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Defensive Spiroketals from *Asceles glaber* (Phasmatodea): Absolute Configuration and Effects on Ants and Mosquitoes

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Abstract Insects are the largest and most diverse group of organisms on earth, with over 1,000,000 species identified to date. Stick insects ("walkingsticks" or "phasmids", Order Phasmatodea) are known for and name-derived from their camouflage that acts as a primary line of defense from predation. However, many species also possess a potent chemical defense spray. Recently we discovered that the spray of *Asceles glaber* contains spiroketals [a confirmed major component: (2S,6R)-(−)(*E*)-2-methyl-1,7-dioxaspiro[5.5]undecane, and a tentatively identified minor component: 2-ethyl-1,6-dioxaspiro[4.5]decane] and glucose. In this paper, we: 1) illustrate the identification of spiroketals and glucose in the defense spray of *A. glaber* by using Nuclear Magnetic Resonance (NMR), Gas Chromatography/Mass Spectrometry (GC/MS), and comparison with a synthetic reference sample; 2) provide the elucidation of the absolute configuration of the major spiroketal in that defense spray; and 3) demonstrate the effect of this compound and its enantiomer on both fire ants (*Solenopsis invicta*) and mosquitoes (*Aedes aegypti*).

Keywords Spiroketal · Phasmatodea · *Asceles glaber* · *Solenopsis invicta* · *Aedes aegypti* · Defense · Phasmatodea.

Introduction

Insects are known for their utilization of chemistry in communication and defense (Blum, 1981; Eisner et al., 2005; Laurent et al., 2005; Dossey, 2010). Stick and leaf insects (walkingstick insects, or phasmids; Order Phasmatodea), a relatively small order of insects composed of around 3,000 named species, are best known for their elaborate use of camouflage as a defense against predators (Bedford, 1978; Brock, 1999, 2009). However, many species produce a

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chemical spray from a pair of tegumental glands in their prothorax when disturbed (Scudder, 1876; Bedford, 1978; Dossey, 2010, 2011) (and references therein). 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; Schmeda-Hirschmann, 2006; Dossey et al., 2006, 2007, 2008, 2009; Prescott et al., 2009; Dossey, 2010, 2011). Besides the various secondary metabolites, glucose has been reported in the defensive sprays of *A. buprestoides* (Dossey et al., 2006), *P. schultei* (Dossey et al., 2006), *P. mocquerysi* (Dossey et al., 2007), *P. westwoodii* (Dossey et al., 2009), and *M. nigrosulfurea* (Prescott et al., 2009).

Asceles glaber (Günther, 1938) (Fig. 1) is a species of phasmid in the Suborder Euphasmatodea, Subfamily Necrosciinae. This is the most diverse Subfamily within the Stick and leaf insects, containing over 600 named species distributed throughout Asian and Australasian tropical forests. The natural history of these phasmids is poorly understood, but most members are winged, appear to be associated with the forest canopy, and exhibit specialized feeding habits (Bragg, 2001). *Asceles glaber* occurs in Myanmar and Thailand, and like many other phasmid species produces a liquid defense spray from a pair of tegumental glands in the anterior portion of their prothorax at minimum disturbance (Fig. 1b).

Spiroketals, sometimes referred to as spiroacetals, make up a large and diverse group of natural products that have been extensively reviewed in the literature (Booth et al., 2009). These have been isolated from a number of insect species (Tengö et al., 1982; Moore et al., 1994; Francke and Kitching, 2001; Goubault et al., 2008; Schwartz et al., 2008; Booth et al., 2009). However, to date, no spiroketals have been reported from stick insects. The first insect spiroketal was chalcogran (2-ethyl-1,6-dioxaspiro[4.4]nonane) isolated from the European spruce bark beetle (*Pityogenes chalcographus*) (Francke et al., 1977; Booth et al., 2009). Various spiroketals possess important biological activities such as pheromone response (Francke et al., 1977; Weston et al., 1997) and chemical defense (Dettner et al., 1992; Zhang et al., 1999) in insects.

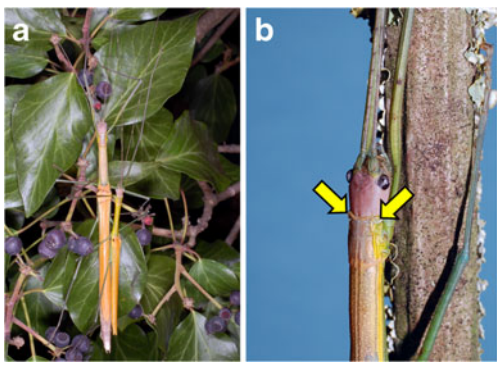

Fig. 1 Photographs of **a** an adult mating pair and **b** a close-up of head and prothorax of an adult female of *Asceles glaber* with arrows showing the position of the openings of its defensive glands. Photographs by Marco Gottardo

Here, we report the identification of two spiroketals (a confirmed major component (2S,6R)-(−)(*E*)-2-methyl-1,7-dioxaspiro(5,5)-undecane **1** and a tentatively identified minor component 2-ethyl-1,6-dioxaspiro[4.5]decane **2**) (Fig. 2) and glucose from the secretion of the prothoracic exocrine glands of both sexes of *A. glaber*. This paper 1) illustrates the identification of spiroketals and glucose in the defense spray of *A. glaber* by using Nuclear Magnetic Resonance (NMR), Gas Chromatography/Mass Spectrometry (GC/MS), and comparison with a synthetic reference sample, 2) provides the elucidation of the absolute configuration of the major spiroketal in that defense spray, and 3) demonstrates the effect of both enantiomers of the major *A. glaber* defensive spiroketal on both fire ants (*Solenopsis invicta*) and mosquitoes (*Aedes aegypti*).

Methods and Materials

General Experimental Procedures NMR experiments were performed with a 600 MHz 5-mm triple resonance cryogenic probe Bruker Biospin. Each sample was loaded into a 2.5-mm NMR tube (Norell, Inc.). During NMR experiments, the tube was held in a standard 10-mm spinner using a Bruker MATCH™ device, and the tube-MATCH-spinner combination was lowered vertically into the magnet on an air-column as usual. Sample temperature was regulated at 29°C. The spectrometer used for all NMR experiments was a Bruker Avance II 600. Additional NMR data acquisition parameters are 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 resonances of the solvent proton impurity (benzene-d_5) to 7.16 for ^{1}H and 128.39 for ^{13}C, respectively. For spectra of samples in D_2O, chemical shift referencing was achieved by setting the anomeric ^{1}H to 5.22 and ^{13}C to 94.8 ppm of alpha glucose based on

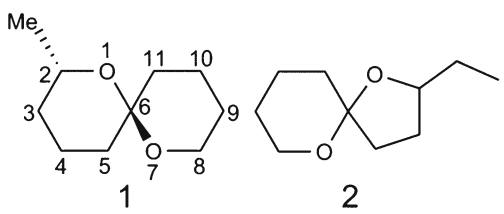

Fig. 2 Spiroketals identified in the defensive spray of the stick insect *Asceles glaber*: Major component (1) (2S,6R)-(−)(*E*)-2-methyl-1,7-dioxaspiro(5,5)-undecane (by NMR, GC/MS and comparison with an authentic synthetic standard) and a tentatively identified minor component (**2**) 2-ethyl-1,6-dioxaspiro[4.5]decane (by GC/MS)

the reported values for these resonances in the BMRB Metabolomic database (http://www.bmrb.wisc.edu/metabolomics/) (Ulrich et al., 2008).

GC/MS analyses were conducted on a TraceGC Ultra DSQ mass spectrometer (Thermo Scientific) equipped with an AT-5 ms column from Alltech (60 m×0.25 μm i.d.× 0.25 μm d_f). The injector was maintained at 280 °C, while the transfer line was set at 250 °C. The ion source was set at 180 °C and operated in electron impact (EI) mode, while He flow rate was at 1 ml/min. Two microliters (2 μl) of each sample were injected into the column in splitless mode at 40 °C for 2 min. The GC oven temperature was increased to 280 °C at a rate of 20 °C/min.

Asceles glaber Rearing and Sample Collection *Asceles glaber* specimens were obtained from descendence of females originally collected in the neighborhoods of Salok, Thailand, in 2003. For the present study, several males and females were reared in the laboratory at room temperature, moderate humidity conditions, and a 12:12 hrL/D photoperiod. Insects were kept in ventilated cages, and fed daily with *Hypericum* spp. leaves. Four independent samples, with volumes of approximately 1–50 μl, of defensive glandular secretion was obtained from a total of 51 milkings of *A. glaber*. Specifically, the following nomenclature will be used to refer to specific samples throughout this paper: *Asceles* 1 (26 milkings from 6 males to 4 females collected between December 2007 and Jenuary 2008), *Asceles* 2 (18 milkings from 3 males to 3 females collected between August 2008 and January 2009), *Asceles* 3 (5 milkings from 3 females collected between February and March 2008), and *Asceles* 4 (2 milkings from 1 female collected between February and March 2008). Sample *Asceles* 2 was larger despite the fewer number of milkings, because it contained the sprays from a large female that produced a large amount of secretion at each milking compared to other female specimens. It is also possible that *Asceles* 1 was quite small because sometimes not all the secretion was sprayed into the vial. For NMR, 1–5 μl of each milking was utilized (see captions of specific NMR figures) and either dissolved in D_2O or extracted with benzene-d_6. For GC/MS, approximately 1 μl of each sample was extracted with 0.5 ml of CH_2Cl_2 for analysis.

Spiroketals The two enantiomers of spiroketal **1** were synthesized as described in Whitaker (2012). Details of the synthesis will be published elsewhere.

(2S,6R)-(−)(E)- and (2R,6R)-(+)(Z)-2-methyl-1,7-dioxaspiro[5.5]undecane (1) Colorless oil; Optical rotations: $[\alpha]_D^{25}$=−62.02 (c 0.99, CH_2Cl_2), $[\alpha]_D^{25}$=+66.72 (c 1.06, CH_2Cl_2); ^{1}H NMR (600 MHz, C_6D_6) δ 3.75 (1H, m), 3.64 (1H, m), 3.54 (1H, m), 2.05 – 1.96 (2H, m), 1.66 – 1.08 (10H, m), 1.15 (3H, d, *J*=6.0 Hz) ppm; ^{13}C NMR (150 MHz, $CDCl_3$) δ 95.6, 65.1, 60.1, 36.2, 35.6, 33.2, 25.7, 22.2, 19.6, 19.1 ppm; The synthetic material was spectroscopically consistent with reported literature (Ghosh et al., 2006).

Quantification of Spiroketal 1 GC/MS was utilized for quantification of spiroketal **1** in each of the *A. glaber* spray samples collected. For these experiments, 1 μl of each sample was dissolved in 500 μl of dichloromethane (DCM) and was then analyzed by GC/MS. Based on preliminary results, *Asceles* 1 and 2 were diluted 3 and 10 fold, respectively, for the quantification. For the external calibration, synthetic spiroketal compound 1, equivalent to 1.25 to 12.5 μg/μl of sample, was prepared fresh. All solutions were injected at least three times in a 24-hr period. The method was validated by injecting the original *Asceles* 2 solution and a day-old standard solution at the end of the work list. Blank analysis consisted of solvent injections using the DCM that was used for the dilutions.

Absolute Configuration of Spiroketal 1 Determination GC/MS using an enantiomer selective column was utilized to determine the absolute configuration of the primary spiroketal component in *A. glaber* defense spray. GC/MS analyses were conducted on the same TraceGC Ultra DSQ mass spectrometer (Thermo Scientific) but using a Beta DEX 120 column from Supelco (30 m×0.25 μm i.d.×0.25 μm d_f). The injector and transfer line were maintained at 200 °C while the EI ion source was set at 180 °C. High purity He was used as carrier gas at a flow rate of 1 ml/min. Four microliters (4 μl) of the same sample solutions used for the quantification were injected into the column in splitless mode at 40 °C for 2 min. The GC oven temperature was increased to 220 °C at a rate of 20 °C/min. The 2(*R*)-(+) and 2(*S*)-(−) synthetic standards were prepared in DCM as 100 μg/ml solutions and analyzed in the same manner using 0.5 μl injections.

Bioassays on Fire Ants

Olfactometer Bioassay The bioassay is similar to that described by Vander Meer et al. (1988). The volatile spiroketal was dissolved in pentane to make an initial test concentration of 1 %. The solvent control was pentane. Each treatment and control (10 μl) was applied to filter paper pieces (Whatman #1; 1×0.3 cm). Filter paper pieces containing the treatment and control were placed inside the entrances of each of the two arms of the Y-tube olfactometer connected to the airflow. Purified compressed air was passed through each of the two sample Y-tube arms at a rate of 100 ml/min (200 ml/min combined). The main body of the olfactometer was 12 cm long×1.5 cm id. Approximately 100 worker ants were placed in a piece of tygon tubing (7 cm long×1.0 cm id) closed with a wire mesh cap at the distal end. The other

end of the tubing was connected to the downwind arm of the Y-tube olfactometer. Worker ants walked to the bifurcation choice point and went to treatment or control. After 20 ants had made a choice, the apparatus was cleaned and returned to the previous position. Treatment and control samples were prepared again, and their positions in the Y-tube arms were reversed. The choice of another 20 workers ants was recorded, and the sum of the two results constituted one replicate (N=40). Due to the volatility of the test compounds, the experiments were terminated after 3 min. The experiment was replicated a minimum of 3 times. The *S. invicta* queen attractant found in the poison sac was used as a positive standard (0.33 queen poison sac equivalents per 1.5 μl hexane) (Vander Meer et al., 1980) to test proper function of the olfactometer. Result significance was measured by *chi-square* analysis with a null hypothesis of equal numbers of ants in each arm. Results where <35 % of the ants chose the treatment indicate significant repellency; whereas results of >65 % represents significant attraction to the treatment. Results between 35 and 65 % indicate neutral activity.

Contact Repellent Bioassay The test tray was comprised of a porcelain pan measuring 180×290×50 mm. The upper 3 mm of the pan was coated with Fluon© to preclude ants from escaping. A Petri dish nest cell (55 mm diam.) was placed at one end of the pan. The Petri dish had a 5 mm layer of Castone© dental cement on the bottom that acted as a moisture reservoir. The lid had a hole placed in the center to allow ant access. To protect the bottom of the pan from contamination by the test materials, 2.5 cm sq. pieces of aluminum foil were placed in the opposite end of the pan from the nest cell at each corner approximately 3.0 cm from the sides of the pan. No food or water was available to the ants during the bioassay. For the execution of the bioassay fifty micro liters of test material were introduced into the test chamber on a 2.0 cm sq. piece of Whatman© silicone treated filter paper (cat. # 2200 125) and was randomly assigned and placed on one of the Aluminum foil squares. The other aluminum square received a filter paper square with 50 μl of pentane as control. Placed on top of each filter paper square was a small wad of cotton soaked in 10 % sucrose to serve as a phagostimulant. Once test materials were in place, approximately 1 g of ants (starved for a minimum of 24 hr) was placed in the nest cell and a stopwatch was started. The number of ants actively feeding on the treatment and control cotton balls was recorded at 1-min intervals for a total of 5 min. Each experiment was replicated three times, each with a unique monogyne colony and in a different test tray.

Bioassays on Mosquitoes Aedes aegypti Bioassays on Mosquitoes (*Aedes aegypti*) were received from the colony maintained at the United States Department of Agriculture-Agricultural Research Service in Gainesville, FL, at the same location as the assays were conducted. Mosquitoes were maintained in the laboratory on water and a solution of 10 % sucrose in water. The laboratory photoperiod was 12:12 L:D. A draw box (Posey and Schreck, 1981) was used to select the appropriate number of female mosquitoes for the assays of repellency, attraction, and attraction-inhibition. Ages of mosquitoes selected for tests ranged from 5 to 12 d old.

Contact Repellency Repellency was assessed by using a cage test (Barnard et al., 2007), and treatments were tested with the "cloth patch" assay method described in depth previously (Katritzky et al., 2010). Treatments consisted of 2(*R*)-(+)-spiroketal and 2(*S*)-(−)-spiroketal dissolved in acetone; N,N-diethyl-3-methylbenzamide (DEET) (Sigma-Aldrich, Milwaukee, WI, USA) was also included as the standard. A 75 mg amount of each spiroketal or DEET was measured and placed in a separate 2-dram vial with 2 ml of acetone and muslin cloth, resulting in an acetonic solution. The removed cloth has a 0.750 mg/cm^2 concentration, and this was the highest concentration tested. Serial dilutions were carried out on each initial vial, and additional sections of muslin cloth were added to produce concentrations of 0.375, 0.187, 0.094, 0.047, 0.23, 0.011, and 0.005 mg/cm^2. Volunteers tested the treated cloth according to exact procedures of (Katritzky et al., 2010). The lowest concentration at which the repellent passed is an estimate of the Minimum Effective Dosage (MED).

Three male and one female volunteers participated in the repellency tests. During the test, all volunteers participated at the same time, each with their own cage. Since only two spiroketals were tested, patches of the same concentration of each enantiomer were tested by two of the volunteers, while the other volunteers were not testing. The patches then rotated to the next volunteer. In this manner, no patch was tested later than about 4 min from the first test. DEET was tested in a separate rotation from the spiroketals. All subjects provided informed written consent. The protocol was approved by the University of Florida Human Use Institutional Review Board-01 (Study #636-2005).

Olfactometer Bioassays A dual-port triple cage olfactometer was used to assess relative attraction and attraction-inhibition to treatments placed in separate test ports (Posey et al., 1998; Bernier et al., 2007b). The unit was constructed such that each cage is operated non-concurrently from the other two, and therefore, mosquitoes in the cage can either select to fly into one of the two ports or remaining in the cage. Approximately 75 (± 10) mosquitoes were placed in each cage 50 (± 10) min prior to experiments. Air was drawn in from the outside, filtered, and conditioned to 27±1 °C

and 60±5 % RH with a velocity of 28±1 cm/s through the ports.

Experiments were designed to test treatments in a competitive manner, where each spiroketal enantiomer added to the port with a synthetic human volatile blend compared to the synthetic human blend in the adjacent port. The stock blend consisted of 0.6 gL-(+)-lactic acid and 100 µl methyl disulfide dissolved in 250 ml acetone (Bernier et al., 2007a). A 500 µl aliquot of the stock solution was placed in a 1.4 ml plastic cap (15 mm i.d.×9.5 mm height) and 50 µl of either the 2(*R*)-(+) or 2(*S*)-(−)-spiroketal were added in a separate 400 µl cap (9 mm i.d.×9 mm height) to one of the ports; caps were placed on a 13×7 cm aluminum tray prior to insertion into the olfactometer port. Each test was 3 min in duration. The selection of cage and port test order was randomized, but the overall experiment designed such that each port within the 3 cages received a specific combined treatment only once. Therefore, 6 replicates of each competitive combination were obtained.

Results

The defensive spray of *A. glaber* is whitish in color, and can be sprayed carefully in the direction of the potential predator attack. Approximately 1 µl of an initial sample of this substance (*Asceles* 1) was extracted with benzene-d_6 for NMR analysis (unpublished results). Also, 1 µl of the same sample was extracted with approximately 100 µl of dichloromethane for GC/MS analysis. For a description and definitions of the *Asceles* sample numbers used, see Methods. From here on that nomenclature will be used to refer to specific samples of *A. glaber* defense spray. Preliminary GC/MS analysis showed one major compound that comprised over 95 % of the chromatogram for all samples (Supplemental Material Figs. S1, S2, S3, S4, S5, S6 and S7). The EI mass spectrum of the tentative minor natural spiroketal **2** (Supplemental Material Fig. S2 C), from the analysis of *Asceles* 2, matched well to a known spiroketal, 2-ethyl-,(2R-trans)-1,6-dioxaspiro[4.5]decane (Supplemental Material Fig. S2 5d; CAS# 76495-09-5), with a reverse-fit score of 891 and a probability score of 94.7 (Supplemental Material Fig. S6). In contrast, the mass spectrum of the major component (Supplemental Material Figs. S1, S2 and S3, Supplemental Material Fig. S4), did not have an equivalent in the National Institutes of Science and Technology (NIST) EI MS library database. The closest match for the major component was not a spiroketal, and it gave a reverse-fit score of below 700 (spiroketal **2** came next) (Supplemental Material Fig. S7). Based on visual inspection of the mass spectrum and its similarity to one found in the literature spiroketal **1** (Tengö et al., 1982), with additional structural information provided by NMR analysis, spiroketal **1** was synthesized and compared to the natural material by both GC/MS and NMR (Fig. 3; Supplemental Material Fig. S8). Identical mass spectra acquired for synthetic spiroketal **1** and the major component from *A. glaber* chemical defense spray provided confirmation of its identification (Supplemental Material Figs. S1 and S2 a and b).

For confirmation purposes and biological assays, both enantiomers of the proposed spiroketal were produced synthetically (Whitaker, 2012). The synthetic spiroketals exhibited roughly equal and opposite optical rotations, and thus are referred to as either a "2(R)-(+)-spiroketal" or "2(*S*)-(−)-spiroketal" as a correlation to the sign of the optical rotation. The enantiomers also displayed unique retention times when subjected to GC analysis utilizing a chiral stationary phase. Spectroscopically, the synthetic spiroketals were identical to data published in previous synthetic efforts (Ghosh et al., 2006). The details of the syntheses will be published separately.

For final verification of the identification for spiroketal **1**, approximately 5 µl of *Asceles* 5 was extracted with 100 µl of benzene-d_6 and a separate 5 µl was dissolved in 100 µl of D_2O for exhaustive NMR analysis to identify the major components (Fig. 3 and Supplemental Material Figs. S8 and S9). One-dimensional (1D) NMR spectral stack plots (Supplemental Material Fig. S8) as well as 2D TOCSY and HSQC spectral overlays (Fig. 3) of natural *A. glaber* defense spray (Supplemental Material Figs. S8 and S9) and synthetic spiroketal **1** (Supplemental Material Figs. S8 and S10) provide robust verification that the major component in that species' chemical defenses is spiroketal **1**. Additionally, the chemical shifts of ^{1}H and ^{13}C resonances observed in all spectra of synthetic spiroketal **1** in benzene-d_6 match closely with those published by Ghosh et al. measured for the same molecule dissolved in chloroform-d (Ghosh et al., 2006).

To determine the absolute configuration of spiroketal **1** as it occurs naturally in *A. glaber*, GC/MS analysis utilizing an enantiomer selective column was performed on the natural material in sample *Asceles* 2 and both synthetic enantiomers of spiroketal **1**. GC/MS results are shown in Fig. 4. The standards were found to be a mixture of two spiroketals and are labeled based on the predominant isomer (Fig. 4). Retention times confirmed that the major sporiketal in *A. glaber* defense spray is the 2(*S*)-(−) enantiomer (Fig. 4). Both peaks at 12.28 min and all three peaks at 12.39 min have identical EI mass spectra (Supplemental Material Fig. S11).

In preparation for ant and mosquito bioassays, the concentration of spiroketal **1** in natural *A. glaber* defense spray was determined by GC/MS utilizing serial dilutions of both natural and synthetic standard material. Integration of peaks from the TICs and molecular ion of all *Asceles* samples (Supplemental Material Fig. S1) along with those of a series

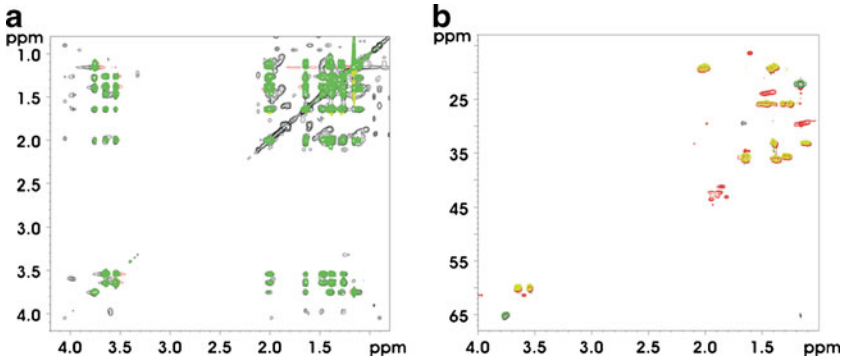

Fig. 3 Two dimensional (2D) NMR spectral overlays of: **a** synthetic spiroketal (1) ((2S,6R)-(−)(*E*)-2-methyl-1,7-dioxaspiro(5,5)-undecane) dissolved in benzene-d_6 (*green and yellow*)on top of ^{1}H-^{1}H TOCSY spectra of *Asceles glaber* defense spray extracted with benzene-d_6 (black and red); **b** synthetic spiroketal (**1**) ((2S,6R)-(−)(*E*)-2-methyl-1,7-dioxaspiro(5,5)-undecane) dissolved in benzene-d_6 (*green and yellow*)on top of ^{1}H-^{13}C HSQC spectra of *Asceles glaber* defense spray extracted with benzene-d_6 (*black and red*)

of dilutions of synthetic spiroketal **1** were compared via standard plot (Supplemental Material Figs. S12 and S13) to calculate the concentrations of that compound in *A. glaber* chemical defense spray reported in Table 1 (see also Supplemental Material Figs. S12 and S13) for the calibration curve and the extended table.

Having verified the identity of spiroketal **1** by GC/MS, NMR and by comparison with authentic synthetic standards, as well as having determined its concentration in *A. glaber* defense spray via GC/MS, its identity in the aqueous environment can be deduced by overlaying spectra (1D ^{1}H as well as 2D ^{1}H TOCSY) of natural and synthetic material both dissolved in D_2O, as shown (Fig. 5, Supplemental Material Fig. S14). All other NMR spectra for both of those samples also showed that all resonances and correlations present in spectra for the sample of synthetic spiroketal **1** matched an identical set of the same in spectra of the natural *Asceles* defense spray sample (*Asceles* 5), all collected in D_2O (Fig. 5, Supplemental Material Figs. S14, S15, S16 and S17). In addition to spiroketal **1**, spectra for the natural sample dissolved in D_2O revealed the presence of sugar-like resonances and correlations (Fig. 5, Supplemental Material Figs. S14 and S15). Since glucose has been reported from several phasmid chemical defense sprays to date (see Discussion), it was chosen as the most likely candidate for the identity of the *A. glaber* defense spray sugar. Indeed, as show in Fig. 5, overlaying spectra of pure authentic commercially obtained glucose show an exact match for glucose (also see Supplemental Material Figs. S14, S15, S16 and S17; S17 contains NMR spectra of an authentic sample of D-Glucose). Thus, *A. glaber* defense spray contains both spiroketal **1** and glucose.

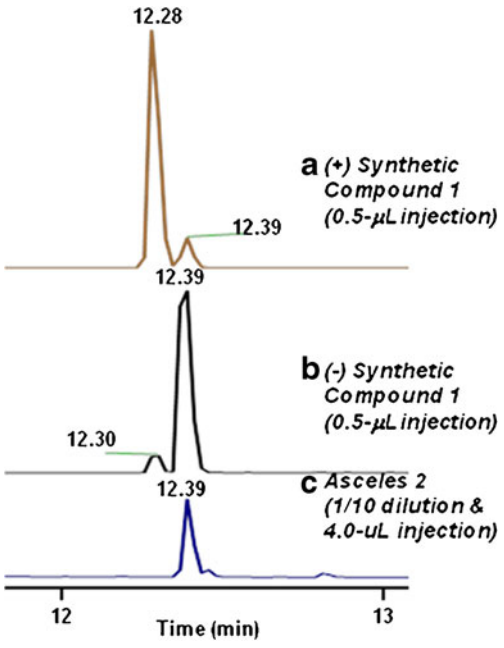

Fig. 4 The overlapping TICs (from the Beta DEX 120 column) for the 2(*R*)-(+) and 2(*S*)-(−) optical isomers of synthetic compound **1** (**a** & **b**) and for *Asceles* **2** (**c**) are normalized against the peak height of the 2(*R*)-(+) optical isomer

We also determined the efficacy of these compounds against potential arthropod predators and other arthropods. In one set of experiments, the compounds were tested for their ability to repel red imported fire ants (*Solenopsis invicta*) as a model predator. Two bioassays were used to evaluate the repellent activity of the spiroketal against *S. invicta*. The Y-tube olfactometer measures the response of worker ants to the spiroketal in the air stream of one choice arm vs. the solvent control in the other choice arm of the olfactometer. The

Table 1 GC/MS quantification: concentration of compound 1 (µg/µl) in various *Asceles* samples (*N*=3)

Samples	Average	Std. Deviation
Asceles-1	11	0.15
Asceles-2	42	1.4
Asceles-3	3.5	0.09
Asceles-4	4.5	0.11

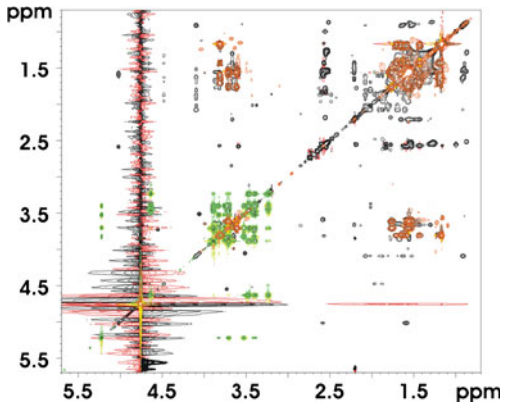

Fig. 5 Identification of Glucose in *Asceles glaber* defense spray: Two dimensional (2D) ^{1}H-^{1}H TOCSY spectra of *Asceles glaber* defense spray (bottom spectrum: *black and red*), synthetic spiroketal (**1**) ((2S,6R)-(−)(*E*)-2-methyl-1,7-dioxaspiro(5,5)-undecane) (top spectrum: *orange and light yellow*) and authentic commercial glucose (*green and greenish-yellow*). All spectra of samples dissolved in D_2O

positive control, queen recognition pheromone, was always highly attractive (mean ± SE=77.5±1.4 %; X^2=12.1, 1*df*, two-tailed *P*=0.001, *N*=3). The results are shown in Fig. 6a as the percent worker ants responding to the spiroketal. Significant repellent activity was only found in the 3.3 % spiroketal

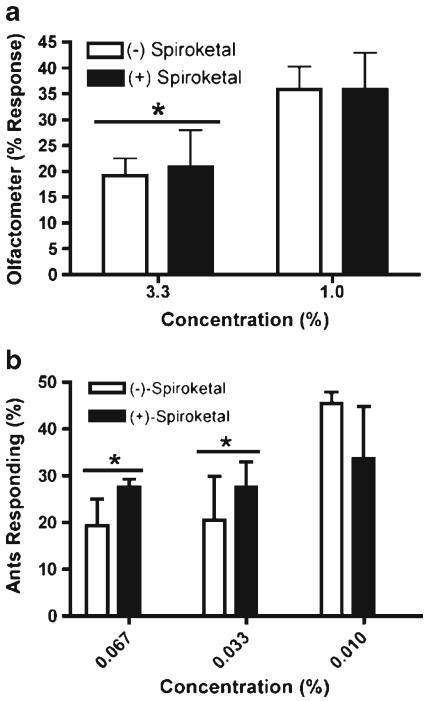

Fig. 6 **a** The Y-tube olfactometer response of fire ant workers to the spiroketal at the percent concentration indicated (pentane, W/V, mean ± SE; *n*=3). A mean response <35 % indicates significant repellent activity (*). Only the 3.3 % concentration showed repellent activity, **b** Contact repellency bioassay results (mean ± SE; *N*=3) for a series of Spiroketal dilutions (% concentration, W/V, in pentane). All concentrations were significantly different (repellent) from the pentane control, except the 0.01 % concentration. (see Results)

concentration (Fig. 6a, mean ± SE=29.2±3.6 %, *N*=3; X^2=6.4, 1*df*, two-tailed *P*=0.011). In previous work that investigated possible repellents against fire ants, the initial concentration of test compounds was 1.0 %. If the compound did not show repellent activity at 1 %, it was dropped from further consideration (Vander Meer et al., 1996). Based on the repellent activity of the spiroketal in the olfactometer bioassay, the compound has activity only at >1 %, or poor repellency to fire ant workers. Since some compounds can exhibit contact repellency (as well as or in addition to through the air repellency), we subjected the spiroketal to a bioassay that measured its effectiveness at preventing worker ants from feeding on a food substance. Compounds that are good volatile repellents can confound this bioassay, since the ants would be prevented from contacting the test compound. Our results (Fig. 6b) demonstrate that the spiroketal is an excellent contact repellent for fire ants. Activity is lost only between 0.033 and 0.01 %, which is 100 times more effective than demonstrated by the olfactometer bioassay, thus through the air activity is unlikely. In addition, observation showed that the ants approached and touched the test material before moving away. All evaluated concentrations (1.0 % though 0.01 % were significantly repellent to fire ant workers (above 0.067 % not shown), except the 0.01 % concentration (Fig. 6b). Therefore, by inference 1 % through 0.033 % activities also were significantly repellent. The dichotomy in results for the two bioassays points out the importance of differentiating contact and through the air repellency.

In addition to the testing of the synthetic spiroketals on ants,, their effects on mosquitoes (*A. aegypti*) also were studied. The repellency of the 2(*R*)-(+) and 2(*S*)-(−)-spiroketal were nearly identical at about 0.500 mg/cm^2. The amount of spiroketal required to repel *A. aegypti* was about 100 times higher than the amount of DEET required (Table 2). The 2(*R*)-(+)-spiroketal suppressed attraction to a synthetic human volatile blend when combined with the blend and tested again in a port containing only the blend. In contrast, the (−)-spiroketal increased the attraction of mosquitoes to the side containing it and the blend (Table 3).

Discussion

The data presented in this report demonstrate that the defensive spray of *Asceles glaber* contains primarily (2S,6R)-(−)(*E*)-2-methyl-1,7-dioxaspiro(5,5)-undecane (spiroketal **1**), 2-ethyl-1,6-dioxaspiro[4.5]decane (spiroketal **2**), and glucose. Additionally, the data show that spiroketal **1** is able to repel red imported fire ants (*Solenopsis invicta*) at concentrations well within the range of those deployed in the chemical defense system of *A. glaber,* and it exhibits a

Table 2 Minimum effective doses (MED)[a] of 2(*R*)-(+) and 2(*S*)-(−)-spiroketal and DEET tested on human volunteers against *Asceles aegypti* mosquitoes

Volunteer No.	2(*R*)-(+)-spiroketal	2(*S*)-(−)-spiroketal	DEET
1[b]	0.094	0.375	0.005
2	0.750	0.750	0.003
3	0.375	0.187	0.005
4	0.750	0.750	0.005
Average (±SE)	0.492 (0.159)	0.516 (0.141)	0.005 (0.001)

[a] MED in mg/cm^2 ; ≥ 5 bites in 1 min exposure to 500 mosquitoes is considered a failure at that compound concentration

[b] Volunteer 1 was a female, the other three volunteers were males

behavioral response from mosquitoes (*Aedes aegypti*). Chemical defense sprays from the same glands in other phasmid species have been shown by others to be effective repellents against potential predators such as ants, beetles, mice, rats, frogs, and birds and thus are assumed to be used for defense (Eisner, 1965; Carlberg, 1985a, 1985b, 1986; Dossey, 2011). However, it is unknown how effective spiroketal **1** is at protecting *A. glaber* in the wild.

Given the biological assay results described above and in previous literature, several conclusions can be made about the mechanism by which the chemical defenses of stick insects, particularly *A. glabor*, function. First, this spray appears to function as a contact repellent, not a volatile odor repellent. For both fire ants and mosquitoes, it is clear that the major spiroketal from *A. glabor* defense spray is a very weak airborne repellent. However, fire ants are turned away quite effectively upon contact with the substance, and it also appears to deter mosquitoes from landing and/or feeding. Additionally, the literature (and Dossey, personal observation) demonstrate that other stick insect defense sprays, like those of *Anisomorpha buprestoides* (mentioned previously), are quite irritating primarily when they come into contact with the eyes, nose, and mouth where sensitive mucus membranes are preset, They also produce a generally irritating response in model predators tested as well as humans and dogs (Dziezyc, 1992; Eisner et al., 1997; Paysse et al., 2001; Dossey, 2010; Brutlag et al., 2011). Thus, stick insect defense sprays such as this appear to function primarily as contact repellents, which are generally irritating to predators.

Table 3 Attraction of female *Asceles aegypti* mosquitoes to a synthetic blend compared to the blend with each spiroketal in a dual-port olfactometer

Only Blend	2(*R*)-(+)-spiroketal and Blend	Only Blend	2(*S*)-(−)-spiroketal and Blend
20.4(3.6)	8.7(3.9)	16.4(6.7)	33.1(3.7)

Attraction is presented as a percentage ± (SE) of approximately 75 female *A. aegypti* that were trapped in each port containing the treatments. Mosquitoes may select to fly into either treated port or to remain in the cage (6 replications were conducted for each spiroketal and blend vs. blend combination)

Since it is unlikely that mosquitoes prey upon *A. glaber*, it would not be expected that the defense secretions and the components contained therein would be mosquito-specific repellents. The weak repellency shown by the spiroketals indicate a deterrence to mosquito landing and biting behavior that could perhaps be a result of these compounds being irritating to the mosquito. While the repellency Minimum Effective Dosage (MED) values of the 2(*R*)-(+) and 2(*S*)-(−)-entantiomers are approximately 0.500 mg/cm^2, the amount of DEET required to repel these mosquitoes is 0.005 mg/cm^2 (100 times lower) (Table 2). However, DEET is known to be potent at low concentrations, typically ranging from 0.011 to 0.094 mg/cm^2 in this type of assay. Thus, the spiroketals identified from *A. glaber* may function as a general irritant to many other arthropods as well as other animal predators. Additionally, Dipterans have been observed as both endo- and ectoparasites on various species of stick insects (Neff and Eisner, 1960; Tilgner and McHugh, 1999). Therefore, these compounds may also affect Dipterans as well as other arthropods posing a threat to stick insects. These hypotheses require further investigation.

While the contact repellent efficacy appeared to be identical for both enantiomers of the spiroketals, there was a noticeable difference between the 2(*R*)-(+) and 2(*S*)-(−) forms when examined for their volatile based effects in competitive assays using a dual-port triple cage olfactometer (Table 3). The 2(R)-(+)-spiroketal decreased the attraction of mosquitoes to the ports that contained this enantiomer, whereas the 2(*S*)-(−)-spiroketal increased the port catches. This result is interesting since no clear difference was observed in contact repellency between the two. However, it has been established previously that there can be significant differences in the behaviors determined as evident in results when using these different types of bioassays (Weldon et al., 2011). Thus, while both enantiomers may show an irritant effect to mosquitoes at a higher concentration then DEET, there apparently is still some mechanism by which host-seeking mosquitoes are differentiating between the two spiroketal forms at a distance of a few meters down to several centimeters.

Interestingly, the spiroketals analyzed in this study are utilized as pheromones by a number of other insect species (Francke et al., 1977; Tengö et al., 1982; Francke and Kitching, 2001; Ghosh et al., 2006; Booth et al., 2009). Additionally, the amounts secreted by *A. glaber* in response to being disturbed are quite small compared to other species with more robust chemical defenses such as in the genera

Anisomorpha, *Peruphasma*, *Megacrania* and others (Eisner, 1965; Smith et al., 1979; Chow and Lin, 1986; Ho and Chow, 1993; Dossey et al., 2006, 2008; Dossey, 2010, 2011). In fact, it has been postulated that phasmids may also utilize their chemical defense systems for other purposes such as pheromonal communication (Tilgner, 2002; Dossey et al., 2008, 2009). In general, several other arthropods are known to utilize their various chemical production systems for multiple purposes, a concept described by Blum as Semiochemical Parsimony (Blum, 1996). Thus, it is possible that *A. glaber*, as well as other phasmids, use these compounds as pheromones or in other forms of chemical communication.

Including the current report, glucose has been found in the defense spray of 6 species of phasmids to date including: *Anisomorpha buprestoides* (Dossey et al., 2006), *Peruphasma schultei* (Dossey et al., 2006), *Parectatosoma mocquerysi* (Dossey et al., 2007), *Phyllium westwoodii* (Dossey et al., 2009), *Megacrania nigrosulfurea* (Prescott et al., 2009), and *Asceles glaber*. It also has been shown that the phasmid *A. buprestoides* can biosynthesize its defensive spray monoterpene *de novo* from ^{13}C-labeled glucose (Dossey et al., 2008). Additionally, beetles (Order Coleoptera) of the family Chrysomelidae (the leaf beetles) use precursors that are conjugated with glucose for larval defensive secretion biosynthesis and transport (Kunert et al., 2008). The chemical simplicity of phasmid chemical defense sprays and lack of other common primary metabolites (amino acids, etc.) besides glucose suggests that glucose plays a critical role in their chemical defense system. By analogy to the pathways elucidated in Chrysomelid beetles so far, glucose likely plays a similar role in phasmids as it does in beetles. The presence of glucose in *A. glaber* defense spray, as well as in that of other phasmid species, suggests that phasmids may utilize glucoconjugate precursors of multiple classes of secondary metabolites for biosynthesis, transport, or both. These hypotheses require further investigation.

The prothoracic exocrine glands of the Phasmatodea represent one key synapomorphy that defines this insect group (Tilgner, 2002; Bradler, 2009). Chemical analyses have shown that the composition of their secretory product differs significantly among the phasmid lineages studied to date (see Introduction), suggesting a potential chemotaxonomic value of the semiochemicals expressed. *Ascales glaber* is a typical representative of the Subfamily Necrosciinae, which also includes *S. sipylus*. This latter species is, according to Bouchard et al. (1997), apparently characterized by a complex mixture of chemicals, comprising limonene, benzothiazole, benzaldehyde, acetic acid, and predominantly diethyl ether as the major compound. The spiroketal here reported from *A. glaber* also has two ether linkages. However, this is not a strong similarity, and we believe that diethyl ether may have been an artifact in the report about *S. sipylus* secretion, since it is both unusual to find it in an animal and it is also a common laboratory solvent. At least, the spray of that species merits re-investigation, using other analytical methods. At any rate, the composition of defensive secretions is notably different in the two Necrosciinae species analyzed so far. This underlines the heterogeneous nature of this phasmid assemblage, which is also evidenced by the analysis of some morphological characters (Sellick, 1997; Bradler, 2009).

In summary, we have demonstrated the stereo-specific identification of spiroketal **1**, as well as glucose, in the chemical defense spray of *Asceles glaber* as well as its effect on ants and mosquitoes. We also have provided a well-supported mechanism by which this and possibly other stick insect chemical defenses function to ward off potential predators, attackers, and sources of other offending stimuli. These results demonstrate that spiroketals such as these found in the defense spray of *A. glaber* merit further investigation as potential components of insect repellents. In general, this report lends support to the demonstration of the chemical biodiversity that exists in insect chemical defense systems as well as the utility of stick insects as models of studying those systems.

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Supplemental Material Available online: S1) GC/MS Chromatograms of all natural samples of *Asceles glaber* defense spray analyzed; S2) Mass spectra of *A. glaber* defense spray samples and synthetic spiroketal **1**; S3) TIC for *A. glaber* chemical defense spray; S4) The EI mass spectra for *A. glaber* chemical defense spray; S5) TIC and mass spectrum of synthetic spiroketal **1**; S6) The NIST EI Mass Spectral Library search identified the minor peak in *A. glaber* defense spray; S7) The NIST EI Mass Spectral Library search for the major peak for *A. glaber* chemical defense spray; S8) 1D ^{1}H NMR spectral overlays of natural *A. glaber* defense spray and synthetic spiroketal **1** In benzene-d_6; S9) NMR spectra of *A. glaber* defense spray extracted with benzene-d_6; S10) NMR spectra of synthetic spiroketal **1**; S11) EI mass spectra from enantiomer selective GC/MS analysis of *A. glaber* chemical defense spray and synthetic spiroketal **1**; S12) External calibration curves from the GC/MS of synthetic spiroketal **1** for quantification of that compound in *A. glaber* chemical defense spray; S13) Extended table of concentrations of spiroketal **1** in *A. glaber* chemical defense spray; S14) 1D ^{1}H NMR spectral stack plots of natural *A. glaber* chemical defense spray and authentic D-Glucose dissolved in D_2O, S15) NMR spectra of *A. glaber* defense spray dissolved in D_2O; S16) NMR spectra of synthetic spiroketal **1** dissolved in D_2O; and S17) NMR spectra of authentic D-Glucose dissolved in D_2O.

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