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STUDIES OF PHYSIOLOGICALLY ACTIVE ARTHROPOD SECRETIONS

X. SEX PHEROMONE OF THE EASTERN SPRUCE BUDWORM, *CHORISTONEURA FUMIFERANA* (LEPIDOPTERA: TORTRICIDAE)[1]

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Abstract

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The sex pheromone of the female eastern spruce budworm was identified as *trans*-11-tetradecenal by a combination of electroantennogram techniques, laboratory bioassays, and chemical analysis. Subsequent field trapping showed that this compound is a potent attractant for both male *Choristoneura fumiferana* (Clem.) and *C. occidentalis* Free. thus supporting its identification as a sex pheromone of the eastern spruce budworm and suggesting that it is also a major component in the pheromone system of *C. occidentalis*.

Résumé

Identification de la phéromone sexuelle sécrétée par le papillon femelle de la Tordeuse des bourgeons de l'Épinette. Il s'agit du *trans*-11-tétradécenal, décelé à l'électro-antennogramme et par des essais biochimiques et des analyses chimiques. Outre le mâle de *Choristoneura fumiferana*, ce composé attire aussi le *C. occidentalis* mâle, suggérant qu'il forme aussi une partie importante du système des phéromones appartenant à *C. occidentalis*.

Introduction

The spruce budworms are among the most widely distributed, destructive forest insects in North America. Of the species in this complex, the eastern spruce budworm, *Choristoneura fumiferana* (Clem.), is the major pest of balsam fir and white spruce in eastern Canada.

The presence of a sex attractant in the eastern spruce budworm was first demonstrated in the field with traps baited with live virgin females (Greenbank 1963). Subsequently Findlay and Macdonald (1966) obtained materials attractive to males by passing air over virgin females and condensing out the volatiles, or by preparing an ether rinse from polyethylene bags that had contained females. Due to both a lack of a satisfactory bioassay and insufficient material these workers were unable to characterize the attractant.

This paper describes the isolation, chemical elucidation, electroantennogram techniques, behavioural bioassays, and field testing that led to the identification of a sex pheromone in female *C. fumiferana*.

Materials and Methods

The insects used in this study were reared in the laboratory on artificial diet (McMorran 1965), sexed as pupae, and maintained separately thereafter. The adult males were held under continuous light conditions whereas the adult females were held under a 17/7 hr light/dark cycle.

The most common method of obtaining pheromone from female Lepidoptera is to excise the sex pheromone gland from the abdominal tips of virgin insects and

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extract these with a suitable solvent. In the case of *C. fumiferana* this was done using a variety of solvents but in every instance the crude extract was shown to be inactive although previous work (Sanders 1969; Weatherston and Percy 1970; Percy and Weatherston 1971) has shown that the gland is located on the abdominal tip as in other tortricids (Roelofs and Feng 1968). Purification of these extracts by column chromatography on various supports yielded many fractions, some of these elicited extremely weak responses from male insects in laboratory bioassays; such responses were considered insignificant. Consequently a new method of collecting pheromone was devised. Virgin females were placed in large Mason jars which contained a piece of cheesecloth on which they could rest (ca. 100 females/jar). After 2 days the moths were removed and the jars and cheesecloth well rinsed with ether to give an ethereal wash which proved to be biologically active by laboratory bioassay. In an attempt to quantify the amounts of material obtained, the term 'female night' (FN) was coined. One FN is equivalent to the amount of material which would be obtained from the ether rinse of a jar that had contained one virgin female budworm for one night.

Biological activity was demonstrated by three types of bioassay: laboratory bioassay involving behavioural response, field attraction tests, and electroantennograms (EAG's). The laboratory bioassays were conducted using the olfactometer previously described by Sanders (1971*a*). Briefly, the olfactometer consisted of 12 circular plastic boxes connected by equal lengths of tubing to a common air stream, producing similar air flow through all the boxes. The air was exhausted from each box into a fume hood. Five 2-day-old males were placed in each box and test compounds injected from medicine droppers through a hole in the lid in the boxes, although filter paper was used in the original experiment. Compounds for laboratory bioassay were made up as solutions of known concentrations in ether. Approximately 0.5 ml of the test solution was then taken up in the medicine dropper and immediately expelled. Air was then blown through the dropper for 5 sec to evaporate the solvent ether. Bioassays were carried out by squirting air from the medicine dropper into one of the boxes containing males in the olfactometer. Response was recorded as the number of males "buzzing," a behavioural pattern involving circling on the substrate with rapidly fanning wings which is only observed in sexually aroused males.

Field tests were carried out by putting the required quantity of chemical in ethereal solution inside polyethylene stoppers (Kimble Products, opticlear 2 dr vials). On evaporation of the ether the stoppers were placed inside dark green Sectar I traps (3M Co., St. Paul, Minn.).

EAG's were recorded using the apparatus and methods previously described (Roelofs and Comeau 1971; Roelofs *et al.* in press; 1971). The 12- and 14-carbon chain acetate and alcohol standards were tested by sending a 1 ml air puff from a 5 ml glass syringe through a test pipette (containing 80 μg of chemical on a piece of filter paper) into the airstream passing over an antenna. Aldehyde standards were similarly run with 1 μg of test chemical on a piece of filter paper. Gas chromatographic effluent for EAG analysis was collected in 12 in. capillary tubes from polar (5% cyclohexanedimethanol succinate on Chromosorb Q) and non-polar (3% OV-1 on Chromosorb Q) columns. EAG activity was ascertained by puffing 1 ml of air through the tubes and into the airstream passing over a male spruce budworm antenna. Standard chemicals were injected onto the columns immediately after the collections were made to correlate retention times.

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Analytical gas–liquid chromatography (GLC) was performed on a Perkin–Elmer 990 instrument fitted with 6 ft × ⅛ in. stainless steel columns packed with 1.5% SE-30 on Chromosorb W (80/100 mesh) and 15% IBP on Gaschrom Q (100/120 mesh).

Thin layer chromatography (TLC) was on 0.25 mm layers of silver nitrate impregnated silica gel (Adsorbosil-1-ADN, Applied Science Laboratories, State College, Pa.) developed with either petroleum ether:ether (5:1) or petroleum ether:ether (19:1). Visualization was effected by spraying the plates with a hot methanolic sulphuric acid solution of 2,4-dinitrophenylhydrazine.

Hydrogenation was carried out at room temperature by stirring the mixture of compounds, in ethanol containing platinum oxide catalyst, in an atmosphere of hydrogen (Farquhar *et al.* 1959). The ozonolysis was performed in carbon disulphide at —70°C. The ozonides were decomposed with triphenylphosphine prior to GLC analysis (Beroza and Bierl 1967). Hydrolyses were carried out by refluxing the crude material with 0.5 *N* potassium hydroxide in 95% ethanol for 15 min. Acetylations were performed at room temperature for 2 hr using acetyl chloride. Treatment with 2,4-dinitrophenylhydrazine was carried out using a stock solution of the reagent (Openshaw 1955). 2,4-Dinitrophenylhydrazine controls were obtained by treating crude material with methanolic sulphuric acid in the absence of the carbonyl reagent. Lithium aluminum hydride reductions were carried out at room temperature in anhydrous ether.

Results and Discussion

Roelofs and Comeau (1970) have reported that the sex attractants of a number of tortricid species are monounsaturated 14-carbon chain alcohols or acetates with the double bond in the 11-position. Tests were therefore carried out to determine if the active component in the female spruce budworm wash contained an alcohol or an acetate. Male antennal responses of over 2 mv were elicited only with collections between 8 and 8½ and between 8½ and 9 min after injection of female wash on the polar (Hi-eff 8BP) column, while all other collections from 3 to 15 min produced antennal responses of less than 0.5 mv. Under the same column conditions dodecyl alcohol, dodecyl acetate, tetradecyl alcohol, and tetradecyl acetate had retention times of 7.0, 6.7, 16.0, and 14.8 min respectively. Male antennal responses of over 2.5 mv were elicited only with collections between 5 and 5½ and between 5½ and 6 min after injection of female wash on the non-polar (OV-1) column, while all other collections from 3 to 15 min produced antennal responses less than 1.0 mv. Under the same column conditions dodecyl alcohol, dodecyl acetate, tetradecyl alcohol, and tetradecyl acetate had retention times of 4.0, 7.8, 10.0, and 17.5 min respectively. It was therefore concluded that the active component is not an alcohol or an acetate.

Male spruce budworm antennal responses to a series of monounsaturated 12- and 14-carbon chain acetate and alcohol standards showed that only *cis*- and *trans*-11-tetradecen-1-ol elicit responses above 1 mv. Although gas chromatograph retention times indicate that the active component is not an alcohol, the EAG data suggest that a 14-carbon compound with unsaturation in the 11-position may be involved.

Functional group analyses (Table I) were carried out on 200 FN portions of the crude wash. From the analyses it was concluded that the biologically active compound in the ether wash was an unsaturated aldehyde or ketone. Considering that the majority of known female lepidopterous pheromones are either primary

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Table I. Functional group analyses of *C. fumiferana* wash

Vial	Reaction	Bioassay	Inference
1	Control	+ve	—
2	Hydrogenation	−ve	Unsaturation present
3	Addition of bromine	−ve	Unsaturation present
4	Hydrolysis	+ve	Ester absent
5	Acetylation	+ve	Primary or secondary alcohol absent
6	Lithium aluminum hydride reduction	−ve	Ester, carboxylic acid, aldehyde, or ketone present
7	Treatment with 2,4-dinitrophenylhydrazine	−ve	Aldehyde or ketone present
8	2,4-Dinitrophenylhydrazine control	+ve	—

acetates of aliphatic alcohols or the alcohols themselves, and that nature is parsimonious in biological conversions, the aldehyde structure was favoured.

Various monounsaturated aldehyde standards were prepared. It was found that 14-carbon aldehydes behaved similarly to the spruce budworm active component on both polar and non-polar columns. Male spruce budworm antennal responses to a series of monounsaturated 14-carbon aldehydes showed that the antennae were very responsive to *trans*-11-tetradecenal (Fig. 1). This compound also had retention times identical with the active material on both polar and non-polar GC columns.

Bioassays were, therefore, conducted with isomers of tetradecenal with the double bond in, or adjacent to, the 11-position. Successive dilutions of the compounds were used on the assumption that a compound identical with the sex pheromone would evoke a response at greater dilutions than the other isomers. Experiments started with concentrations of 10^{-7} g/ml and proceeded by successive 10-fold dilutions to 10^{-11} g/ml (Table II). Of the compounds tested, *trans*-11-tetradecenal evoked a higher response than any other isomer at all dilutions, and still retained activity at 10^{-10} and 10^{-11} g/ml, at which levels all the other isomers tested were not active. This indicated that *trans*-11-tetradecenal is a potent stimulant of male spruce budworm in the laboratory.

A large laboratory rearing of the eastern spruce budworm was undertaken and 120,000 FN of crude wash obtained. Purification by column chromatography on florisil resulted in the active material being eluted from the column with petroleum ether:ether (85:15) and petroleum ether:ether (1:1). These fractions were re-

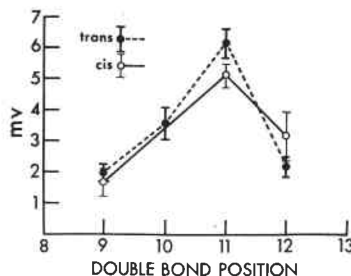

Fig. 1. Antennal responses (six replicates) of male spruce budworm to a series of isomers of tetradecenal.

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Table II. Per cent response of male *C. fumiferana* to various concentrations of six isomers of tetradecenal*

Concn. (g/ml)	Tetradecenal					
	trans-10	*cis*-10	*trans*-11	*cis*-11	*trans*-12	*cis*-12
10^{-7}	16	6	28	3	8	22
10^{-8}	4	2	47	0	1	8
10^{-9}	1	0	20	1	1	1
10^{-10}	0	0	10	0	0	0
10^{-11}	0	0	4	0	0	0

*One hundred males were used for the bioassay of each concentration of each isomer.

combined and subjected to re-chromatography on a second florisil column, the activity coming off the column in petroleum ether:ether (4:1). Subsequent column chromatography on 25% silver nitrate impregnated silica gel (Adsorbosil CABN, Applied Science Laboratories, State College, Pa.) yielded the activity in fractions eluted with petroleum ether:ether (3:1). The next stage involved a vacuum distillation of the residue of the active fractions from column chromatography; the activity was in the fraction distilling at 134°/0.8 mm Hg. The final stage in the purification was TLC eluting with petroleum ether:ether (19:1). A comparison of TLC data (Table III) of the purified natural material and several geometric isomers of tetradecenal indicates that the purified aldehyde has the *trans* configuration. This was verified by testing material scraped from the TLC plate for EAG activity. Activity was found only in the area with an R_F value corresponding to the *trans* isomer. If the pheromone is *trans*-11-tetradecenal, ozonolysis would yield undecandial as the high molecular weight moiety. The purified natural material was ozonized and the products analyzed by GLC on two columns. Two compounds were observed, one eluting with the solvent and the other having retention times of 142 sec (SE-30 at 145°) and 1256 sec (IBP at 155°). Authentic undecandial chromatographed under identical conditions gave retention times of 146 and 1260 sec respectively.

The synthesis of *trans*-11-tetradecenal was carried out in seven stages starting with decamethylene glycol. 11-Tetradecyn-1-ol was prepared as previously reported by Roelofs and Arn (1968). This alcohol was reduced with sodium in liquid ammonia to give *trans*-11-tetradecen-1-ol which was converted into the corresponding aldehyde by the method of Gunstone and Lie Ken Jie (1970). That the natural attractant and the synthetic material were chemically identical was shown by GLC at 155° on two columns, the natural attractant giving retention times of 149 sec (SE-30) and 310 sec (IBP) while authentic *trans*-11-tetradecenal had retention times of 150 sec (SE-30) and 312 sec (IBP). These data indicate that the sex attractant of the eastern spruce budworm is *trans*-11-tetradecenal.

Table III TLC of spruce budworm sex pheromone

Compound	R_F value × 100
cis–12–tetradecenal	40.0
trans–12–tetradecenal	48.0
cis–11–tetradecenal	39.1
trans–11–tetradecenal	47.4
Natural attractant	47.2

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Table IV. Average number of male budworm caught in traps baited with 100 μg *trans*-11-tetradecenal, compared with catches in virgin female-baited traps and empty traps (controls) placed in populations of *C. fumiferana* and *C. occidentalis*

	C. fumiferana		*C. occidentalis*	
	Ontario	Alberta	B.C.	Oregon
Replicates	6	6	3	6
trans–11–tetradecenal	50.2	53.7	53.7	49.5
Virgin females	88.3	45.8	40.0	45.8
Controls	1.8	1.0	0.3	0.5

Proof of the attractant properties of *trans*-11-tetradecenal was obtained from field experiments using the chemical to lure males to a trap.

The tests involved 100 μg of *trans*-11-tetradecenal per stopper, virgin females housed in screen cages and empty traps as controls. Tests were conducted in known outbreaks of *C. fumiferana* in Ontario, and also, with the cooperation of H. Cerezke, Canadian Forestry Service, Edmonton, Alta., in northern Alberta. Previous work (Sanders 1971*b*) has shown that the same primary pheromone is apparently shared by *C. fumiferana* and *C. occidentalis* Free., the western 1-yr-cycle budworm. Therefore tests with the same chemical were also conducted in populations of *C. occidentalis* in British Columbia with the cooperation of R. F. Shepherd, Canadian Forestry Service, Victoria, B.C., and in Oregon with the cooperation of G. Daterman, U.S. Forest Service, Corvallis, Ore. The results (Table IV) indicate that *trans*-11-tetradecenal is a potent attractant for both male *C. fumiferana* and male *C. occidentalis,* thus supporting the identification of this chemical as a sex pheromone of the eastern spruce budworm, *C. fumiferana,* and suggesting that it is also a major component in the pheromone system of *C. occidentalis*.

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