Published in final edited form as:

J Chem Ecol. 2009 August ; 35(8): 861–870. doi:10.1007/s10886-009-9666-9.

ALKYLDIMETHYLPYRAZINES IN THE DEFENSIVE SPRAY OF *Phyllium westwoodii*: A FIRST FOR ORDER PHASMATODEA

AARON T. DOSSEY^{1,*}, MARCO GOTTARDO², JOHN M. WHITAKER³, WILLIAM R. ROUSH³, and ARTHUR S. EDISON^{1,4,5}

¹Department of Biochemistry & Molecular Biology, University of Florida, Gainesville FL 32610-0245

²Museo Civico di Storia Naturale di Ferrara, Via De Pisis 24, I-44100 Ferrara, Italy

³Department of Chemistry, Scripps Florida, Jupiter, Florida 33458

⁴McKnight Brain Institute, University of Florida, Gainesville FL 32610

⁵National High Magnetic Field Laboratory, University of Florida

Abstract

Phyllium westwoodii is a species of phasmid insect (Order Phasmatodea) in the more specific group known as leaf insects (Family Phylliidae). These rather large and ornate creatures are known for their morphological resemblance to plant leaves for camouflage. Pyrazines are a common class of compounds used or produced by a wide variety of organisms, even humans. When an individual of *P. westwoodii* is disturbed, it sprays an opaque liquid from a pair of prothoracic glands which are utilized by other phasmid species for defense. The current study has found that this liquid contains glucose and a mixture of 3-isobutyl-2,5-dimethylpyrazine, 2,5-dimethyl-3-(2-methylbutyl)pyrazine, and 2,5-dimethyl-3-(3-methylbutyl)pyrazine. This is the first report of pyrazines found in the defensive gland spray of phasmid insects and the first chemical analysis of glandular material from family Phylliidae.

Keywords

Insect; *Phyllium westwoodii*; Phasmatodea; Phasmid; Phylliidae; Chemical ecology; Defense; Dimethyl alkylpyrazine; 3-Isobutyl-2,5-dimethylpyrazine; 2,5-Dimethyl-3-(2-methylbutyl)pyrazine; 2,5-Dimethyl-3-(3-methylbutyl)pyrazine; Glucose

INTRODUCTION

Chemical defense in walkingstick insects (also called stick insects, or “phasmids”; Order Phasmatodea) has been studied for many decades. However, the chemical composition of defensive sprays from only a few species has been analyzed to date (Bouchard et al. 1997; Chow and Lin 1986; Dossey et al. 2007; Dossey et al. 2006; Eisner et al. 1997; Eisner 1965; Ho and Chow 1993; Meinwald et al. 1962; Schmeda-Hirschmann 2006; Schneider 1934; Smith et al. 1979). The first analysis of a phasmid defensive spray was published in 1934 on

* Correspondence should be addressed to: Dr. Aaron T. Dossey, BugoChem@gmail.com.

Supporting Information Available - S1) EIMS database matching data for natural pyrazines **1**, **2**, and **3** from *Phyllium westwoodii* defensive spray; S2-S5) 2D COSY, TOCSY, HSQC, and HMBC spectra of *P. westwoodii* defensive spray; S6) Alkyl ¹H - aromatic ¹³C expansion of HMBC spectra from synthetic compounds **1**, **2**, and **3**; and S7) A video illustrating the defensive behavior of *P. westwoodii*.

Agathemera crassa (Schneider 1934) (referred to by Schneider as *Paradoxomorpha crassa*). Possibly due to analytical limitations of that time, the chemical structure obtained in that study was likely incorrect, as the defense compound of another species in that genus (*Agathemera elegans*) was more recently determined to be 4-methyl-1-hepten-3-one (Schmeda-Hirschmann 2006). Subsequent to the work by Schneider in 1934, the next species studied was *Anisomorpha buprestoides* in classic works by Eisner, Meinwald, and co-workers (Eisner 1965; Meinwald et al. 1962). *A. buprestoides* produces at least three stereoisomers of a monoterpene first identified by Meinwald et al. in 1962 and named anisomorphal (Meinwald et al. 1962). Since then, other phasmids have been shown to produce monoterpenes such as: iridodoal (*Graeffea crouani*) (Smith et al. 1979), nepetalactone (*Graeffea crouani*) (Smith et al. 1979), actinidine (*Megacrania alpheus* (Chow and Lin 1986) and *Megacrania tsudai* (Ho and Chow 1993)), limonene (*Sipyloidea sipyilus*) (Bouchard et al. 1997), parectadial (a novel monoterpene first identified from the phasmid *Parectatosoma mocquerysi* (Dossey et al. 2007)), dolichodial (isomer of anisomorphal found in young *A. buprestoides* (Dossey et al. 2008)), peruphasmal (isomer of anisomorphal from *Peruphasma schultei*) (Dossey et al. 2006), and others as minor components (Bouchard et al. 1997; Ho and Chow 1993). In addition to monoterpenes, 4-methyl-1-hepten-3-one (as mentioned above) (Schmeda-Hirschmann 2006) and quinoline (from *Oreophoetes peruana*) (Eisner et al. 1997) have also been found in phasmid insect defense gland sprays. Besides these secondary metabolites, glucose has also been reported in the defensive spray of *A. buprestoides* (Dossey et al. 2007; Dossey et al. 2006; Zhang et al. 2007), *P. schultei* (Dossey et al. 2006), and *P. mocquerysi* (Dossey et al. 2007). The observation of glucose may be of significance in the biosynthesis and/or transport of defense substances into the glandular reservoir of these insects (see Discussion section below) (Dossey et al. 2007; Dossey et al. 2006). It has also been shown that *A. buprestoides* has the ability to biosynthesize its defensive monoterpenes from normal terpene precursors (Meinwald et al. 1966) and de-novo from glucose (Dossey et al. 2008). To date, in spite of the range of small molecules already found in phasmids, no pyrazines have been isolated from insects in the order Phasmatodea.

Leaf insects (Order Phasmatodea, Family Phylliidae) are a small group of anciently diverged phasmids comprising 46 known species, all characterized by remarkable cryptic morphological and behavioral adaptations. They have dorso-ventrally flattened bodies and foliaceous lobes on legs which resemble leaves. Females are always much longer and proportionally broader than the males, and their antennae are also particularly dimorphic. Adult males have long and densely setose antennae, while adult females possess very short antennae which bear on the third segment a stridulatory apparatus used for the production of defensive sounds (Bedford 1978; Henry 1922; Zompro and Grösser 2003) (and references within). Members of this family now occur in tropical Asia and Australia, with presumably introduced populations in eastern Africa, Madagascar and adjacent islands, but once lived as far away as Europe (Wedmann et al. 2007).

Phyllium (*Phyllium*) *westwoodii* (Wood-Mason 1875) (Fig. 1A) is a leaf insect that occurs in the Andaman Islands, China, Laos, Myanmar, Thailand and Vietnam. When threatened, females of *P. westwoodii* enter into an immobile behavioral state known as catalepsy. If the threatening stimulus persists they begin to stridulate with the antennae and release an opaque-milky spray from a pair of glands at the anterior of their prothorax (Fig. 1B). This secretion is sprayed at moderate distance over the dorsal surface of the head and thorax. Many other phasmid species produce chemical sprays from homologous glands in response to being disturbed (Bedford 1978; Bouchard et al. 1997; Chow and Lin 1986; Dossey et al. 2007; Dossey et al. 2006; Eisner et al. 1997; Eisner 1965; Ho and Chow 1993; Meinwald et al. 1962; Schmeda-Hirschmann 2006; Schneider 1934; Smith et al. 1979; Thomas 2001), and in certain cases it has been shown that these sprays function to repel potential predators

(Bouchard et al. 1997; Carlberg 1985a; Carlberg 1985b; Carlberg 1986; Carlberg 1987; Chow and Lin 1986; Eisner et al. 1997; Eisner 1965). A. T. Dossey notes that upon his first exposure to the sample sent by M. Gottardo, there was a striking similarity of the odor of *P. westwoodii* defensive spray to that of chocolate.

Pyrazines are produced by a wide range of organisms (Blum 1981; Brown and Moore 1979; Cross et al. 1979; Dickschat et al. 2005a; Dickschat et al. 2005b; Moore et al. 1990; Wheeler and Blum 1973). For insects they often function as pheromones. Alkyl dimethylpyrazines are used as pheromones by a number of insect species in the Order Hymenoptera (ants, bees, and wasps). Specifically, they are used as trail (leaf cutter ant, *Atta sexdens rubropilosa* (Cross et al. 1979)) and alarm (Brown and Moore 1979; Wheeler and Blum 1973) pheromones by some ant species and are commonly found in ant glandular secretions (Blum 1981). Other pyrazines, such as methoxyalkylpyrazines, also protect some insects from predation by functioning as anti-feedants in combination with bright coloration in aposematic species (Moore et al. 1990). These have been found in crude extracts of insects other than phasmids such as grasshoppers (genus *Poekilocerus*, for example (Moore et al. 1990)) which are in Order Orthoptera, a distinct order from Phasmatodea (Terry and Whiting 2005).

Pyrazines have very different uses for humans. They are often associated with characteristic aromas and flavors of some of our favorite foods (Adams et al. 2002; Maga and Sizer 1973). The Flavor and Extract Manufacturer's Association (FEMA) reports that 2135 kg of pyrazine flavor additives are used in the United States annually (Adams et al. 2002). Alkyl dimethylpyrazines function as odorants in foods such as chocolate (Welty et al. 2001) and are produced in the Maillard reaction during the roasting of cocoa beans (Arnoldi et al. 1988). Pyrazines also contribute to the flavor and aroma of roasted peanuts, beef, and other cooked foods (Maga and Sizer 1973).

This study reports the identification of three alkyl dimethylpyrazines, along with glucose, from less than 1 μ L of the defensive spray from a single female *P. westwoodii*. Specifically, 3-isobutyl-2,5-dimethylpyrazine (**1**), 2,5-dimethyl-3-(2-methylbutyl)pyrazine (**2**), and 2,5-dimethyl-3-(3-methylbutyl)pyrazine (**3**) (Fig. 2) were identified in the mixture. The chemical identification was performed by GC-MS and verified by NMR and chemical synthesis. This work demonstrates the increasingly apparent diversity of compounds produced by phasmid insects and the uniqueness of Phylliidae among the Phasmatodea.

MATERIALS AND METHODS

Animal Material and Sample Collection

Laboratory culture of *P. westwoodii* originated from Chiang Rai Province in northern Thailand, where the species thrives in tropical rain forest ecosystems. Rearing was conducted in a ventilated cage at a mean temperature of 23.5°C, moderate humidity conditions, and a photoperiod of 12 hr. The leaf insects were fed on *Quercus robur* and *Rubus ulmifolius*. To obtain the original sample of defensive spray (less than 1 μ L), one adult female *P. westwoodii* was milked by M. Gottardo two times by placing a clean 1.5 ml glass vial over the glandular openings, and slightly agitating the insects. A second sample (approximately 2-4 μ L), consisting of seven milkings, was obtained the same way from two adult females.

Less than 1 μ L of defensive glandular spray was obtained from a single female *P. westwoodii*. This sample was dissolved in approximately 15 μ L of D₂O and used for NMR analysis. Approximately 2 μ L were removed from this sample after collection of NMR data and extracted with CH₂Cl₂ for GC-MS analysis. Since it was unknown whether the

compounds in the mixture contained exchangeable protons, an additional 2 μL sample was mixed with $\sim 100 \mu\text{L}$ of H_2O and extracted with CH_2Cl_2 for GC-MS analysis.

Analytical Procedures

NMR experiments were performed using a 600 MHz 1-mm triple resonance high temperature superconducting (HTS) cryogenic probe that was developed through collaboration between the University of Florida, the National High Magnetic Field Laboratory (NHMFL), and Bruker Biospin (Brey et al. 2006). Each sample was loaded into a 1-mm $\times$ 100-mm capillary NMR tube (Norell, Inc.) using a 10 μL syringe with a removable 110-mm $\times$ 30-gauge blunt needle. During NMR experiments, the capillary tube was held in a standard 10-mm spinner using a Bruker MATCHTM device, and the capillary-MATCH-spinner combination was lowered vertically into the magnet on an air-column as usual. The sample temperature was maintained at 20°C. The spectrometer used for all NMR experiments was a Bruker Avance II 600. Additional NMR data acquisition parameters can be found in the Supporting Information with their respective spectra. All data acquisition, processing, and analysis were done using Bruker TopSpin[®] 2.0 software. Chemical shift assignments were made by referencing the natural *P. westwoodii* ^1H and ^{13}C chemical shifts of the anomeric ^1H to 5.22 and ^{13}C to 94.8 ppm of alpha glucose based on the reported values for these resonances in the BMRB Metabolomic database (<http://www.bmrb.wisc.edu/metabolomics/>) (Ulrich et al. 2008). In order to verify the components in *P. westwoodii* defensive spray tentatively identified by GC-EIMS and assign ^1H and ^{13}C chemical shifts to those compounds (Tables 1-3), one- and two-dimensional (1D and 2D, respectively) datasets were recorded (Figs 5-7, Tables 1-3, and Supporting Information Figs S2-S5). Specifically, COSY, TOCSY, NOESY, and natural abundance ^{13}C HMQC and HMBC datasets were collected.

GC-MS analyses were performed with a Thermo Scientific Trace DSQ mass spectrometer, equipped with a Restek RxiTM-5ms column (15 m $\times$ 0.25 mm ID $\times$ 0.25 μm df) and with helium as carrier gas (flow rate = 1 mL/min). Four μL of each sample were injected in splitless mode, for 2 minutes, followed by a split flow rate of 50 mL/min. The GC oven temperature was maintained 40°C for 3 minutes, and then increased at a rate of 20°C/min to 260°C. The injector and transfer line were set at 250°C while the ion source was at 180°C. Compounds eluting from the column underwent electron ionization (EI) at 70 eV and ions from m/z 50 to 700 were detected. The electron-ionization mass spectra (EI-MS) of these peaks were used to query the NIST/EPA/NIH Mass Spectral Library of known compounds using the NIST MS Search (ver. 2) software (Stein et al. 1987-2002).

Synthesis of Pyrazines: General Procedure

The Grignard reagent was prepared by slow addition of the appropriate alkyl bromide (1.0 equiv.) to a stirred mixture of magnesium turnings (1.2 equiv.) in dry ether (1.0 M) at room temperature. The resulting mixture was stirred for 2 h at room temperature and then titrated with 2-hydroxybenzaldehyde phenylhydrazon (Love and Jones 1999). Commercial 3-chloro-2,5-dimethylpyrazine (1.0 equiv.) was added to a solution of $\text{Fe}(\text{acac})_3$ (0.05 equiv.) in THF-NMP (9:1, 0.25 M) and the mixture was cooled to 0°C in an ice-water bath. To this solution was added, dropwise, a solution of the Grignard reagent (1.5 equiv) in ether. The resulting mixture was stirred for an additional 30 min and then quenched by the addition of water. The aqueous layer was extracted with diethyl ether. The combined organic extracts were dried over MgSO_4 , clarified with charcoal, filtered and solvent removed under reduced pressure. The crude product mixtures were purified by silica gel column chromatography, using a solution of 5% ethyl acetate in hexanes.

3-Isobutyl-2,5-dimethylpyrazine (1)

Yield: 42% (3.463 g, 21.08 mmol). ^1H NMR (400 MHz, CDCl_3): 0.94 (d, $J = 6.8$ Hz, 6 H), 2.12 (non, $J = 6.8$ Hz, 1 H), 2.48 (s, 3 H), 2.51 (s, 3 H), 2.65 (d, $J = 7.2$ Hz, 2 H), 8.14 (s, 1 H); ^{13}C NMR (100 MHz, CDCl_3): 21.3, 21.7, 22.6, 28.8, 43.7, 140.7, 149.0, 150.1, 154.4; IR (film): 2957, 2869, 1450, 1370, 1288, 1168, 1074 cm^{-1} ; EI-MS 165.1386, HRMS-ESI (m/z): $[\text{M}^+]$ calcd for $\text{C}_{10}\text{H}_{16}\text{N}_2$ 165.1386, found 165.1390. Spectroscopic values for synthetic **1** were in agreement with reported literature values (Dickschat et al. 2005a).

(S)-2,5-Dimethyl-3-(2-methylbutyl)pyrazine (2)

Yield. 46% (546 mg, 3.06 mmol). ^1H NMR (400 MHz, CDCl_3): 0.87 (d, $J = 6.8$ Hz, 3 H), 0.92 (t, $J = 7.6$ Hz, 3 H), 1.26 (m, 1 H), 1.42 (m, 1 H), 1.90 (m, 1 H), 2.49 (s, 3 H), 2.51 (s, 3 H), 2.56 (dd, $J = 8.4, 13.2$ Hz, 1 H) 2.78 (dd, $J = 6.0, 13.2$ Hz, 1 H), 8.13 (s, 1 H); ^{13}C NMR (100 MHz, CDCl_3): 11.6, 19.0, 21.3, 21.6, 29.7, 34.9, 42.0, 140.7, 149.0, 150.1, 154.5; HRMS-ESI (m/z): $[\text{M}^+]$ calcd for $\text{C}_{11}\text{H}_{18}\text{N}_2$ 179.1543, found 179.1545, specific optical rotation: $[\alpha]_{\text{D}}^{25} = +8.8$ ($c = 2.535$, CH_2Cl_2). Spectroscopic values for synthetic **2** were in agreement with reported literature values (Dickschat et al. 2005a).

2,5-Dimethyl-3-(3-methylbutyl)pyrazine (3)

Yield: 55% (2.458 g, 13.79 mmol). ^1H NMR (400 MHz, CDCl_3): 0.97 (d, $J = 6.8$ Hz, 6 H), 1.53 (m, 2 H), 1.67 (sept, $J = 6.8$ Hz, 1 H), 2.47 (s, 1 H), 2.51 (s, 1 H), 2.75 (m, 2 H), 8.13 (s, 1 H); ^{13}C NMR (100 MHz, CDCl_3): 21.2, 22.6, 28.5, 33.4, 37.8, 140.8, 148.5, 150.2, 155.3; IR (film): 2956, 2927, 2870, 1536, 1452, 1320, 1278, 1254, 1169, 1080, 1034, 999, 942, 738; HRMS-ESI (m/z): $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{11}\text{H}_{18}\text{N}_2$ 179.1543, found 179.1546. Spectroscopic values of synthetic **3** were in agreement with reported literature values (Dickschat et al. 2005b).

RESULTS

In the GC-MS chromatogram of *Phyllium westwoodii* defensive spray, three main peaks were detected (Fig. 3). Two samples extracted from D_2O and H_2O preparations both showed nearly identical mass spectra, suggesting that they contained no exchangeable protons (Fig. 4 and data not shown).

Mass spectra from all three peaks were matched to known alkyl dimethylpyrazines by high scoring database hits (Fig. 4 and Supporting Information Fig. S1) (Stein et al. 1987-2002). Additionally, as can be seen in Fig. 4, the mass spectra match those of synthetic standards (Fig. 4). However, some isomers of the highest scoring compounds were also close matches (Supporting Information Fig S1). Additionally, similar mass spectra could be obtained from molecules with different relative placement of the alkyl chains and methyl groups on the pyrazine ring. Also, previous studies have shown phasmid defensive sprays to contain glucose (Dossey et al. 2007; Dossey et al. 2006; Zhang et al. 2007), which is not directly amenable to study by GC-MS. Thus, NMR experiments were performed on both the natural defensive spray of *P. westwoodii* and synthetic reference standards in order to alleviate ambiguity in the identification of chemical components and identify non-volatiles.

First, as with other phasmids, *P. westwoodii* defensive spray contained a significant amount of glucose: approximately 27% for one sample and 42% for an independent replicate sample of the total material observed by integration of the 1D ^1H NMR spectrum: anomeric resonances of glucose compared to the vinyl and ring methyl resonances for pyrazines (Fig. 5 and data not shown). Additionally, three sets of spin systems consistent with the aliphatic constituents of the alkyl pyrazines identified by GC-MS were the major peaks observed in 2D TOCSY NMR spectra (Fig. 7). Overlaying of two-dimensional NMR spectra can be a

powerful and robust way to identify individual known substances in natural product mixtures. However, considerable overlap was observed between some key resonances common to the three compounds (Fig. 6). Thus, all three substances were synthesized and examined in pure form in D₂O by NMR to verify their identity in the spectra of the natural product sample. 2,5-Dimethyl-3-alkylpyrazines **1**, **2** and **3** were synthesized *via* iron-catalyzed cross-coupling (Furstner et al. 2002) of 3-chloro-2,5-dimethylpyrazine with the appropriate Grignard reagent, as described by Dickschat et al. (Fig. 8) (Dickschat et al. 2005a; Dickschat et al. 2005b). Enantiopure (*S*)-(+)-1-bromo-2-methylbutane was used for formation of the Grignard reagent in the synthesis of **2** under the presumption that the pyrazine derives biosynthetically from *L*-isoleucine.

One-dimensional (1D) NMR spectra of the natural mixture from *P. westwoodii* and the three synthetic pyrazines in D₂O are compared in Fig. 6. The alkyl and aromatic methyl region of the spectrum from 0.6-3.1 ppm (Fig. 6B) shows reasonably well-resolved resonances for the alkyl sidechains. Comparing the spectrum of the mixture to those of the synthetic pyrazines in Fig. 6 indicates that the aromatic methyl resonances for all three compounds have nearly identical chemical shifts in the 2.4-2.5 ppm region. Additionally, the aromatic ¹H region at about 8.15 ppm shows at least three resonances, two of which are almost completely overlapped. Thus, analysis of 2D spectra for the natural mixture and of the individual synthetic compounds was employed to resolve the ambiguity caused by the overlapping regions and obtain complete ¹H and ¹³C chemical shift assignments for all three pyrazines.

Fig. 7 shows the aromatic methyl and alkyl sidechain resonances of TOCSY spectra of the natural mixture (black) and the three synthetic pyrazines in color overlays (**1** green, **2** blue, and **3** red). In the TOCSY spectrum of the mixture, three distinct alkyl spin systems were observed. For compound **1**, TOCSY correlations were observed between resonances at 2.68 ppm and the following: 2.00, and 0.90 ppm. COSY correlations were observed between resonances at 2.68 ppm and 2.00 ppm and between 2.00 ppm and 0.90, but not between 2.68 ppm and 0.90 ppm. For compound **2**, TOCSY correlations were observed between the resonance at 2.82 ppm and the following resonances: 2.58, 1.78, 1.36, 1.25, 0.88, and 0.83 ppm. Strong COSY correlations were observed between 2.82 and 2.58 ppm; 1.36 and 1.25 ppm; 1.36 ppm and 0.88 ppm; and 1.25 ppm and 0.88 ppm. Weaker COSY correlations are seen between the following resonances: 2.82 and 1.78; 2.58 and 1.78; 1.78 and 0.83 ppm. Oddly, no COSY ¹H-¹H correlations were observed in spectra from either natural or synthetic compound **2** between the methine 2' resonance (1.78 ppm) and methylenes at 1.36 or 1.25 ppm (Supporting Information Fig. S3). However, distinct TOCSY correlations were observed between these pairs of resonances as expected (Fig. 7 and Supporting Information Fig. S2). For compound **3**, TOCSY correlations were observed between 2.78 ppm and the following resonances: 1.47, 1.63, and 0.93 ppm. A strong COSY correlation was observed between the methylenes at 2.78 and 1.47 ppm and between the methyl and methylene resonances (1.47 and 0.93, respectively). However, only very weak COSY correlations were observed between the methylene resonance at 1.47 ppm and methyne at 1.63 ppm.

An overlapped set of spin systems with correlations between resonances at about 2.4-2.5 ppm and 8.15 ppm were also observed in TOCSY spectra. There were no clear ¹H-¹H correlations between those spin systems and the three alkyl ones (Fig. 7). However, the HMBC of the three pure synthetic pyrazines analyzed showed correlations between the 1' ¹H and the 2 and 3 ¹³C resonances (Supporting Information S6). Additionally, the HMBC of the mixture showed a weak correlation between 2.78 and 158.5 ppm, demonstrating connectivity between the alkyl and other spin systems (Supporting Information S5). Finally, the sign (+/-) of the HSQC correlations are sensitive to and consistent with the proposed methine, methylene, and methyl groups (Supporting Information S4).

In addition to chemical analysis, a video was recorded which illustrates some features of the defensive behavior of *P. westwoodii* (Supporting Information Fig. S7). This behavior is well established in the literature for other species in the same genus (Bedford 1978; Henry 1922; Zompro and Grösser 2003)(and references within). First, the insect was removed from the cage and placed on a stick for observation. This action sufficiently disturbed the insect causing it froze in a pose as if to play dead or possibly to resemble a leaf (a behavior known as catalepsy). The legs are positioned in a seemingly disordered conformation and not all are used to hold onto the stick. Next, if the offending stimulus is continued more aggressively and the insect is grasped or compressed, it becomes more active, attempts to escape, and produces stridulatory sounds using its antennae. It is at this point that the insect releases its defensive spray. Although the amount of secretion produced by *P. westwoodii* is small, to the human observer its intense odor can be strongly perceived even at a distance of at least 30 cm from the insect. M. Gottardo notes that he has never perceived this odor in behavioral contexts of *P. westwoodii* besides defense.

DISCUSSION

These data demonstrate that the defensive spray of *Phyllium westwoodii* contains 3-isobutyl-2,5-dimethylpyrazine (**1**), 2,5-dimethyl-3-(2-methylbutyl)pyrazine (**2**), and 2,5-dimethyl-3-(3-methylbutyl)pyrazine (**3**), and glucose. The release of this spray from *P. westwoodii* happens concurrently with a characteristic defense behavior (Supporting Information Fig. S7 and described in the Introduction section above) when approached by a human, which likely resembles an attack of natural predators of this species such as slow lorises and other primates (Francis Seow-Choen, personal communication). Additionally, chemical sprays from homologous glands in other phasmid species have been experimentally shown to be effective in repelling potential predators such as ants, frogs, and birds (Bouchard et al. 1997; Carlberg 1985a; Carlberg 1985b; Carlberg 1986; Carlberg 1987; Chow and Lin 1986; Eisner et al. 1997; Eisner 1965). Thus, the prothoracic glandular spray from *P. westwoodii* is likely to be used predominantly for defense. However, it has not been shown if **1**, **2**, or **3** function as repellants of predators in the natural habitat of *P. westwoodii*. Interestingly, these compounds are widely found in the aromas and flavors of foods which humans find very pleasing (Maga and Sizer 1973). Some insects use these and other similar pyrazines as pheromones (Blum 1981; Cavill and Houghton 1974; Cross et al. 1979; Tengo et al. 1982). Also, the amount produced by *P. westwoodii* in response to being disturbed is quite small. In fact, it has been postulated that because many phasmids that produce only very small quantities of prothoracic glandular spray (Tilgner 2002), and because the chemistry of their spray can vary over development (Dossey et al. 2008), some species may use these substances for functions other than defense. Thus, it is possible that *P. westwoodii*, as well as other Phylliidae, use these compounds as pheromones or in other forms of chemical communication. However, these hypotheses are beyond the scope of this study and, as far as we are aware, have not yet been tested.

Other phasmid species produce monoterpenes (Bouchard et al. 1997; Chow and Lin 1986; Dossey et al. 2007; Dossey et al. 2006; Ho and Chow 1993; Meinwald et al. 1962; Smith et al. 1979), a straight chain ketone (Schmeda-Hirschmann 2006), quinoline (Eisner et al. 1997), and other small secondary metabolites in their prothoracic defensive glands. However, this is the first report of pyrazines from these glands in any phasmid. In addition to *P. westwoodii* in the current study, glucose has also been found in defensive sprays from other species of phasmids (Dossey et al. 2007; Dossey et al. 2006; Zhang et al. 2007). In fact, some beetles (Order Coleoptera) of the family Chrysomelidae (the leaf beetles) use precursors that are conjugated with glucose for monoterpene defensive secretion biosynthesis and transport into the defense glands (Burse et al. 2007; Feld et al. 2001; Kunert et al. 2008; Laurent et al. 2003). The common occurrence of glucose in insect

defensive secretions, especially phasmids such as *P. westwoodii*, suggests that it may be similarly utilized in defensive secondary metabolite processing and/or transport. If so, this may represent a previously unreported mechanism for pyrazine production. However, such a hypothesis requires further investigation.

The peculiar morphological features characterizing fossil and extant leaf insects (Wedmann et al. 2007; Zompro 2004), along with the results of this article, contribute to the evidence that Phylliidae occupy a unique position among Phasmatodea. This study demonstrates yet another distinguishing feature of this unique group of phasmids. Many other phasmids that have been studied produce monoterpenes or other secondary metabolites in their defensive spray, but the apparently homologous glands in *P. westwoodii* produce pyrazines, a class of compounds not previously reported from Phasmatodea. Pyrazines found in the chemical defense glands of *P. westwoodii* might even be considered an evolutionarily derived (apomorphic) feature of Phylliidae that further supports the monophyly of the taxon. The presence of glucose in walkingstick defensive sprays, along with the large size and ease of culture for these creatures, suggests potential value of these insects as a model for the study of their biosynthetic pathways. Additionally, discovery of the functional role of pyrazines in *P. westwoodii* may shed new light on the role of secreted small molecules used by insects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank James R. Rocca at the Advanced Magnetic Resonance Imaging and Spectroscopy, University of Florida, for very helpful technical assistance on NMR experiments. The GC-MS analysis reported in this study was developed and conducted by Dr. Maria Cristina Dancel of the Mass Spectrometry Facility, Department of Chemistry, University of Florida, who also helped with data interpretation. Funding was generously provided by NIH P41RR016105, the Human Frontier Science Program (ASE), and the NSF-supported National High Magnetic Field Laboratory, and Scripps Florida (WRR). NMR data were collected in the AMRIS facility in the McKnight Brain Institute of the University of Florida.

REFERENCES

- ADAMS TB, DOULL J, FERON VJ, GOODMAN JI, MARNETT LJ, MUNRO IC, NEWBERNE PM, PORTOGHESE PS, SMITH RL, WADDELL WJ, WAGNER BM. The FEMA GRAS assessment of pyrazine derivatives used as flavor ingredients. Flavor and Extract Manufacturers Association. Food Chem. Toxicol. 2002; 40:429–51. [PubMed: 11893403]
- ARNOLDI A, ARNOLDI C, BALDI O, GRIFFINI A. Flavor Components in the Maillard Reaction of Different Amino-Acids with Fructose in Cocoa Butter Water - Qualitative and Quantitative-Analysis of Pyrazines. Journal of Agricultural and Food Chemistry. 1988; 36:988–992.
- BEDFORD GO. Biology and Ecology of the Phasmatodea. Ann. Rev. Entomol. 1978; 23:125–149.
- BLUM, MS. Chemical defenses of arthropods. Academic Press; New York: 1981. p. xiip. 562
- BOUCHARD P, HSIUNG CC, YAYLAYAN VA. Chemical analysis of defense secretions of *Sipyloidea sipylos* and their potential use as repellents against rats. J. Chem. Ecol. 1997; 23:2049–2057.
- BREY WW, EDISON AS, NAST RE, ROCCA JR, SAHA S, WITHERS RS. Design, construction, and validation of a 1-mm triple-resonance high-temperature-superconducting probe for NMR. J. Magn. Reson. 2006; 179:290–3. [PubMed: 16423543]
- BROWN WV, MOORE BP. Volatile secretory products of an australian formicine ant of the genus *Calomyrmex* (Hymenoptera: Formicidae). Insect Biochem. 1979; 9:451–460.
- BURSE A, SCHMIDT A, FRICK S, KUHN J, GERSHENZON J, BOLAND W. Iridoid biosynthesis in Chrysomelina larvae: Fat body produces early terpenoid precursors. Insect Biochem. Molec. Biol. 2007; 37:255–265. [PubMed: 17296500]

- CARLBERG U. Chemical Defense in *Anisomorpha buprestoides* (Houttuyn in Stoll) (Insecta, Phasmida). Zool. Anz. 1985a; 215:177–188.
- CARLBERG U. Chemical Defense in *Extatosoma tiaratum* (Macleay) (Insecta, Phasmida). Zool. Anz. 1985b; 214:185–192.
- CARLBERG U. Chemical Defense in *Sipyloidea sipyilus* (Westwood) (Insecta, Phasmida). Zool. Anz. 1986; 217:31–38.
- CARLBERG U. Chemical Defense in Phasmida Vs Mantodea (Insecta). Zool. Anz. 1987; 218:369–373.
- CAVILL GWK, HOUGHTON E. Some Pyrazine Derivatives from Argentine Ant, *Iridomyrmex humilis*. Aust. J. Chem. 1974; 27:879–889.
- CHOW YS, LIN YM. Actinidine, a Defensive Secretion of Stick Insect, *Megacrana alpheus* Westwood (Orthoptera, Phasmatidae). J. Entomol. Sci. 1986; 21:97–101.
- CROSS JH, BYLER RC, RAVID U, SILVERSTEIN RM, ROBINSON SW, BAKER PM, SABINODEOLIVEIRA J, JUTSUM AR, CHERRETT JM. Major Component of the Trail Pheromone of the Leaf-Cutting Ant, *Atta sexdens rubropilosa* Forel - 3-Ethyl-2,5-Dimethylpyrazine. J. Chem. Ecol. 1979; 5:187–203.
- DICKSCHAT JS, REICHENBACH H, WAGNER-DOBLER I, SCHULZ S. Novel pyrazines from the myxobacterium *Chondromyces crocatus* and marine bacteria. Eur. J. Org. Chem. 2005a; 4141–4153.
- DICKSCHAT JS, WAGNER-DOBLER I, SCHULZ S. The chafer pheromone buibulactone and ant pyrazines are also produced by marine bacteria. J. Chem. Ecol. 2005b; 31:925–947. [PubMed: 16124260]
- DOSSEY AT, WALSE SS, CONLE OV, EDISON AS. Parectadial, a Monoterpenoid from the Defensive Spray of *Parectatosoma mocquerysi*. J. Nat. Prod. 2007; 70:1335–1338. [PubMed: 17661519]
- DOSSEY AT, WALSE SS, EDISON AS. Developmental and geographical variation in the chemical defense of the walkingstick insect *Anisomorpha buprestoides*. J. Chem. Ecol. 2008; 34:584–590. [PubMed: 18401661]
- DOSSEY AT, WALSE SS, ROCCA JR, EDISON AS. Single insect NMR: a new tool to probe chemical biodiversity. ACS Chem. Biol. 2006; 1:511–4. [PubMed: 17168538]
- EISNER, MORGAN RC, ATTYGALLE AB, SMEDLEY SR, HERATH KB, MEINWALD J. Defensive production of quinoline by a phasmid insect (*Oreophoetes peruana*). J. Exp. Biol. 1997; 200:2493–500. [PubMed: 9366083]
- EISNER T. Defensive Spray of a Phasmid Insect. Science. 1965; 148:966. [PubMed: 17751052]
- FELD BK, PASTEELS JM, BOLAND W. *Phaedon cochleariae* and *Gastrophysa viridula* (Coleoptera : Chrysomelidae) produce defensive iridoid monoterpenes de novo and are able to sequester glycosidically bound terpenoid precursors. Chemoecol. 2001; 11:191–198.
- FURSTNER A, LEITNER A, MENDEZ M, KRAUSE H. Iron-catalyzed cross-coupling reactions. J. Am. Chem. Soc. 2002; 124:13856–13863. [PubMed: 12431116]
- HENRY GM. Stridulation in the leaf insect. Spolia Zeylan. 1922; 12:217–219.
- HO HY, CHOW YS. Chemical-Identification of Defensive Secretion of Stick Insect, *Megacrana tsudai* Shiraki. J. Chem. Ecol. 1993; 19:39–46.
- KUNERT M, SOE A, BARTRAM S, DISCHER S, TOLZIN-BANASCH K, NIE L, DAVID A, PASTEELS J, BOLAND W. De novo biosynthesis versus sequestration: A network of transport systems supports in iridoid producing leaf beetle larvae both modes of defense. Insect Biochem. Molec. Biol. 2008; 38:895–904. [PubMed: 18687400]
- LAURENT P, BRAEKMAN JC, DALOZE D, PASTEELS J. Biosynthesis of defensive compounds from beetles and ants. Eur. J. Org. Chem. 2003; 2733–2743.
- LOVE BE, JONES EG. The use of salicylaldehyde phenylhydrazine as an indicator for the titration of organometallic reagents. J. Org. Chem. 1999; 64:3755–3756. [PubMed: 11674512]
- MAGA JA, SIZER CE. Pyrazines in Foods - Review. J. Agric. Food Chem. 1973; 21:22–30.
- MEINWALD J, CHADHA MS, HURST JJ, EISNER T. Defense Mechanisms of Arthropods .9. Anisomorphal, the Secretion of a Phasmid Insect. Tet. Lett. 1962:29–33.

- MEINWALD J, HAPP GM, LABOWS J, EISNER T. Cyclopentanoid Terpene Biosynthesis in a Phasmid Insect and in Catmint. *Science*. 1966; 151:79–80. [PubMed: 5908966]
- MOORE BP, BROWN WV, ROTHSCILD M. Methyalkylpyrazines in aposematic insects. *Chemoecol*. 1990; 1:43–51.
- SCHMEDA-HIRSCHMANN G. 4-Methyl-1-hepten-3-one, the defensive compound from *Agathemera elegans* (Philippi) (Phasmatidae) insecta. *Zeitschrift Fur Natur. C - J. Biosci*. 2006; 61:592–594.
- SCHNEIDER CO. Las emanaciones del chinchemayo *Paradoxomorpha crassa*. *Rev. Chil. Hist. Nat*. 1934; 38:44–46.
- SMITH RM, BROPHY JJ, CAVILL GWK, DAVIES NW. Iridodials and Nepetalactone in the Defensive Secretion of the Coconut Stick Insects, *Graeffea crouani*. *J. Chem. Ecol*. 1979; 5:727–735.
- STEIN, S.; MIROKHIN, Y.; TCHEKHOVSKOI, D.; MALLARD, G. NIST Mass Spectral Search Program. Version July 1, 2002 a build. National Institute of Standards and Technology, Standard Reference Data Program, ChemSW (Chemistry Software for Windows); Fairfield, CA: 1987-2002.
- TENGO J, BERGSTROM G, BORGKARLSON AK, GROTH I, FRANCKE W. Volatile Compounds from Cephalic Secretions of Females in 2 Cleptoparasite Bee Genera, *Epeolus* (Hym, Anthophoridae) and *Coelioxys* (Hym, Megachilidae). *Zeitschrift Fur Naturforschung C-a Journal of Biosciences*. 1982; 37:376–380.
- TERRY MD, WHITING MF. Mantophasmatodea and phylogeny of the lower neopterous insects. *Cladistics*. 2005; 21:240–257.
- THOMAS MC. The Twostriped Walkingstick, *Anisomorpha buprestoides* (Stoll), (Phasmatodea: Pseudophasmatidae). Fla. Dept. Agri. & Consumer Svcs. Division of Plant Industry - Entomology Circular. 2001:408.
- TILGNER, EH. PhD Dissertation. University of Georgia; Athens: 2002. Systematics of Phasmida..
- ULRICH EL, AKUTSU H, DORELEIJERS JF, HARANO Y, IOANNIDIS YE, LIN J, LIVNY M, MADING S, MAZIUK D, MILLER Z, NAKATANI E, SCHULTE CF, TOLMIE DE, KENT WENGER R, YAO H, MARKLEY JL. BioMagResBank. *Nucleic Acids Res*. 2008; 36:D402–8. [PubMed: 17984079]
- WEDMANN S, BRADLER S, RUST J. The first fossil leaf insect: 47 million years of specialized cryptic morphology and behavior. *Proc. Natl. Acad. Sci. USA*. 2007; 104:565–569. [PubMed: 17197423]
- WELTY WM, MARSHALL RT, GRUN IU, ELLERSIECK MR. Effects of milk fat, cocoa butter, or selected fat replacers on flavor volatiles of chocolate ice cream. *J. Dairy Sci*. 2001; 84:21–30. [PubMed: 11210035]
- WHEELER JW, BLUM MS. Alkylpyrazine Alarm Pheromones in Ponerine Ants. *Science*. 1973; 182:501–503. [PubMed: 17832469]
- WOOD-MASON J. On new or little-known species of Phasmidae, with a brief preliminary notice of the occurrence of a clasping apparatus in the males throughout the family. *J. Asiatic Soc. Bengal*. 1875; 44:215–220.
- ZHANG F, DOSSEY AT, ZACHARIAH C, EDISON AS, BRUSCHWEILER R. Strategy for automated analysis of dynamic metabolic mixtures by NMR. Application to an insect venom. *Anal. Chem*. 2007; 79:7748–7752. [PubMed: 17822309]
- ZOMPRO, O. Revision of the genera of the Areolatae, including the status of *Timema* and *Agathemera* (Insecta, Phasmatodea).. Verlag Goecke & Evers.; Weiler, Germany: 2004. p. 327
- ZOMPRO O, GRÖSSER D. A generic revision of the insect order Phasmatodea: The genera of the areolate stick insect family Phylliidae (Walking Leaves) (Insecta, Orthoptera). *Spixiana*. 2003; 26:130.

Fig. 1. Photographs of an adult female *Phyllium westwoodii*. A) Whole insect shown sitting on *Sambucus nigra* (not native food plant), and B) close-up of head and prothorax of same insect as in (A) with red arrows showing the position of the openings of its defensive glands. Photographs by Marco Gottardo

Fig.2.

Three alkyl dimethylpyrazines identified in the defensive spray of *Phyllium westwoodii*. 3-isobutyl-2,5-dimethylpyrazine (**1**), 2,5-dimethyl-3-(2-methylbutyl)pyrazine (**2**), and 2,5-dimethyl-3-(3-methylbutyl)pyrazine (**3**). Each structure is shown with corresponding carbon atom labeling.

Fig. 3. GC-CIMS total ion traces of natural *P. westwoodii* defensive spray compared with authentic synthetic standards. Traces shown are of natural *P. westwoodii* defensive spray (black), synthetic 3-isobutyl-2,5-dimethylpyrazine (green), synthetic (*S*)-2,5-dimethyl-3-(2-methylbutyl)pyrazine (blue), and synthetic 2,5-dimethyl-3-(3-methylbutyl)pyrazine (red). Each trace represents the injection into the GC apparatus of 4 μL of a sample made by extracting 2 μL of a D_2O NMR sample with 50 μL of methylene chloride (CH_2Cl_2). The NMR samples of synthetic reference compounds were 1 μL of pure neat material dissolved in 1 mL of D_2O .

Fig. 4. Electron ionization mass spectra (GC-EIMS) of *Phyllium westwoodii* defensive spray extracted with CH_2Cl_2 (methylene chloride). The top row of spectra are from natural material and the bottom row are from authentic synthesized standards.

Fig. 5.
1D ^1H NMR spectrum of *P. westwoodii* defensive spray dissolved in deuterium oxide (D_2O). Resonances from glucose are shown under the red bracket.

Fig. 6.
1D ^1H spectra of natural *P. westwoodii* defensive spray compared with authentic synthetic standards: A) aromatic region and B) aliphatic region. Spectra shown are of natural *P. westwoodii* defensive spray (black), synthetic 3-isobutyl-2,5-dimethylpyrazine (**1**) (green), synthetic (*S*)-2,5-dimethyl-3-(2-methylbutyl)pyrazine (**2**) (blue), and synthetic 2,5-dimethyl-3-(3-methylbutyl)pyrazine (**3**) (red). All samples were analyzed in D_2O . The synthetic NMR samples were 1 μL of pure neat material dissolved in 1 mL of D_2O .

Fig. 7.

Aliphatic sidechain region of 2D ^1H TOCSY spectra compared to authentic synthetic standards by overlaying the spectra. Spectra shown are of natural *P. westwoodii* defensive spray (black), synthetic 3-isobutyl-2,5-dimethylpyrazine (**1**) (green), synthetic (*S*)-2,5-dimethyl-3-(2-methylbutyl)pyrazine (**2**) (blue), and synthetic 2,5-dimethyl-3-(3-methylbutyl)pyrazine (**3**) (red). All samples were analyzed in D_2O . The synthetic NMR samples were 1 μL of pure neat material dissolved in 1 mL of D_2O .

Fig. 8.
Synthetic scheme for alkyldimethylpyrazines **1-3**.

Table 1NMR chemical shift assignments Of synthetic **1** (3-isobutyl-2,5-dimethylpyrazine) dissolved in D₂O

	Position	¹ H (ppm)	¹³ C (ppm)	<i>J</i> _{H-H} (Hz)
2	-	151.9	-
	3	-	157.2	-
	5	-	152.7	-
	6	8.17	143.0	(s)
	2-Me	2.51	22.6	(s)
	5-Me	2.45	22.2	(s)
	1'	2.68	45.3	7.46 (d)
	2'	2.00	31.2	(m)
	3'a/b	0.90	24.3	6.62 (d)

Table 2

NMR chemical shift assignments of synthetic **2** ((*S*)-2,5-dimethyl-3-(2-methylbutyl)pyrazine) dissolved in D₂O

	Position	¹ H (ppm)	¹³ C (ppm)	<i>J</i> _{H-H} (Hz)
	2	-	152.3	-
	3	-	157.8	-
	5	-	153.0	-
	6	8.16	143.0	(s)
	2-Me	2.51	22.6	(s)
	5-Me	2.45	22.18	(s)
	1'	2.82	43.7	(m)
		2.58	-	(m)
	2'	1.78	37.6	(m)
	2'-Me	0.83	20.8	6.69 (d)
	3'	1.36	31.9	(m)
		1.25	-	(m)
	4'	0.88	13.5	7.44 (t)

Table 3NMR chemical shift assignments **3** (2,5-dimethyl-3-(3-methylbutyl)pyrazine) dissolved in D₂O

	Position	¹ H (ppm)	¹³ C (ppm)	<i>J</i> _{H-H} (Hz)
2	-	149.5	-
	3	-	156.3	-
	5	-	150.9	-
	6	8.15	140.5	(s)
	2-Me	2.51	20.1	(s)
	5-Me	2.44	20.0	(s)
	1'	2.78	32.6	(m)
	2'	1.47	38.0	(m)
	3'	1.63	28.2	6.6 (m)
	4'a/b	0.93	22.1	6.71 (d)