Identification, Synthesis, and Field Evaluation of the Sex Pheromone from the Ten-Lined June Beetle

Project No.: **08-ENTO5-Leal**

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Objectives:

- Extract the natural sex pheromone from field-collected female beetles
- Isolate active fraction(s) and isolate pheromone to purity by Preparative Fraction Collection
- Determine structural features of the sex pheromone by gas chromatography (GC)-electroantennographic detection (EAD)-based chemical derivatizations
- Elucidate structure by GCT Premier Gas Chromotograpgy/Mass Spectroscopy, Gas Chromatograpgy-fourier transform infrared (FTIR), and micro-analytical chemistry
- Synthesize new pheromone

Interpretive Summary:

The long-term goal of this project is the development of sex pheromone-based new technologies for monitoring and controlling populations of the Ten-Lined June beetle (TLJB). TLJB is a chronic agricultural pest problem where it occurs. There is no effective method of control, because the immature insects submerge so deep in the soil that they are out of the reach of conventional pesticide treatments. TLJB is most susceptible to control measures at the adult stage from early June to late August. During this limited period, female emerge out of the soil, and advertise their readiness to mate by releasing a volatile and very potent sex pheromone. Males can detect this sex

pheromone even from a distance while the female's body is still partially or totally buried in the soil. The pheromone is so active that female body debris left in the field remains attractive to males the following night. Mated females return to the soil to lay numerous eggs, the progeny of which proceed to voraciously feed on roots of almonds and other commercial trees and vine crops. Interrupting mating and/or controlling adult populations would have a significant effect on the number of offspring.

To develop a synthetic pheromone system for monitoring and/or controlling TLJB populations, we must first identify the natural compound. We are very close to the finish line, but progress has been slow because this is a unique pheromone system that combines the complexity of a novel chemistry with production of very small amounts of pheromone. While these features make identification of the natural pheromone a challenging task, the fact that the pheromone is so potent (thus requiring minimal amounts to be produced) will pay off later when the synthetic pheromone becomes available for practical applications. An analytical protocol for the isolation and identification of the sex pheromone has already been developed. However, TLJB females produce so minute amounts of pheromone that conventional analytical instrumentation employed in chemical ecology research turned out to be too insensitive. We estimated that each female produce less than 1 picogram of sex pheromone.

Materials and Methods:

To collect enough pheromone for structure elucidation we took a tour-de-force approach. From June to August, professors, postdoctoral scholars, SRAs, graduate and undergraduate students take trips to the field almost every night to catch female beetles for pheromone extraction. This year we have improved our approach by hiring local students so that collections were made in Manteca almost daily during the short flight season.

Gas chromatography with electroantennographic detection (GC-EAD) was performed on a Hewlett-Packard 5890 Series II plus GC equipped with an HP-5MS column (30 m x 0.25 mm; 0.25 µm; Agilent Technologies, Palo Alto, CA) and connected with the transfer line and temperature control unit from Syntech (Kirchzarten, Germany). The temperature program started at 70°C for 1 min, increased to 250°C at a rate of 10°C/min. The GC was operated under splitless mode with the injection port at 250°C and a post run at 290°C for 10 min. Effluent from the capillary column was split in to EAD and flame ionization detector (FID) in 3:1 ratio. Antennal signals were fed into Syntech AMS-01 amplifiers. The analog signals were fed into A/D 35900E interface (Agilent) and acquired simultaneously with a FID signal on an Agilent Chemstation. Gas chromatography-mass spectrometry (low resolution GC-MS) was performed on a 6890 Series GC and a 5973 Network Mass Selective Detector (Agilent Technologies) operated under Electron Impact (EI) mode. GC was equipped with a HP-5MS capillary column (see above) and operated as in GC-EAD.

Results and Discussion:

Using our previously developed methods, we have re-isolated the active compound, confirmed its electrophysiological activity by GC-EAD, and proceeded with structural elucidation of the sex pheromone. Earlier, we were able to pool samples and obtain a low resolution mass spectrum of the active compound. Unfortunately, the structure is unusual thus requiring more identifying features for molecular characterization. We have now employed a state-of-the-art analytical instrument, the GCT Premier Orthogonal Acceleration Time of the Flight Mass Spectrometer with Electron Impact and Chemical Ionization Sources and Dynamic Range Enhancement Capability (GCT Premier GC/MS in short). This is a GC-MS system developed by Waters Co. with extremely high sensitivity and high-resolution capability. We have employed this technology to help us obtain additional structural features of the elusive pheromone. We were able not only to detect the sex pheromone peak with small amounts of samples that were below the detection limit of conventional analytical techniques, but also to obtain high-resolution, accurate mass spectral data. As we determined that the high resolution molecular weight of the pheromone in the ionization mode is 299.2589 Da, we were able to determine the molecular formula $C_{18}H_{35}O_3$ (calculated 299.2586 Da). With sample collected this flight season and re-isolated using our previously developed protocol and monitoring activity by GC-EAD, we determined the molecular weight of the base peak in the mass spectrum of the pheromone. With two independent measurements, we obtained the molecular weight that suggests a molecular formula $C_{10}H_{19}O_2$ (**Table 1**). Out of three possibilities, two were ruled out because the compound contains no nitrogen.

Table 1. Molecular Formula for Base Peak of TLJB Sex Pheromone

Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula
171.1367	171.1385	-1.8	-10.5	1.5	65.9	C10H19O2
	171.1372	-0.5	-2.9	2	70.7	C8H17N3O
	171.1358	0.9	5.3	2.5	79.2	C6H15N6

Next, we attempted to obtain an infrared (IR) spectrum of the compound to gather additional molecular features for structure elucidation. Despite the modifications in our vapor-phase GC-IR system to optimize sensitivity, we were unable to obtain a signal-to-noise high enough to generate reliable IR data. We then explored a more sensitive instrument, DiscovIR-GC (Spectra Analysis Inc.), which - as opposed to the time-of-flight detection of vapor-phase IR - utilizes a deposition and detection system. We recorded GC-IR data (**Figure 1**) for the isolated active sample.

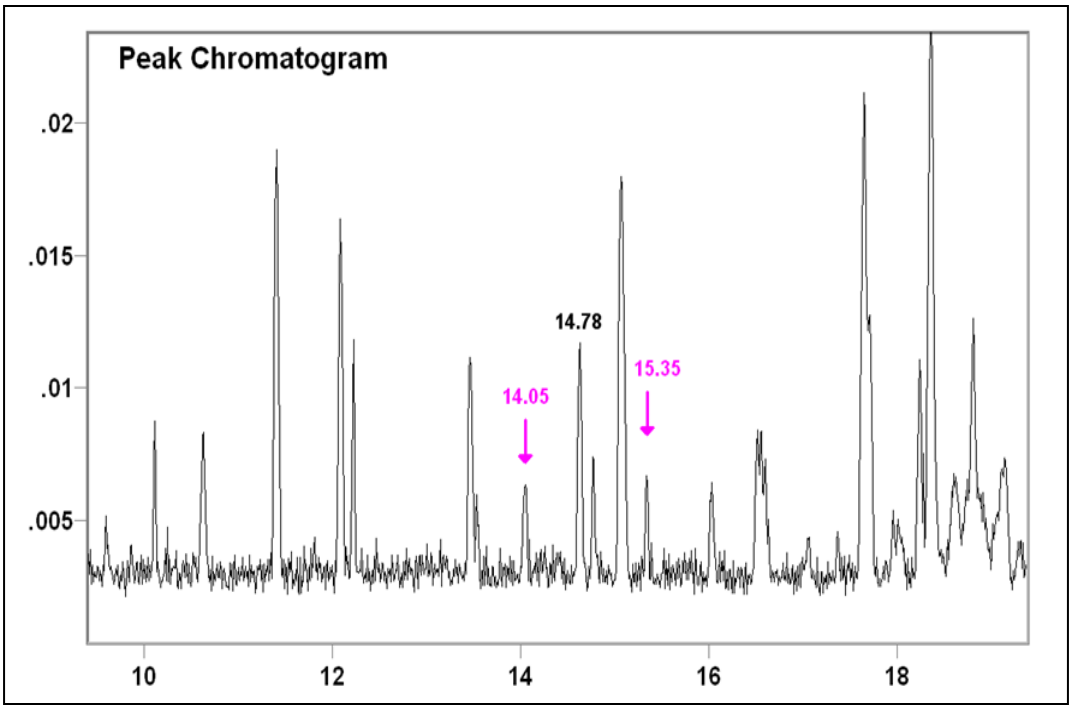

Figure 1. Peak chromatogram generated by GC-IR with the tentative target peak and two “markers” highlighted in black and violet, respectively.

To identify the retention time of the target peak we used hydrocarbon markers in the sample. Although the signals of these markers were weak, we tentatively identified the sex pheromone as the compound eluting at 14.78 min for which we obtained an IR spectrum (**Figure 2**).

Intriguingly, this spectrum suggests that the compound eluting at 14.78 min is either a ketone or aldehyde, which is branched with several methyl groups. These findings are not consistent with the MS data, particularly because there is no fragmentation pattern suggesting the existence of branched methyl groups. In addition, GC-EAD-assisted chemical derivatizations are not consistent with an aldehyde or ketone as the sex pheromone did not undergo reaction with lithium aluminum hydride (LAH). These compounds would be readily reduced by LAH. It is not unconceivable that the IR data is from a compound other than the target sex pheromone thus making structure elucidation a confounding task. Although we monitor the elution of the sex pheromone on the basis of two hydrocarbon markers, the signals of the formers were weak thus leading to possible misidentification. Moreover, the in house GC-MS data and the DiscovIR-GC data were obtained with different capillary columns. While we employed a HP-5MS 25 m capillary column in GC-MS analysis, for IR data the vendor run the sample on an Alltech AT5 15 m capillary column.

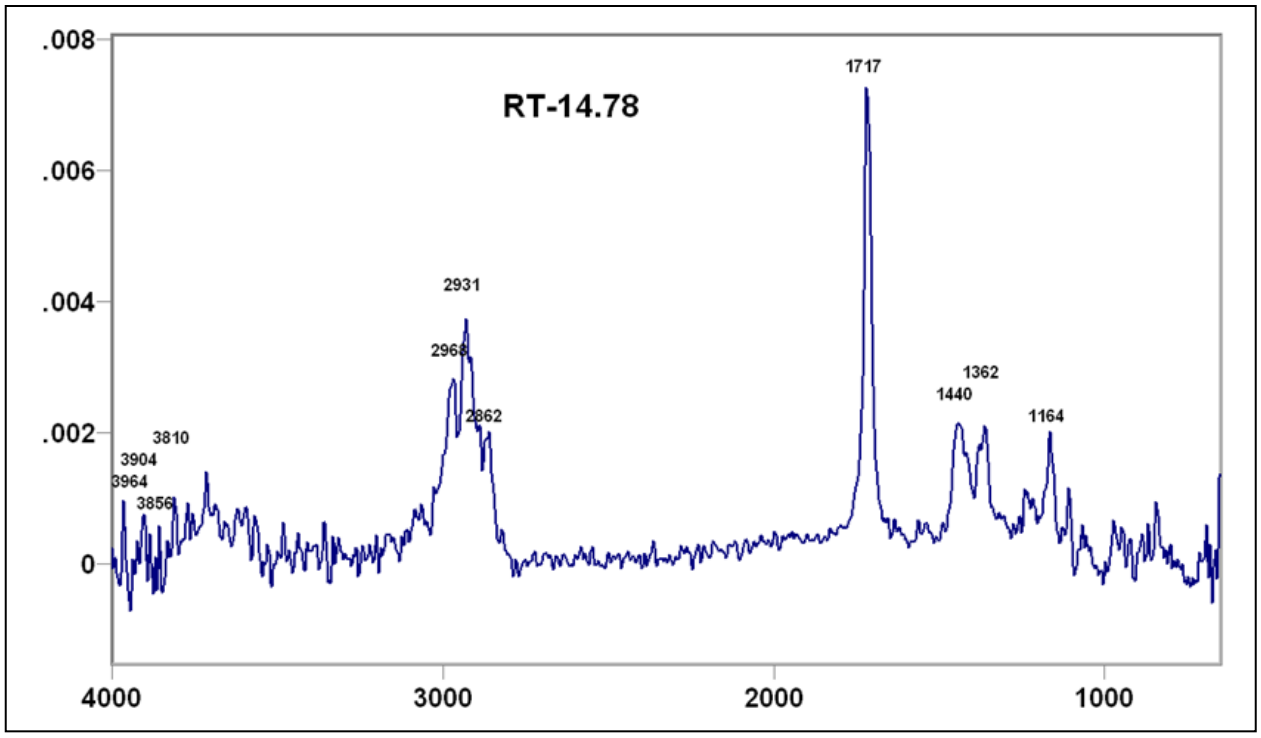

Figure. 2. IR spectrum from the tentative target peak showing a strong carbonyl band at 1717 cm^{-1} and weak C-H stretching bonds.

To unambiguously determine the IR for the sex pheromone and complete the chemical characterization of the elusive pheromone we will next spike the sample with hydrocarbon markers and re-run GC-IR.

We pooled satellite fractions (3% and 5%), which were leftovers from previous fractionations kept at -80^{o}C, and re-isolated the pheromone using GC-MS to identify the active peak. With this newly isolated sample, we obtained additional insights on the molecular structure of the sex pheromone. Clearly, proton exchange took place (**Figure 3**) when the isolated sample was re-dissolved in deuterated methanol, CD_3OD.

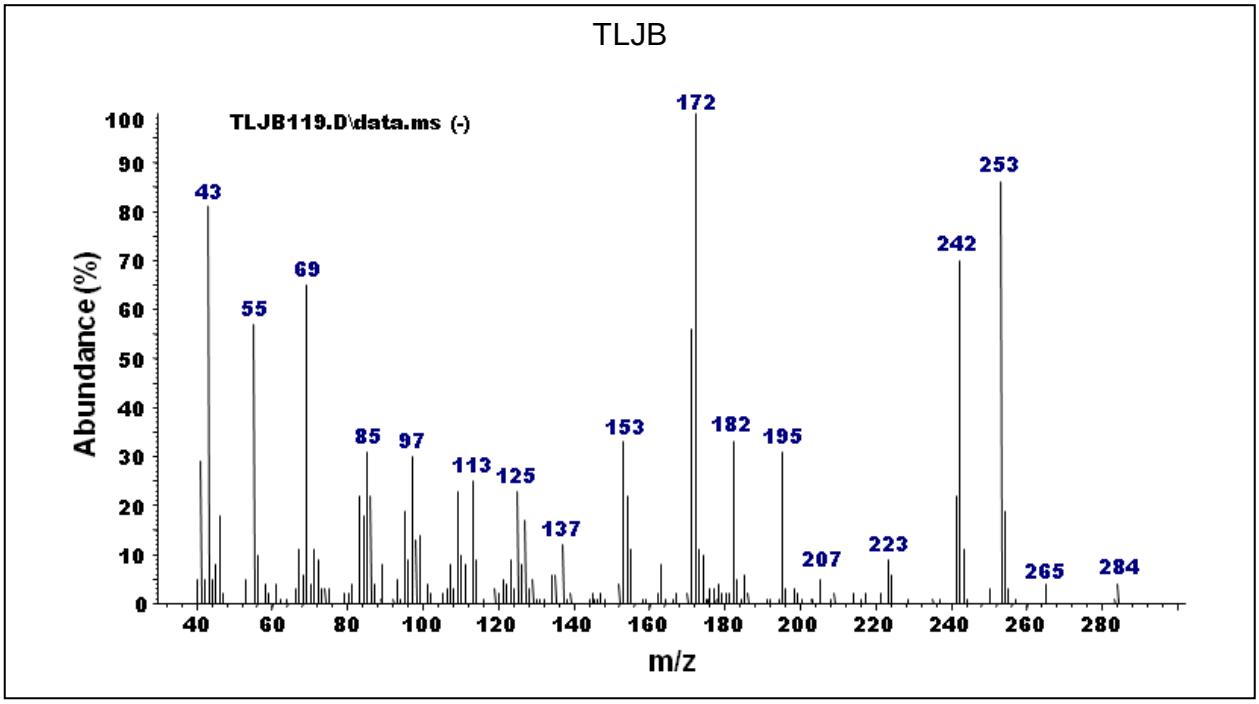

Figure 3. GC-MS data for the sex pheromone when dissolved in deuterated methanol. Note the increase (1 m/z) in some peaks thus indicating an H→D exchange.

The increase in mass-to-charge ratios (m/z) of various fragments strongly suggests that the pheromone molecule has one free hydroxy group. Comparison with the original MS indicates shifts in m/z 283→284, 241→242, and 171→172 (base peak). Although this new information alone is not enough for structure elucidation, it adds new key pieces to the puzzle, the most important being the occurrence of a free OH group. The polarity of this hydroxy group might be stabilized by intramolecular hydrogen bonding, as indicated by the low polarity of the compound (difference in Kotatz indexes, Δpolar - non-polar, 295). This low polarity is consistent with elution from silica gel column with 4% ether in hexane. Unfortunately, we are dealing with a rather unusual sex pheromone and, therefore, progress has been painfully slow, but the blue skies are just ahead.

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