Sex Pheromone of the Comstock Mealybug, *Pseudococcus comstocki* KUWANA : Isolation and Identification

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The sex pheromone produced by females of the Comstock mealybug, *Pseudococcus Comstocki* KUWANA, was isolated and identified as 2,6-dimethyl-3-acetoxy-1,5-heptadiene. Synthetic pheromone showed a potent activity in laboratory bioassay and field test.

INTRODUCTION

The Comstock mealybug, *Pseudococcus comstocki* KUWANA, is one of the most important pests of apple, pear and other agricultural crops. It is very difficult to detect their infestation at early stage by visual inspection, because they lurk, develop and reproduce in inaccessible sites. Especially, a bag-culture of apple and pear in Japan make the detection of the pests infestation troublesome. For an effective control of this insect pest, a better method of detection is required.

MORENO et al. (1972) reported that traps baited with virgin females of Comstock mealybug attracted males in the field. But, the chemical structure of the sex pheromone was not determined. Identification of the mealybug sex pheromone would make possible the use of a pheromone trap to detect the infestation of the pest and control the insect population.

Bioassay methods and behaviouristic responses of the males to the female pheromone extract have been reported (NEGISHI et al., 1980). This paper deals with the isolation and identification of the Comstock mealybug sex pheromone.

MATERIALS AND METHODS

The Comstock mealybug, *Pseudococcus comstocki* KUWANA, used in this study was reared on pumpkins under conditions of 9 hr light (8:00–17:00)/15 hr dark photoperiod, 25°C–28°C and 50±10% r.h. The males were isolated from the females in their

nymphal stage. Only male nymphs, in their final stage, began to crawl vigorously on the surface of pumpkins. At this time, inoculated pumpkins were wrapped with tissue papers for 2 to 3 days to make many male nymphs crawl into the double folded tissue papers (NEGISHI et al., 1980). The collected papers carrying male nymphs were hung with clips in a bioassay room (9L-15D, 25°C–28°C).

After having reached adult stage, virgin females were removed from pumpkins and maintained for 4 days in petri dishes in group of about 1000, in which four filter papers (Toyo roshi no. 2, 9 cm in diameter) were placed to absorb the sex pheromone. The collected filter papers were submerged in distilled pentane. The pentane extract was concentrated by rotary evaporator without heating, filtered through filter paper after freezing at -20°C for 20 to 30 minutes to remove waxy substances, and then dried over anhydrous Na_2SO_4 to obtain a crude extract.

The crude extract was subjected to preliminary purification by chromatography of Florisil column (50 cm in length 1.0 cm i.d.). Fractions were collected by stepwise elution with the following solvent: 100 ml each of pentane, 5%, 10%, 50% ethyl ether in pentane, and ethyl ether. Silicic acid (Wakogel C-100) impregnated with silver nitrate (16.7%) was used in column chromatography for further purification. Fractions were collected by stepwise elution with following solvent: 200 ml each of 2%, 5%, 30%, 50% ethyl ether in pentane and ethyl ether. High-pressure liquid chromatography (HPLC) was performed with a Waters model ALC/GPC 244 chromatograph. Active fraction of AgNO_3 -silicic acid column chromatography was injected and eluted with 1% ethyl ether in hexane at 1 ml/min. through a μ Porasil column (0.4×30 cm). Chromatographic peaks were recorded with Hitachi recorder model 056 and fractions were collected as the peaks were observed.

Pheromone component was analyzed and isolated with a Hewlett Packard model A 5710A gas chromatograph equipped with a flame ionization detector and a $1.8 \text{ m} \times 3 \text{ mm}$ glass column (1.5% OV-1 or 10% DEGS, 95°C). Gas chromatographic fraction were collected in glass capillary tubes ($60 \text{ cm} \times 2 \text{ mm}$) inserted into the outlet of an effluent splitter (split ratios, 25:1).

The active GLC fraction was injected on a gas chromatograph-mass spectrometer, JEOL JMS-D300 using a $1 \text{ m} \times 2 \text{ mm}$ column of 3% OV-17. The column temperature was 127°C . Electron impact (EI) spectrum of the sex pheromone was recorded at 70 eV over a mass range of 30–300 m/e with helium as carrier gas. Chemical ionization (CI) spectrum was recorded over a mass range of 100–300 m/e. Ammonia was used as a reagent gas. NMR spectrum was recorded on a JEOL JNM PS-100 (100 MHz) spectrometer using CDCl_3 as solvent (tube: 1.8 mm, 15,000 pulses).

Throughout the isolation of the pheromone, bioassay was conducted by a laboratory trap test (NEGISHI et al., 1980) for free flying males in a bioassay room (9L-15D, 25–28°C). Male adults crawled out from tissue papers after light on and they took off in a few minutes. Flying males responded to the pheromone and were caught with traps. Fractionated pheromone extracts were put on the paper disc (0.8 cm in diameter) and placed on the slide-glass coated with sticky substances (Tack Trap, Animal Repellents, Inc.). Number of males caught with the sticky slide glass were recorded. In the field test, synthetic sex pheromone was injected into a polyethylene capsule (BEEM capsule, Nisshin EM) and placed in a triangular trap of 6 cm height and 7.5 cm width (Otsuka pharmaceutical Corp.).

RESULTS AND DISCUSSION

The crude extract was chromatographed on Florisil and eluted with ether-pentane mixtures as shown in Table 1. Biological activity for the males appeared in the fraction eluted with 5% ethyl ether in pentane. This active fraction was concentrated and chromatographed on a silicic acid impregnated with silver nitrate for further purification. Biological activity was present only in the fraction eluted with 30% ether in pentane. The active material was separated by HPLC employing 1 per cent ethyl ether in hexane solvent and a μ Porasil column. Strong activity was found in one fraction (fraction no. 9) (Table 1). The fraction was combined with fraction no. 8 and 10 and examined by GLC. The GLC active fraction on OV-1 (95°C) showed a peak at 6.8 minutes (fraction no. 9). The active material collected from OV-1 was further purified on DEGS (95°C). An active peak appeared at 7.3 minutes (fraction no. 5).

Biological activity could not be increased by recombination of all other fractions in each purification step (Florisil column chromatography, AgNO₃-silicic acid column chromatography, HPLC and GLC). From these results, we concluded that the compound corresponding to the peak at 7.3 minutes on DEGS was the Comstock mealybug sex pheromone. Preliminary experiment on saponification (5% methanolic KOH)-acetylation (acetic anhydride and pyridine) of crude extract of the sex pheromone revealed the Comstock mealybug sex pheromone to be an alcohol acetate.

An active sample collected from GLC (on DEGS) (ca. 1.2 μ g from about 150,000 females by GLC analysis) was submitted to GC-MS analysis. The EI mass spectrum of the sex pheromone showed the following diagnostic peak: m/e 122, 113, 107, 69 and 43 (base peak) (Fig. 1). Peak at m/e 107 (107.0848) led to the fragment ion C₈H₁₁⁺. No parent ion could be detected in the EI mass spectrum. The background subtracted CI mass spectrum of the sex pheromone showed the highest mass at m/e

Table 1. BIOASSAY RESULTS DURING PHEROMONE PURIFICATION : MALE CATCHES
IN LABORATORY BIOASSAY

Florisil ^a		AgNO ₃ ^b		HPLC ^c		GLC			
Fraction	No. males	Fraction	No. males	Fraction	No. males	OV-1 ^d		DEGS ^e	
						Fraction	No. males	Fraction	No. males
pentane	3	2% ether/ pentane	1	—6	8	—6	1	1	5
5% ether/ pentane	51	5% ether/ pentane	1	7	3	7	7	2	7
10% ether/ pentane	2	30% ether/ pentane	54	8	15	8	3	3	3
50% ether/ pentane	2	50% ether/ pentane	1	9	77	9	108	4	5
ether	3	ether	1	10	11	10	0	5	68
				11	3	11	5	6	6
				12—	6	12—	2	—	—

^a crude extract ^b 5% ether fraction from Florisil ^c 30% ether fraction from AgNO₃

^d active fraction from HPLC ^e active fraction from OV-1

Fig. 1. EI mass spectrum of the comstock mealybug sex pheromone.

Fig. 2. CI mass spectrum of the comstock mealybug sex pheromone.

200 (Fig. 2). The CI mass spectrum suggested that the molecular weight of the Comstock mealybug sex pheromone was 182 ($200-\text{NH}_4$). Prominent peaks at m/e 122, 113, and 107 of EI mass spectrum would imply as fragment M-60, M-69 and M-60-15, respectively. The peak at m/e 122 appeared to correspond to C_9H_{14} . These two mass spectra and the results of functional tests suggested that the Comstock mealybug sex pheromone was an acetate of doubly unsaturated 9-carbon alcohol.

The amount of purified material finally available for NMR analysis was ca. 6–7 μg from about 600,000 females. The presence of a terminal methylene group is in accordance with the signal of hydrogen atoms at 4.70–5.3 ppm in the spectrum and the signal at 1.63–1.73 ppm suggested methyl branches on double bonds.

At the beginning, (a) 2,6-dimethyl-hept-5-en-1-al enol acetate or (b) 1-acetoxy-2,6-dimethyl-2,6-heptadiene were assumed as the chemical structure of the sex pheromone. But these two synthesized chemicals had very much longer retention times on GLC (OV-1) compared with that of the natural pheromone (a: 11.3 min., b: 11.1

min., natural pheromone: 6.2 min.). These results and the NMR spectrum data suggested that the sex pheromone was not the primary alcohol acetate, but probably, the secondary alcohol acetate.

From these interpretations, the chemical structure of the sex pheromone was assumed to be either: 1) 2,6-dimethyl-3-acetoxy-1,5-heptadiene 2) 2,6-dimethyl-4-acetoxy-1,5-heptadiene 3) 2,5-dimethyl-3-acetoxy-1,5-heptadiene 4) 2,6-dimethyl-3-acetoxy-1,6-heptadiene 5) 2,5-dimethyl-4-acetoxy-1,5-heptadiene 6) 2,6-dimethyl-4-acetoxy-1,6-heptadiene. In these chemicals, synthetic 2,6-dimethyl-4-acetoxy-1,5-heptadiene (2) had shorter retention times on DEGS and OV-1 compared with that of the natural pheromone. These results excluded the possibility that the sex pheromone was the chemicals with acetoxy at C-4 position. Therefore the synthesis of the C-4 acetoxy chemicals (5 and 6) were not undertaken, and the three C-3 acetoxy chemicals (1,3,4) were synthesized (Uchida et al., unpublished).

2,6-dimethyl-3-acetoxy-1,5-heptadiene(1) had the same retention times on OV-1 and DEGS as the natural sex pheromone (Table 2). EI mass spectrum of 2,6-dimethyl-3-acetoxy-1,5-heptadiene exhibited a high degree of similarity to the spectrum of the natural pheromone. Based on these findings [the identical GLC retention times and EI spectrum], we concluded the sex pheromone of the Comstock mealybug to be 2,6-dimethyl-3-acetoxy-1,5-heptadiene. This is the first identification of the secondary alcohol acetate as a sex pheromone of Coccid species.

A preliminary trap test with laboratory reared population was conducted. The synthesized sex pheromone in polyethylene capsule (0.1 mg/capsule) was much more attractive to the males (7.2/trap/day) than the crude extract of 2,400 female-day-equivalents (0.5 males/trap/day). Field test was also conducted in Tokushima. Two triangular traps baited with pheromone capsules were placed on two citrus trees and empty traps were also placed as a control. An apple tree was located near the pheromone traps. The synthesized sex pheromone was attractive to native males. The two traps caught 81 and 127 males from August 11 to 31 in 1979. Empty traps caught no males.

Recently, sex pheromone with asymmetric carbons were identified from other Coccid-species, *Aonidilla aurantii* MASKELL and *A. citrina* COQUILLET (ROELOFS et al., 1977, 1978; GIESELMANN et al., 1979). The sex pheromone of the Comstock mealybug possess also an asymmetric carbon at the C-3 position. Synthesis of the enantiomers is in progress.

Table 2. GLC ANALYSIS OF PHEROMONE AND SYNTHESIZED CHEMICALS

Compound	Retention time	
	OV-1 ^a	DEGS ^b
Pheromone	6.8 min	7.3 min
2,6-dimethyl-3-acetoxy-1,5-heptadiene	6.8	7.3
2,6-dimethyl-4-acetoxy-1,5-heptadiene	5.9	5.7
2,5-dimethyl-3-acetoxy-1,5-heptadiene	6.6	6.4
2,6-dimethyl-3-acetoxy-1,6-heptadiene	7.0	7.7

^a 1.5% OV-1 on Chromosorb W.

^b 10% Diethylene glycol succinate on Chromosorb W. (3 mm × 1.8 m, 95 °C)

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