The isolation, identification and synthesis of the alarm pheromone of *Vespula squamosa* (Drury) (Hymenoptera: Vespidae) and associated behavior [1]

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Summary. A material that elicits alarm and attack behavior by *Vespula squamosa* (Drury) workers was isolated from venom extracts and identified by spectroscopic methods as N-3-methylbutylacetamide. This compound elicited attack responses from worker wasps identical to those responses observed when venom was applied at the same dosage. This is the first behavioral role reported for this compound.
Key words. Vespula squamosa; alarm and attack behavior; N-3-methylbutylacetamide; southern yellowjacket.

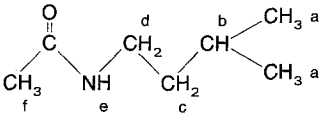
The southern yellowjacket is distributed widely throughout much of the eastern United States and south into Guatemala. It often forms large perennial colonies in subtropical to tropical areas of its range. This wasp is an extremely dangerous insect because of its abundance, large colony size and aggressiveness. Although separate figures are not available for this species, at least several dozen people are killed per year by yellowjackets in the U.S. [2]. We previously described alarm and attack behavior in the southern yellowjacket, *Vespula squamosa* (Drury), elicited by a pheromone in venom [2]. Although chemically mediated alarm behavior has been reported for several other social wasps [3–8], *V. squamosa* is the first known to use an alarm pheromone to orient to the source and to focus attacks. We report here the isolation, identification, and synthesis of the alarm pheromone of *V. squamosa*, and demonstrate the biological activity of this compound.

Alarm pheromone activity in this study was determined by the numbers of attacking wasps caught on a baited trap versus an unbaited trap [3]. We used this bioassay to test for responses to natural and synthetic materials by wasps from perennial underground *V. squamosa* colonies in Alachua County, Florida. Samples were applied to filter paper (5.5 cm diameter) on top of a 4.6-l, black paper cannister positioned 2 m from the entrance of a *V. squamosa* colony. Wasps were caught in a coating of Stick-um® applied to the outside of the cannister. Each test lasted 2 min.

Worker wasps were vacuumed from a nest entrance and were then frozen. Their venom sacs were dissected and placed in 200-µl aliquots of methylene chloride in glass vials. After 15–30 min the solvent was transferred to new vials. Initial gas chromatographic analysis of venom extracts was conducted using a Hewlett-Packard Model 5890 GC equipped with a splitless capillary injector and flame ionization detector, with a 50-m (0.25 mm i.d.; 0.25-µm film of BP-1®, Supelco Corp., Belfonte, PA) apolar fused silica capillary column. Helium was used as the carrier gas at a linear flow of 18 cm/sec. Two peaks were evident in these chromatograms. The first eluting peak had a Kovats [9] index of 1000 and the second peak had a Kovats index of 1102. These two peaks accounted for 30 and 64%, respectively, of the total peaks integrated in the Kovats range of 800–2100. Venom sac extracts were subsequently purified using a gas liquid chromatograph equipped with a cool on column injector and a 30-m (0.5 mm i.d., 1-µm film; methyl silicone, Quadrex Corp., New Haven, CN) apolar capillary column. Effluent from the column was split with 98% of the material vented and collected in U-shaped capillaries in which the bottom portion of the collecting capillary was immersed in liquid nitrogen. The remaining 2% of the effluent was directed to a flame ionization detector. Fractions were thus obtained of material eluting before and after the major peak area and the major peak itself. Bioassays of each of these three fractions and of a recombined fraction sample at five wasp equivalents demonstrated alarm pheromone activity only in the fraction containing the major peak.

The structure of the active compound, N-3-methylbutylacetamide, was determined by a combination of spectroscopic methods. Methane chemical ionization mass spectra (CI-MS) obtained using a Nermag Model R 1010® spectrometer resulted in a major ion (85%) with a mass-to-charge ratio (m/z) of 130 (M + 1). The molecular weight of 129 was confirmed by the presence of adduct ions at m/z 158 (M + 29), 170 (M + 41) and 187 (M + 58). Electron impact mass spectra (EI-MS) of the active peak resulted in a molecular ion at 129 M^+ (15%), and diagnostic ions at m/z 129 (14%), 114 (14%), 86 (30%), 73 (60%), 72 (62%), 60 (28%), and 43 (100%) The EI spectra obtained were in agreement with the spectra reported for N-3-methylbutylacetamide [10].

Proton magnetic resonance (PMR) spectra obtained on ca. 15 µg of the active material using a Nicolet® 300 MHz spectrometer was consistent with the proposed structure



The chemical shifts relative to benzene (7.15 ppm) of the spectrum obtained in C_6D_6 were: a (6 H, doublet at 0.78 ppm), b (1 H, multiplet at 1.32 ppm), c (2 H, quartet at 1.07 ppm), d (2 H, quartet at 3.04 ppm), e (1 H, broad signal at 0.47 ppm varies with concentration) and f (3 H, singlet at 1.49 ppm). Assignments were confirmed using ^1H decoupling techniques.

Synthetic N-3-methylbutylacetamide was prepared by reaction of 3-methylbutylamine with acetyl chloride at 0° (in the presence of triethylamine). The synthetic amide was purified by packed column GC before use. The CI-MS, EI-MS, and PMR spectra of the synthetic amide were identical to the spectra obtained from extracts of the venom sacs. Retention times of the natural material and synthetic amide were identical on a 50-m polar (0.25 mm i.d.; 0.25-µm film, CPS-1) and the apolar capillary columns.

Quantitative gas chromatographic analysis of venom sac extracts from five individual *V. squamosa* workers showed an average of 575 ± 60.4 ($\bar{x} \pm SE$) ng of N-3-methylbutylacetamide per wasp. Synthetic N-3-methylbutylacetamide was tested in the bioassay described at a range of dosages to see if it elicited wasp alarm behavior. In five replicate tests, a 15-ng dosage was applied initially and was doubled until a response occurred. First response occurred in one replicate each at the 30-, 250-, and 500-ng dosages. In two replicates, attacks did not occur until the 1000-ng dosage was applied. Average number of wasps caught on the trap at the dosage that resulted in an attack was 24.8. Synthetic N-3-methylbutylacetamide also was tested on three separate colonies of *V. squamosa* at 250–400 ng per application. While no wasps responded to methylene chloride controls, 190, 26, and 36 wasps were caught on the traps within 2 min following application at each of the three colonies, respectively. These ex-

perimental results confirm the presence of N-3-methylbutylacetamide in the venom of the southern yellowjacket and demonstrate alarm pheromone activity of this compound similar to that obtained with venom extracts. This is only the second alarm pheromone of a social wasp identified to date. The alarm pheromone of *Vespa crabro* L. is reported to be 2-methyl-3-butene-2-ol[7].

N-3-Methylbutylacetamide is reportedly a volatile constituent of tobacco[11], wines and cheeses[13]. Although the amounts of this chemical in these products are not known, the possibility exists of eliciting an attack from a yellowjacket colony with these or other natural materials containing N-3-methylbutylacetamide. This compound also has been found in the rectal glands of the Queensland fruit fly, *Dacus tryoni* (Froggatt), although its function in gland secretions is unknown[10].

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Goniothalamicin and annonacin: Bioactive acetogenins from *Goniothalamus giganteus* (Annonaceae)

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Summary. Using brine shrimp lethality for activity-directed fractionation, goniothalamicin (**I**), a new tetrahydroxy-mono-tetrahydrofuran fatty acid γ-lactone (acetogenin), has been isolated from ethanolic extracts of the stem bark of *Goniothalamus giganteus* Hook. f., Thomas (Annonaceae). This novel compound was found to be cytotoxic and insecticidal and inhibited the formation of crown gall tumors on potato discs. Annonacin (**II**), the only other reported mono-tetrahydrofuran acetogenin, was also isolated; the previously reported 9ASK (astrocytoma reversal) activity of **II** was confirmed, and **II** is now also found to be weakly active against 3PS murine leukemia.

Key words. Goniothalamicin; annonacin; acetogenins; *Goniothalamus giganteus*; Annonaceae; brine shrimp; insecticidal; cytotoxic; antileukemic activity.

Previous phytochemical studies of the genus *Goniothalamus* have yielded the bioactive compounds altholactone (goniothalenol)[1], a furano-2-pyrone, and goniothalamin[1, 2], a styrylpyrone, as well as pinocembrin[1] (5,7-dihydroxyflavone) and a number of 5,6-dihydro-2-pyrones[3]. During our continuing investigation of higher plants as sources of novel biologically active secondary metabolites, we noted that ethanolic extracts of the stem bark of *Goniothalamus giganteus* Hook. f., Thomas (Annonaceae) exhibited significant murine toxicity in the 3PS lymphocytic leukemia system[4]. The fractionation of the ethanolic extract was guided by a convenient bioassay involving brine shrimp lethality[5]. Through multiple solvent partitionings and chromatographic steps, monitoring the fractions with thin-layer chromatography (TLC) and brine shrimp lethality, goniothalamicin (**I**), a novel acetogenin and the recently reported mono-tetrahydrofuran acetogenin, annonacin[6] (**II**), were isolated and subsequently characterized.

Goniothalamicin (**I**) was found to be cytotoxic in the 9KB (human nasopharyngeal carcinoma, $ED_{50} < 10^{-2}$ μg/ml) and the 9PS (murine lymphocytic leukemia, $ED_{50} < 10^{-1}$ μg/ml)[4] systems. This cytotoxic activity paralleled the brine shrimp lethality throughout the fractionation of the stem bark. Goniothalamicin was toxic to the brine shrimp [LC_{50} 37 ppm, 95% confidence intervals: 13–295 ppm] and also toxic to blowfly larvae[7] (100% mortality at 1% conc.). In a plant tumor system[8, 9], **I** significantly inhibited the formation of crown gall tumors on potato discs (32% and 28%); this plant tumor system[8, 9] has shown a positive correlation with the 3PS murine lymphocytic leukemia system[4] in vivo, indicating that **I** might possess antitumor activity; however, no 3PS activity was observed at