Slāma et al., 1974; Gilbert, L.J., 1975; Menn and Pallos, 1975).

Both groups of compounds have their own specific advantages and disadvantages. The juvenile hormones and their analogs are not highly selective, although certain compounds effect certain categories of insects more than others (Bowers, 1969; Slāma et al., 1974).

Although the physiological properties of the insect growth regulators seem very attractive to be incorporated in insect controlling agents, their oecological properties are less favourable. They do not eliminate the larval stages but merely prevent the appearance of the next generation. Nevertheless, some of the insect growth regulators have reached the stage of practical application and one of them is formally registered by the Food and Drug Administration.

As regards the pheromones, their selectivity is much higher than that of the growth regulators. This selectivity is often enhanced by the existance of several components in specific concentration ratios. They only interfere with specific elements of the behaviour of those species from which they have been isolated. Whereas among the insect growth regulators some synthetic analogs have been found that are more effective than the natural juvenile hormones (Bowers, 1968; Pallos et al., 1971), with pheromones the natural compounds are invariably far superior. In pheromone research not a single case has been reported in which the activity of the natural pheromone has been surpassed by that of a para-pheromone. Having a very high selectivity and reaching their target organs via the natural way (which makes the development of resistance less probable), pheromones do not have the disadvantages which were reported above occurring with growth regulators. Oecologically, their properties are very interesting and they are already of great help in monitoring insect populations. Their practical application as a tool in insect control has, however, not sufficiently been worked out. Many insect pheromones have been isolated and chemically identified, and many types of communications have been detected in which they function. Studies to manipulate insect behaviour by the use of pheromones should therefore be strongly stimulated.

Apart from the subjects described here, the author has participated in the isolation and identification of several other insect pheromones. The emphasis now lies on the development of their practical application. On the basis of their pheromones monitoring systems have been developed for the leafroller moths *Adoxophyes orana* and *Archips podana* (Minks and de Jong, 1975; Minks,1975). Combined with temperature recordings (serving to estimate the development stage

of the young larvae in the eggs) this method is successfully being applied in timing the exact moment of insecticide application. By this method the number of insecticidal sprays has drastically been reduced. Similar techniques have been developed for the codling moth (Batiste et al., 1973,and Riedl et al., 1976). In the case of the summerfruit tortrix moth, *A. orana*, a few attempts have been made to apply the confusion technique, with compounds that interfere with pheromone perception (pheromone inhibitors). So far the results are promising (Minks et al., 1976). Boness (1976) claims a reduction in fruit damage in apple from 20-30 % to 5.1 % with mass trapping of *A. podana*.

More fundamental knowledge of the behavioural responses of the moths to their pheromones is necessary to render the method more consistent. The main aspects of behavioural responses that should be studied are:

- the sexual cycle of males and females as influenced by pheromones;
- the influence of biotic and non-biotic factors on the responses;
- orientation mechanisms functioning in attraction, and
- pheromone concentrations at which gradient responses occur, acting in ultimate partner finding.

As regards the practical application of the pheromones of the false codling moth, *Cryptophlebia leucotreta*, and the potato tuberworm moth, *Phthorimaea operculella*, it is still too early to judge their potential values. From the interest shown by several research workers, especially from South Africa, it may be expected that the practical use of the pheromone of the false codling moth in the direct control of this insect will be subject to intensive testing. So far its application has been limited to monitoring insect populations. No research has been carried out with this insect to investigate the possibilities of applying the confusion technique by maintaining a standing concentration of either the pheromone or pheromone inhibitors in the air. The latter have not been discovered yet in *C. leucotreta* and the above experiments therefore can only be initiated after these compounds become available. This is valid for the majority of cases studied up till now.

Evaluation of the practical use of the sex pheromone of the potato tuberworm moth is even in a less advanced stage: so far the synthetic compounds have been tested only to evaluate their attractiveness under field conditions. Experiments to determine the optimal ratio of the two compounds under natural conditions are currently being carried out. The extremely high catches of these moths indicate that mass trapping is a real possibility of using this pheromone as an effective method to control this insect, either in potato cultures or in potatoes under storage. Especially for effectuating quarantaine regulations the pheromone of the potato tuberworm moth might be very helpful.

The practical prospects of the pheromone of the American cockroach, *Periplaneta americana*, cannot at present be evaluated. Data in literature about the attractiveness of the pheromone of this insect are scarce. The experiments so far have all been carried out with crude extracts all of which contain active components. No experiments have been reported in which the attractive power of the individual purified compounds was evaluated. Therefore one has to be sceptic about the data in literature. Our experiments still give only a limited amount of information in this respect. Large scale experiments can only be carried out as soon as the synthetic compound(s) become available.

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8. SUMMARY

The use of pheromones is one of the methods currently being investigated intensively as an alternative method of insect control. The various ways in which pheromones might be used in insect control programmes are briefly discussed in Chapter 1.

Chapter 2 gives a detailed description of the isolation and identification of the sex pheromone of the American cockroach, *Periplaneta americana*. The various techniques that have been used for the structure elucidation are described.

The sex pheromone of *P. americana* contains two major components (periplanone-A and periplanone-B), as well as minute amounts of another four.

Periplanone-B could be identified as (1 Z, 5 E)-1,10(14)-diepoxy-4(15), 5-germacradiene-9-one. Periplanone-A proved to be very unstable, isomerizing to a biologically inactive compound. The instability of the compound rendered a complete structure elucidation impossible.

The four minor compounds could not be collected in sufficient amounts to carry out a complete structure elucidation.

Of the compounds reported to mimic the natural pheromone, only germacrene-D was found to do so. However, its threshold is very much higher than that of the natural pheromone. The same holds for the other reported mimics, even they do elicit electroantennogram responses. Regardless of concentration, none of the mimics elicit responses as marked as the natural pheromone.

Apart from causing sexual excitation, periplanone-B also can attract males of *P. americana*.

The isolation, identification and field evaluation of the sex pheromone of the leafroller moth, *Archips podana*, is described in Chapter 3. The pheromone consists of a mixture of equal amounts of *cis*-11-tetradecenyl acetate and *trans*-11-tetradecenyl acetate. Although the individual compounds are not attractive in field experiments, a 1:1 mixture of the two is capable of attracting many males of this species.

Investigation of the sex pheromone of the false codling moth, *Cryptophlebia leucotreta* (Chapters 4 and 5) revealed that the pheromone is a mixture of equal amounts of *cis*-8-dodecenyl acetate and *trans*-8-dodecenyl acetate. This finding contradicts other reports which state that the pheromone of this insect is *trans*-7-dodecenyl acetate. Mixtures of *cis*-8- and *trans*-8-dodecenyl acetate in various ratios proved to be very attractive to males of this species.

In Chapter 6 the isolation, identification and field evaluation of the sex pheromone of the potato tuberworm moth, *Phthorimaea operculella*, is described. The sex pheromone of this moth is a mixture of about equal amounts of *trans*-4, *cis*-7-tridecadienyl acetate and *trans*-4,*cis*-7,*cis*-10-tridecatrienyl acetate. These findings were confirmed by synthesis and by trapping experiments in the field.

9. SAMENVATTING

De toenemende mate van optreden van resistentie bij verschillende insecten en de steeds groter wordende aanslag op het milieu door insecticiden zijn aanleiding geworden dat intensieve researchprogramma's zijn opgezet om een aantal alternatieve bestrijdingsmogelijkheden op hun practische toepasbaarheid te onderzoeken. Eén van deze alternatieve methoden is de toepassing van feromonen. Hun toepassingsmogelijkheden bij de geïntegreerde bestrijding van plagen worden in het kort weergegeven in hoofdstuk 1.

De isolatie en identificatie van het sexferomoon van de Amerikaanse kakkerlak, *Periplaneta americana*, wordt gegeven in hoofdstuk 2. Zowel de diverse zuiveringstechnieken als de verschillende identificatietechnieken worden uitvoerig beschreven. Het feromoon van *P. americana* blijkt te bestaan uit twee hoofdcomponenten plus een 4-tal bijproducten. Eén van deze hoofdcomponenten (periplanon-B) kon worden geïdentificeerd als (1 Z, 5 E)-1,10(14)-diepoxy-4(15), 5-germacradieen-9-on.

De tweede hoofdcomponent (periplanon-A) bleek instabiel te zijn en isomeriseerde tot een biologisch inactieve verbinding. Mede hierdoor kon een volledige structuuropheldering van deze verbinding niet worden uitgevoerd.

De vier bijproducten konden niet in voldoende hoeveelheden verzameld worden om een volledige structuuranalyse uit te voeren.

Hoewel in de literatuur een aantal verbindingen wordt genoemd als mimetica van het feromoon, kon dit alleen voor germacreen-D worden bevestigd. De drempelwaarde van dit mimeticum ligt echter vele malen hoger dan die van het natuurlijke feromoon. Deze mimetica geven weliswaar een electroantennogram, doch ook hier ligt de drempelwaarde veel hoger dan die van het natuurlijke feromoon. Ongeacht de dosering wordt de intensiteit van de respons, die door het natuurlijke feromoon wordt opgewekt, nooit bereikt.

Hoewel de gebruikte gedragstoets uitsluitend is gebaseerd op sexuele excitatie, blijkt periplanon-B ook een aantrekkende werking te hebben op mannetjes van deze soort.

Het sexferomoon van de bladroller, *Archips podana*, (hoofdstuk 3), blijkt te bestaan uit een mengsel van *cis*-11-tetradecenyl acetaat en *trans*-11-tetradecenyl acetaat. Mengsels van deze beide stoffen in de verhouding 1:1 zijn in veldproeven bijzonder attractief voor mannetjes van deze soort.

Het sexferomoon van de "false codling moth", *Cryptophlebia leucotreta*, (hoofdstukken 4 en 5), blijkt te bestaan uit een mengsel van *trans*-8-dodecenyl acetaat en *cis*-8-dodecenyl acetaat (1:1). Dit in tegenstelling tot de literatuurgegevens, volgens welke het feromoon van dit insect *trans*-7-dodecenyl acetaat zou zijn. De identificaties konden door veldproeven worden bevestigd.

In hoofdstuk 6 wordt de structuuropheldering beschreven van het sexferomoon van het aardappelmotje, *Phthorimaea operculella*. Ook hier blijkt het feromoon te bestaan uit twee componenten: *trans*-4,*cis*-7-tridecadienyl acetaat en *trans*-4,*cis*-7,*cis*-10-tridecatrienyl acetaat. De identificaties konden worden bevestigd door synthese en veldproeven.

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