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Alkyldimethylpyrazines in the Defensive Spray of *Phyllium westwoodii*: A First for Order Phasmatodea

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Abstract *Phyllium westwoodii* is a phasmid insect (Order Phasmatodea) belonging to the Family Phylliidae (leaf insects). 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 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 decades. However, the chemical composition of defensive sprays from only a few species has been analyzed (Schneider 1934; Meinwald et al. 1962; Smith et al. 1979; Chow and Lin 1986; Ho and Chow 1993; Bouchard et al. 1997; Eisner et al. 1997; Dossey et al. 2006, 2007; Schmeda-Hirschmann 2006). The first analysis of a phasmid defensive spray was published in 1934 on *Agathemera crassa* (Schneider 1934) (referred to by Schneider as *Paradoxomorpha crassa*). Possibly, due to analytical limitations of that time, the chemical structure 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 (Meinwald et al. 1962; Eisner 1965). *Anisomorpha buprestoides* produces at least three stereoisomers of a monoterpene first identified by Meinwald et al. (Meinwald et al. 1962; Dossey et al. 2006; Dossey et al.

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2008) and named anisomorphal. Since then, other phasmids have been shown to produce monoterpenes such as: iridodial (*Graeffea crouani*) (Smith et al. 1979), nepetalactone (*G. crouani*) (Smith et al. 1979), actinidine [*Megacrania alpheus* (Chow and Lin 1986) and *Megacrania* tsudai (Ho and Chow 1993)], limonene (*Sipyloidea sipylus*) (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, 2008), and others as minor components (Ho and Chow 1993; Bouchard et al. 1997). 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) also have been found in phasmid insect defense gland sprays.

Besides these secondary metabolites, glucose has been reported in the defensive spray of *A. buprestoides* (Dossey et al. 2006; Zhang et al. 2007), *P. schultei* (Dossey et al. 2006), and *P. mocquerysi* (Dossey et al. 2007). The presence of glucose may be of significance in the biosynthesis and/or transport of defensive substances into the glandular reservoir of these insects (see "Discussion") (Dossey et al. 2006, 2007). It also has been shown that *A. buprestoides* has the ability to biosynthesize its defensive monoterpenes from acetate and mevalonate (Meinwald et al. 1966) and *de-novo* from glucose (Dossey et al. 2008). To date, despite 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) make up a small group of anciently diverged phasmids that comprises 46 known species, all characterized by remarkable cryptic morphological and behavioral adaptations. They have dorso-ventrally flattened bodies and foliaceus lobes on legs that resemble leaves. Females are always much longer and proportionally broader than males, and their antennae also are particularly dimorphic. Adult males have long and densely setose antennae, while adult females possess very short antennae that bear on the third segment a stridulatory apparatus used for the production of defensive sounds (Henry 1922; Bedford 1978; Zompro and Grösser 2003, and references therein). Members of this family now occur in tropical Asia and Australia, with presumably introduced populations in eastern Africa, Madagascar, and adjacent islands, but they 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 (Schneider 1934; Meinwald et al. 1962; Eisner 1965; Bedford 1978; Smith et al. 1979; Chow and Lin 1986; Ho and Chow 1993; Bouchard et al. 1997; Eisner et al. 1997; Thomas 2001; Dossey et al. 2006, 2007; Schmeda-Hirschmann 2006), and in certain cases these sprays function to repel potential predators (Eisner 1965; Carlberg 1985a b, 1986, 1987; Chow and Lin 1986; Bouchard et al. 1997; Eisner et al. 1997). The odor of *P. westwoodii* defensive spray has a striking similarity to that of chocolate.

Pyrazines are produced by a wide range of organisms (Wheeler and Blum 1973; Brown and Moore 1979; Cross et al. 1979; Blum 1981; Moore et al. 1990; Dickschat et al. 2005a, b). For insects, they often function as pheromones. Alkyldimethyl-pyrazines are used as pheromones by a number of insect species in the Order Hymenoptera (ants, bees, and wasps). Specifically, as trail [leaf cutter ant, *Atta sexdens rubropilosa* (Cross et al. 1979)] and alarm (Wheeler and Blum 1973; Brown and Moore 1979) pheromones by some ant species, and they are found commonly 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*, (Moore et al. 1990)] belonging to the Order Orthoptera, a distinct taxon from Order Phasmatodea (Terry and Whiting 2005).

Pyrazines have different uses for humans. They are often associated with characteristic aromas and flavors of some of our favorite foods (Maga and Sizer 1973; Adams et al. 2002). The Flavor and Extract Manufacturer's Association (FEMA) reports that 2,135 kg of pyrazine flavor additives are used in the United States annually (Adams et al. 2002). Alkyldimethylpyrazines 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 alkyldimethylpyrazines (Fig. 2), along with glucose, in less than 1 μl 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. 3) were

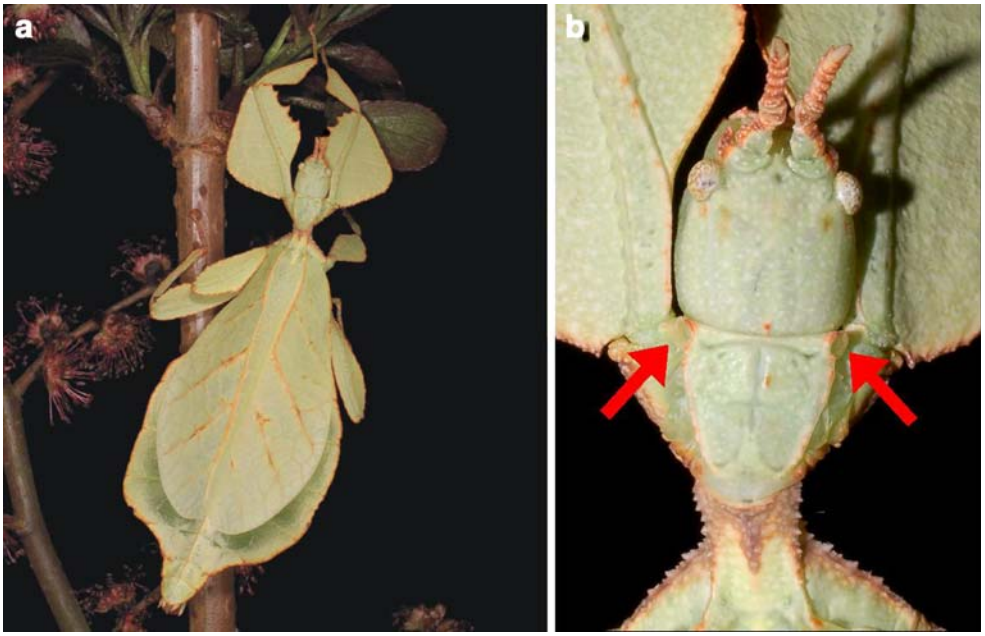


Fig. 1 Photographs of an adult female *Phyllium westwoodii*. **a** Whole insect sitting on *Sambucus nigra* (not native food plant), and **b** close-up of head and prothorax of same insect as in (**a**) with arrows showing the position of the openings of its defensive glands

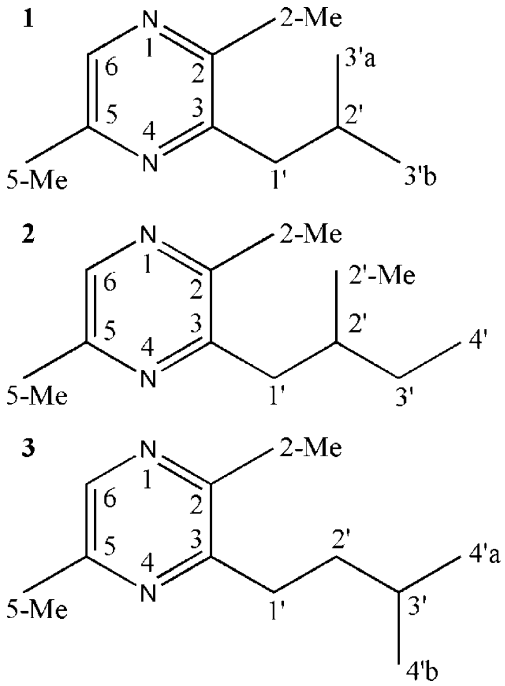


Fig. 2 Three alkyl dimethylpyrazines identified in the defensive spray of *Phyllium westwoodii*. 3-isobutyl-2,5-dimethylpyrazine (**1**), 2,5-dimethyl-3-(2-methylbutyl)pyrazine (**2**), and 2,5-dimethyl-3-(3-methylbutyl)pyrazine (**3**). Each structure is shown with corresponding carbon atom labeling

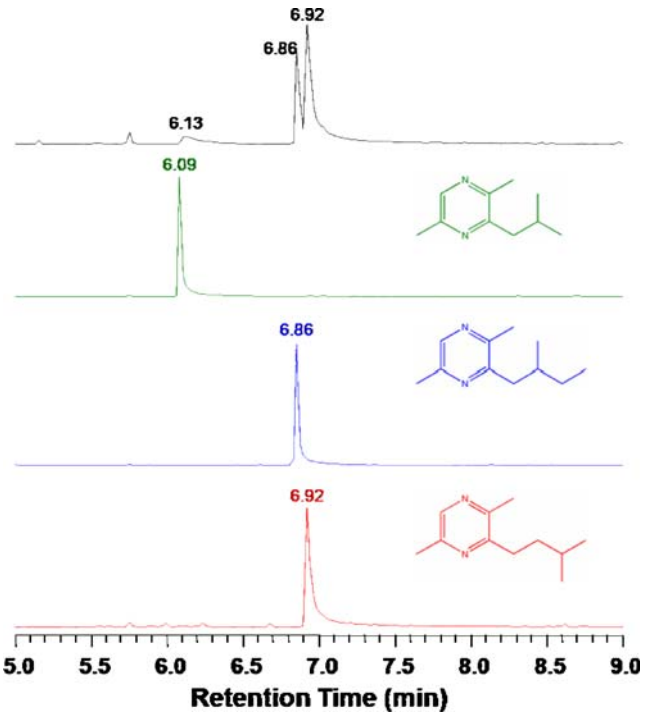


Fig. 3 GC-CI-MS total ion current chromatograms of natural *Phyllium westwoodii* defensive spray (black) compared with authentic synthetic standards: 3-isobutyl-2,5-dimethylpyrazine (top), synthetic (*S*)-2,5-dimethyl-3-(2-methylbutyl)pyrazine (middle), and synthetic 2,5-dimethyl-3-(3-methylbutyl)pyrazine (bottom). 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

identified in the mixture using synthesis and gas chromatography (Fig. 3), mass spectrometry (Fig. 4), and NMR (Figs. 5, 6 and 7). This work demonstrates the increasingly apparent diversity of compounds produced by phasmid insects and the uniqueness of Phylliidae among the Phasmatodea.

Methods and Materials

Animal Material and Sample Collection A 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 h. Leaf insects were fed on *Quercus robur* and *Rubus ulmifolius*. To obtain the original sample of defensive spray (less than 1 µl), one adult female *P. westwoodii* was milked 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 µl), that consisted of seven milkings was obtained the same way from two adult females.

The smaller of the two samples mentioned above (less than 1 µl from a single female *P. westwoodii*) was dissolved in approximately 15 µl of D_2O and used for NMR analysis. Approximately 2 µl were removed from this sample after collection of NMR data, and extracted with CH_2Cl_2 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 ~100 µl of H_2O and extracted with CH_2Cl_2 for GC-MS analysis.

Analytical Procedures NMR experiments were performed with 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×100-mm capillary NMR tube (Norell, Inc.) with a 10 µl syringe with a removable 110-mm×30-gauge blunt needle. During NMR experiments, the capillary tube was held in a standard 10-mm spinner by using a Bruker MATCH™ device, and the capillary-MATCH-spinner combination was lowered vertically into the magnet on an air-column. 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 Supplemental Material with their respective spectra. All data acquisition, processing, and analysis were done with Bruker TopSpin© 2.0 software. Chemical shift assignments were made by referencing the natural *P. westwoodii* ^{1}H and ^{13}C chemical shifts of the anomeric ^{1}H 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 that were tentatively identified by

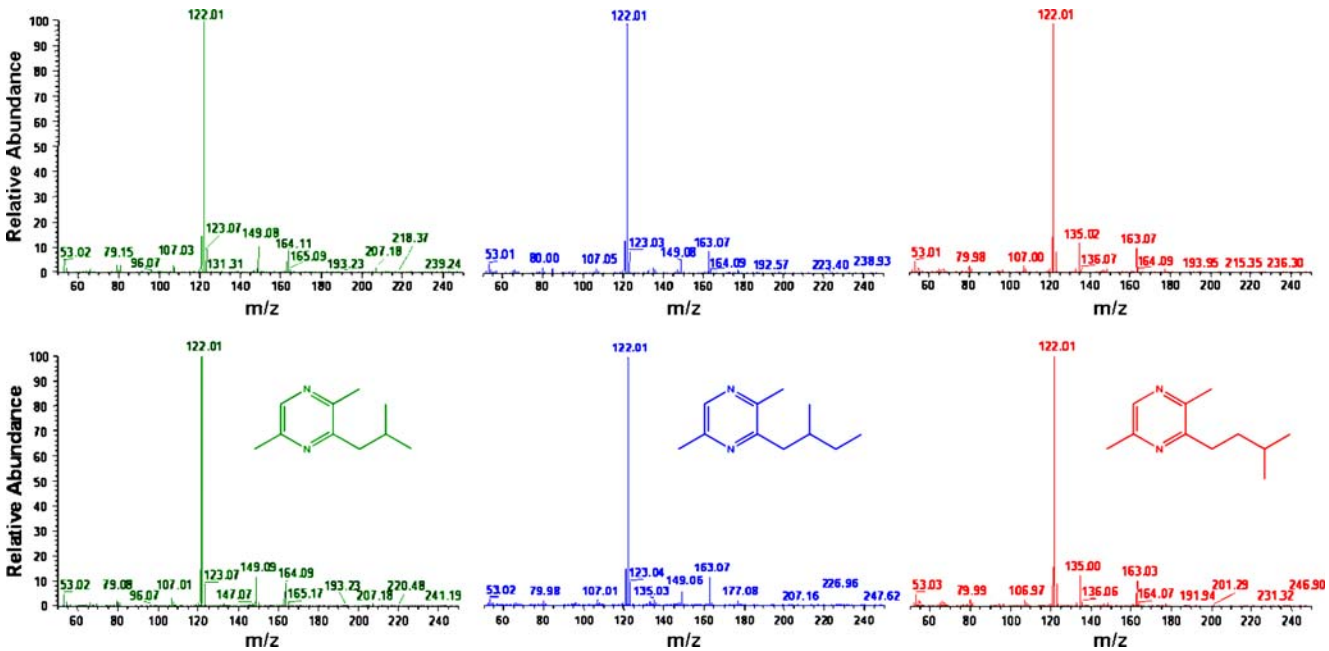


Fig. 4 Electron ionization mass spectra (GC-EI-MS) of compounds detected in *Phyllium westwoodii* defensive spray extracted with CH_2Cl_2 (methylene chloride) (top row; compare Fig. 3, peaks at 6.12, 6.86, and 6.92 min). Bottom row: Mass spectra from authentic synthesized standards

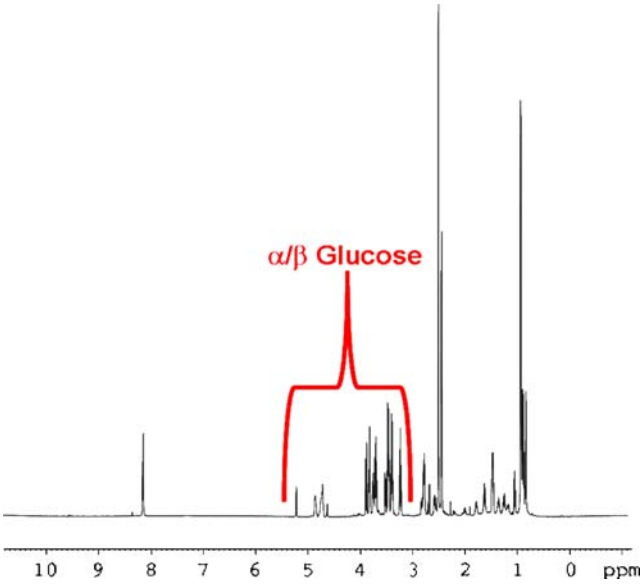


Fig. 5 1D ^{1}H NMR spectrum of *Phyllium westwoodii* defensive spray dissolved in deuterium oxide (D_2O). Resonances from glucose are shown under the bracket

GC-EIMS and to assign ^{1}H and ^{13}C chemical shifts to those compounds (Tables 1, 2 and 3), one- and two-dimensional (1D and 2D, respectively) datasets were recorded (Figs. 5, 6 and 7, and Supplemental Material 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 Rxi™-5 ms column (15 m×0.25 mm ID×0.25 um df) and with helium as carrier gas (flow rate=1 ml/min). Four µl of each sample were injected in splitless mode, for 2 min, followed by a split flow rate of 50 ml/min. The GC oven temperature was maintained at 40 °C for 3 min, 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 that eluted 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 phenylhydrazone (Love and Jones 1999). Commercial 3-chloro-2,5-dimethylpyrazine (1.0 equiv.) was added to a solution of Fe$(acac)_3$ (0.05 equiv.) in THF-NMP (9:1, 0.25 M), and the mixture was cooled to 0 °C in an ice-water bath. Grignard reagent (1.5 equiv) in ether was added dropwise to this solution. 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 the solvent was removed under

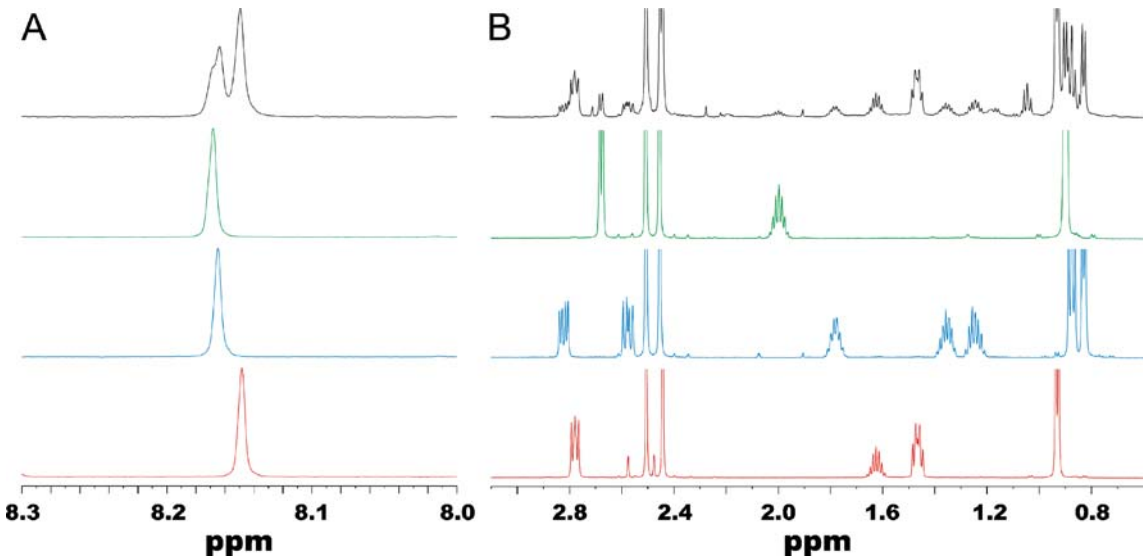


Fig. 6 1D ^{1}H spectra of natural *Phyllium westwoodii* defensive spray compared with authentic synthetic standards: **a** aromatic region and **b** aliphatic region. Spectra shown are of natural *P. westwoodii* defensive spray (top), synthetic 3-isobutyl-2,5-dimethylpyrazine (**1**) (2nd from top), synthetic (*S*)-2,5-dimethyl-3-(2-methylbutyl)pyrazine (**2**) (3rd from top), and synthetic 2,5-dimethyl-3-(3-methylbutyl)pyrazine (**3**) (bottom). 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

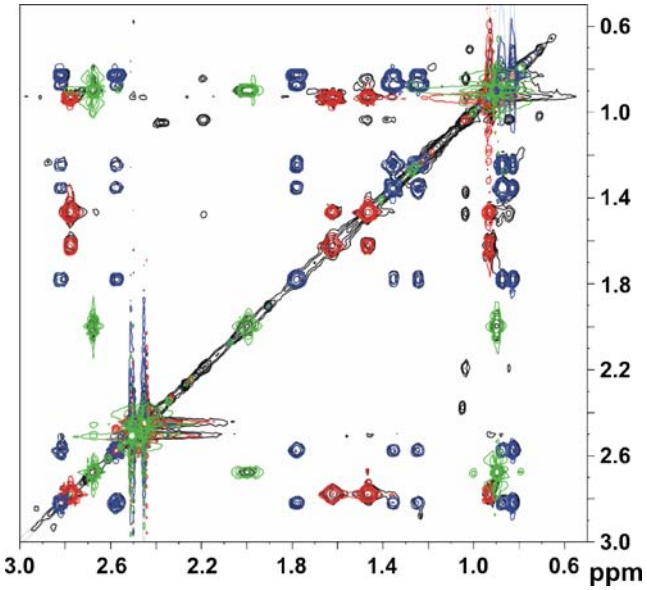


Fig. 7 Aliphatic sidechain region of 2D ^{1}H TOCSY spectra compared to authentic synthetic standards by overlaying the spectra. Spectra shown are of natural *Phyllium westwoodii* defensive spray (black), synthetic 3-isobutyl-2,5-dimethylpyrazine (**1**) (green), synthetic (*S*)-2,5-dimethyl-3-(2-methylbutyl)pyrazine (**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

reduced pressure. The crude product mixtures were purified by silica gel column chromatography, using a solution of 5% ethyl acetate in hexane.

3-Isobutyl-2,5-dimethylpyrazine (1) Yield: 42% (3.463 g, 21.08 mmol). ^{1}H NMR (400 MHz, $CDCl_3$): δ 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, $CDCl_3$): δ 21.3, 21.7, 22.6, 28.8, 43.7, 140.7, 149.0, 150.1, 154.4; IR (film): *v* 2957, 2869, 1450, 1370, 1288, 1168, 1074 cm−1; EI-MS 165.1386, HRMS-ESI (*m/z*): [M+] calcd for $C_{10}H_{16}N_2$ 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). ^{1}H NMR (400 MHz, $CDCl_3$): δ 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, $CDCl_3$): δ 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*): [M+] calcd for $C_{11}H_{18}N_2$ 179.1543, found 179.1545, specific optical rotation: $[\alpha]_D^{25}$ = +8.8 (*c*=2.535, CH_2Cl_2). Spectroscopic values for synthetic **2** were in agreement with reported literature values (Dickschat et al. 2005a).

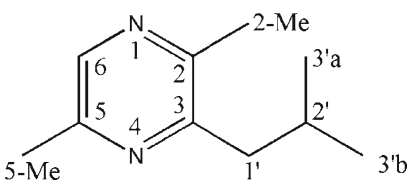
2,5-Dimethyl-3-(3-methylbutyl)pyrazine (3) Yield: 55% (2.458 g, 13.79 mmol). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.97 (d, *J*=6.8 Hz, 6 H), 1.53 (m, 2 H), 1.67 (sept, *J* = 6.8 Hz, 1 H), 2.47 (s, 1 H), 2.51 (s, 1 H), 2.75 (m, 2 H), 8.13 (s, 1 H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 21.2, 22.6, 28.5, 33.4, 37.8, 140.8, 148.5, 150.2, 155.3; IR (film): *v* 2956, 2927, 2870, 1536, 1452, 1320, 1278, 1254, 1169, 1080, 1034, 999, 942, 738; HRMS-ESI (*m/z*): $[M+H]^+$ calcd for $C_{11}H_{18}N_2$ 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).

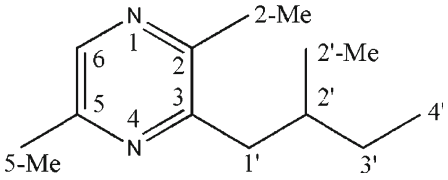
Mass spectra from all three peaks matched those of known alkyldimethylpyrazines with high scoring database

Table 1 NMR chemical shift assignments for synthetic **1** (3-isobutyl-2,5-dimethylpyrazine) dissolved in D_2O



Position	δ ^{1}H (ppm)	δ ^{13}C (ppm)	$J_{H\text{-}H}$ (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 for synthetic **2** ((*S*)-2,5-dimethyl-3-(2-methylbutyl)pyrazine) dissolved in D_2O



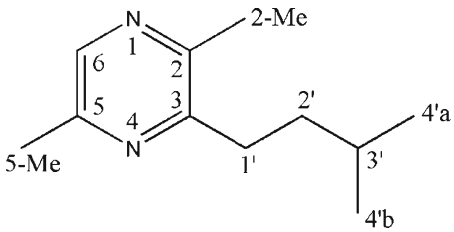
Position	δ ^{1}H (ppm)	δ ^{13}C (ppm)	$J_{H\text{-}H}$ (Hz)
2	-	152.3	-
3	-	157.8	-
5	-	153.0	-
6	8.16	143.0	(s)
2-Me	2.51	22.6	(s)
5-Me	2.45	22.18	(s)
1’	2.82	43.7	(m)
	2.58	-	(m)
2’	1.78	37.6	(m)
2’-Me	0.83	20.8	6.69 (d)
3’	1.36	31.9	(m)
	1.25	-	(m)
4’	0.88	13.5	7.44 (t)

hits (Fig. 4 and Supplemental Material Fig. S1) (Stein et al. 1987–2002). Additionally, the mass spectra match those of synthetic standards (Fig. 4). However, some isomers of the highest scoring compounds also were close matches (Supplemental Material Fig. S1). Additionally, similar mass spectra might be obtained from molecules with different relative placement of the alkyl chains and methyl groups on the pyrazine ring. Previous studies have shown phasmid defensive sprays to contain glucose (Dossey et al. 2006, 2007; 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 to 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 ^{1}H 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). 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 D_2O 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, b). 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 defensive mixture from *P. westwoodii* and the three synthetic pyrazines in D_2O are compared in Fig. 6. The

Table 3 NMR chemical shift assignments for synthetic **3** (2,5-dimethyl-3-(3-methylbutyl)pyrazine) dissolved in D_2O



Position	δ ^{1}H (ppm)	δ ^{13}C (ppm)	$J_{H\text{-}H}$ (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)

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. A comparison of 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 ^{1}H region at about 8.15 ppm shows at least three resonances, two of which 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 ^{1}H and ^{13}C chemical shift assignments for all three pyrazines.

Figure 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). Overlaying of two-dimensional NMR spectra can be a powerful and robust way to identify individual known substances in natural product mixtures. 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 ^{1}H-^{1}H 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 pm (Supplemental Material Fig. S3). However, distinct TOCSY correlations were observed between these pairs of resonances as expected (Fig. 7 and Supplemental Material 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.

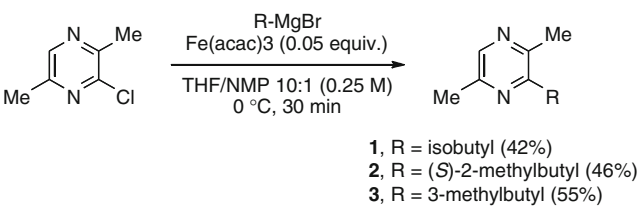


Fig. 8 Synthetic scheme for alkyldimethylpyrazines **1**–**3**

An overlapped set of spin systems with correlations between resonances at about 2.4–2.5 ppm and 8.15 ppm also were observed in TOCSY spectra. There were no clear ^{1}H-^{1}H 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′ ^{1}H and the 2 and 3 ^{13}C resonances (Supplemental Material Fig. 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 (Supplemental Material Fig. S5). Finally, in the HSQC experiment which was used, the signs (+/−) of the correlations were both sensitive to and consistent with the proposed methine, methylene, and methyl groups (Supplemental Material Fig. S4).

In addition to chemical analysis, a video was recorded which illustrates some features of the defensive behavior of *P. westwoodii* (Supplemental Material Fig. S7). This behavior is well established in the literature for other species in the same genus (Henry 1922; Bedford 1978; Zompro and Grösser 2003, and references therein). First, the insect was removed from the cage and placed on a stick for observation. This action sufficiently disturbed the insect causing it to freeze in a pose as if to play dead or possibly to resemble a leaf. The legs were positioned in a seemingly disordered conformation and were not all used to hold onto the stick. Next, when the offending stimulus was continued more aggressively, the insect compressed, became more active, attempted to escape, and produced stridulatory sounds by using its antennae. It is at this point that the insect released 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. This odor was noticed only when *P. westwoodii* defended against attack or disturbance.

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 α- and β-glucose. The release of this spray from *P. westwoodii* happens concurrently with a characteristic defense behavior (Supplemental Material Fig. S7 and described in the "Introduction") 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 shown experimentally to be effective in repelling potential predators such as ants, frogs, and birds (Eisner 1965; Carlberg 1985a, b, 1986, 1987; Chow and Lin 1986; Bouchard et al. 1997; Eisner et al. 1997). 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 found widely in the aromas and flavors of foods that humans find pleasing (Maga and Sizer 1973). Some insects use these and other similar pyrazines as pheromones (Cavill and Houghton 1974; Cross et al. 1979; Blum 1981; Tengo et al. 1982). Also, the amount produced by *P. westwoodii* in response to being disturbed is small. Many phasmids produce only very small quantities of prothoracic glandular spray (Tilgner 2002), and the chemistry of their spray can vary over development (Dossey et al. 2008). Thus, it has been postulated that some species may use these substances for functions other than defense (Tilgner 2002; Dossey et al. 2008). It cannot be excluded that *P. westwoodii*, as well as other Phylliidae, use them for intraspecific communication. However, such a hypothesis is beyond the scope of this study and, as far as we are aware, has not yet been tested.

Other phasmid species produce monoterpenes (Meinwald et al. 1962; Smith et al. 1979; Chow and Lin 1986; Ho and Chow 1993; Bouchard et al. 1997; Dossey et al. 2006, 2007), 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 this study, glucose also has been found in defensive sprays from other phasmids (Dossey et al. 2006, 2007; Zhang et al. 2007). Some Coleoptera of the family Chrysomelidae (leaf beetles) use precursors that are conjugated with glucose for monoterpene biosynthesis and transport into the defense glands (Feld et al. 2001; Laurent et al. 2003; Burse et al. 2007;Kunert et al. 2008). 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. This hypothesis requires further investigation.

The peculiar morphological features that characterize fossil and extant leaf insects (Zompro 2004; Wedmann et al. 2007) as well as the findings presented here, support the view that Phylliidae occupy a unique position among Phasmatodea. This study demonstrates yet another distinguishing feature of this unique group of phasmids. Many other phasmids 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 their potential value 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.

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