Published in final edited form as:

J Chem Ecol. 2012 September ; 38(9): 1105–1115. doi:10.1007/s10886-012-0183-x.

DEFENSIVE SPIROKETALS FROM *Asceles glaber* (PHASMATODEA): ABSOLUTE CONFIGURATION AND EFFECTS ON ANTS AND MOSQUITOES

AARON T. DOSSEY^{1,*}, JOHN M. WHITAKER², MARIA CRISTINA A. DANCEL³, ROBERT K. VANDER MEER⁴, ULRICH R. BERNIER⁵, MARCO GOTTARDO⁶, and WILLIAM R. ROUSH²
13751 SW 20th AVE, APT# 1, Gainesville, FL 32607-4301

²Department of Chemistry, Scripps Florida, Jupiter, Florida 33458

³Mass Spectrometry Facility, Department of Chemistry, University of Florida, 32611-7200

⁴Imported Fire Ant and Household Insects (IFAHl) research unit, Center for Medical, Agricultural, and Veterinary Entomology, ARS/USDA, Gainesville, FL 3260

⁵Mosquito & Fly Research Unit, Center for Medical, Agricultural, and Veterinary Entomology, ARS/USDA, Gainesville, FL 32608

⁶Department of Evolutionary Biology, University of Siena, Via Aldo Moro 2, I-53100 Siena, Italy

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 well known for and name derived from their uncanny stereotypical use of camouflage as a primary line of defense from predation. However, many species also possess a potent chemical defense spray. Recently we discovered that the defensive spray of *Asceles glaber* contains spiroketals (confirmed major component: (2S,6R)-(-)-(E)-2-methyl-1,7-dioxaspiro[5.5]undecane and tentative 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* 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

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

Supporting Information Available: 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 ¹H NMR spectral overlays of natural *A. glaber* defense spray and synthetic spiroketal **1** in benzene-d₆; S9) NMR spectra of *A. glaber* defense spray extracted with benzene-d₆; S10) NMR spectra of synthetic spiroketal **1**; S11) EI mass spectra from chiral 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) NMR spectra of *A. glaber* defense spray dissolved in D₂O; S15) NMR spectra of synthetic spiroketal **1** dissolved in D₂O; and S16) NMR spectra of authentic D-Glucose dissolved in D₂O.

INTRODUCTION

Insects are well known for their utilization of chemistry in various aspects of their livelihoods such as communication and defense (Blum 1981; Dossey 2010; Eisner et al. 2005; Laurent et al. 2005). 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; Brock 2009). However, many species produce a chemical spray from a pair of tegumental glands in their prothorax when disturbed (Bedford 1978; Dossey 2010; Dossey 2011; Scudder 1876) (and references therein). The chemical composition of defensive sprays from only a few species has been analyzed (Bouchard et al. 1997; Chow and Lin 1986; Dossey 2011; Dossey et al. 2009; Dossey et al. 2007; Dossey et al. 2006; Eisner et al. 1997; Ho and Chow 1993; Meinwald et al. 1962; Prescott et al. 2009; Schmeda-Hirschmann 2006; Schneider 1934; Smith et al. 1979). 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. mocquersyi* (Dossey et al. 2007), *P. westwoodii* (Dossey et al. 2009) and *M. nigrosulfurea* (Prescott et al. 2009).

Asceles glaber Günther, 1938 (Günther 1938) (Figure 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). *A. 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 (Figure 1B).

Spiroketals, sometimes referred to as spiroacetals, make up a large and very diverse group of natural products which have been extensively reviewed in the literature (Booth et al. 2009) (and references therein). These compounds have been isolated from a number of insect species (Booth et al. 2009; Dettner et al. 1992; Francke et al. 1977; Francke and Kitching 2001; Goubault et al. 2008; Moore et al. 1994; Schwartz et al. 2008; Tengö et al. 1982). 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*) (Booth et al. 2009; Francke et al. 1977). Various spiroketals have been shown to 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.

Here we report the identification of two spiroketals (confirmed major component (2S,6R)-(-)-(E)-2-methyl-1,7-dioxaspiro(5,5)-undecane **1** and tentative minor component 2-ethyl-1,6-dioxaspiro[4.5]decane **2**) (Figure 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* 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 using 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. The 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 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 resonances of the solvent proton impurity (benzene-d₅) to 7.16 for ¹H and 128.39 for ¹³C, respectively. For spectra of samples in D₂O, chemical shift referencing was achieved by setting the anomeric ¹H to 5.22 and ¹³C 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)

GC/MS analyses were conducted on a TraceGC Ultra DSQ mass spectrometer (Thermo Scientific) equipped with an AT-5ms column from Alltech (60m × 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 and 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 eggs laid by females 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 h light-dark photoperiod. The 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 six males and four females collected between December 2007 and January 2008), *Asceles* 2 (18 milkings from three males and three females collected between August 2008 and January 2009), *Asceles* 3 (five milkings from three females collected between February and March 2008), and *Asceles* 4 (two milkings from one 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 which 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 D₂O or extracted with benzene-d₆. For GC/MS, approximately 1 μL of each sample was extracted with 0.5 mL of CH₂Cl₂ for analysis.

Spiroketals

The two enantiomers of spiroketal **1** were synthesized as described elsewhere (Whitaker, J. M., Dossey, A. T., Dancel, M. C. A., and Roush, W. R. (2011) Manuscript in Preparation on the synthesis of (2S,6R)-(–)(*E*)-2-methyl-1,7-dioxaspiro(5,5)-undecane.

(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, CH₂Cl₂), $[\alpha]_D^{25} = +66.72$ (c 1.06, CH₂Cl₂); ¹H NMR (600 MHz, C₆D₆) δ 3.75 (1 H, m), 3.64 (1 H, m), 3.54 (1 H, m), 2.05 – 1.96 (2 H, m), 1.66 – 1.08 (10 H, m), 1.15 (3 H, d, *J* = 6.0 Hz) ppm; ¹³C NMR (150 MHz, CDCl₃) 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 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* defensive 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 folds, respectively, for the quantification. For the external calibration, synthetic spiroketal compound **1**, equivalent to 1.25 to 12.5 μg/μL of sample, were prepared fresh. All the solutions were injected at least three times in a 24-hour 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 df). 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 and 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) (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). The 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 exact previous position. The 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 minutes. 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 is 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 180mm × 290mm × 50mm. The upper 3 mm of the pan was coated with Fluon[®] to preclude ants from escaping. A Petri dish nest cell (55mm dia.) was placed at one end of the pan. The Petrie dish had a 5mm layer of Castone[®] dental cement on the bottom that acted as a moisture reservoir. The lid of the Petri dish 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.

Bioassay—Fifty micro liters of test material was introduced to 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 a 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 gram of ants (starved for a minimum of 24 hours) 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-minute intervals for a total of 5 minutes. Each experiment was replicated three times, each with a unique monogyne colony and in a different test tray.

Bioassays on Mosquitoes

Aedes aegypti (L.) (Orlando, 1952) were received from the colony maintained at the United States Department of Agriculture-Agricultural Research Service in Gainesville, FL. 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. The ages of mosquitoes selected for tests ranged from 5–12 days old for experiments.

Contact Repellency—Repellency was assessed using a cage test (Barnard et al. 2007) and treatments were tested using the “cloth patch” assay method described in depth previously (Katritzky et al. 2010). The treatments consisted of 2(*R*)-(+)-spiroketal and 2(*S*)-(-)-spiroketal dissolved in acetone; N, N-diethyl-3-methylbenzamide (DEET) (Sigma-Aldrich, Milwaukee, WI) 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 acetonetic solution. The removed cloth has a 0.750 mg/cm² application to the muslin cloth 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/cm² of each test compound on muslin cloth. 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 four 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 (Bernier et al. 2007b; Posey et al. 1998). 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 $75 (\pm 10)$ mosquitoes were placed in each cage $50 (\pm 10)$ min prior to experiments. Air is drawn in from the outside, filtered and conditioned to $27 \pm 1^\circ\text{C}$ and $60 \pm 5\%$ 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 g L-(+)-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. $\times$ 9.5 mm height) and 50 μL of either the 2(R)-(+)- or 2(S)-(–)-spiroketal was added in a separate 400 μL cap (9 mm i.d. $\times$ 9 mm height) to one of the ports; caps were placed on a 13 $\times$ 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 carefully sprayed 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 GCMS analysis. For a description and definitions of the *Asceles* sample numbers used, see the Methods section. 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 (Supporting Information Figure S1–7). The EI mass spectrum of the tentative minor natural spiroketal **2** (Supporting Information Figure S2 C), from the analysis of *Asceles* 2, matched well to a known spiroketal, 2-ethyl-, (2R-trans)-1,6-dioxaspiro[4.5]decane (Supporting Information Figure S2 5d; CAS# 76495-09-5), with a reverse-fit score of 891 and a probability score of 94.7 (Supporting Information Figure S6). In contrast, the mass spectrum of the major component (Supporting Information Figures S1–3, Supporting Information Figure 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 gave a reverse-fit score of below 700 (spiroketal **2** came in a close second) (Supporting Information Figure 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 (Figure 3; Supporting Information Figure S8). Identical mass spectra acquired for synthetic spiroketal **1** and the major component from *A. glaber* chemical defense spray provided confirmation of its identification (Supporting Information Figures S1, S2 a and b).

For confirmation purposes and biological assays, both enantiomers of the proposed spiroketal were produced synthetically in the laboratory. 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 our synthetic efforts will be presented in a separate publication (Whitaker, J. M., Dossey, A. T., Dancel, M. C. A., and Roush, W. R. (2011) Manuscript in Preparation on the synthesis of (2S,6R)-(-)(E)-2-methyl-1,7-dioxaspiro(5,5)-undecane.

For final verification of the identification for spiroketal **1**, approximately 5 μ L of *Asceles* 5 was extracted with 100 μ L of benzene-d₆ and a separate 5 μ L was dissolved in 100 μ L of D₂O for exhaustive NMR analysis to identify the major components (Figure 3 and Supporting Information Figures S8–9). One-dimensional (1D) NMR spectral stack plots (Supporting Information Figure S8) as well as 2D TOCSY and HSQC spectral overlays (Figure 3) of natural *A. glaber* defense spray (Supporting Information Figures S8–9) and synthetic spiroketal **1** (Supporting Information Figure S8 and S10) provide robust verification that the major component in that species’ chemical defenses is spiroketal **1**. Additionally, the chemical shifts of ¹H and ¹³C resonances observed in all spectra of synthetic spiroketal **1** in benzene-d₆ 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 a chiral column was performed on the natural material in sample *Asceles* 2 and both synthetic enantiomers of spiroketal **1**. Chiral GC/MS results are shown in Figure 4. The standards were found to be a mixture of two spiroketals and are labeled based on the predominant isomer (Figure 4). Retention times confirmed that the major spiroketal in *A. glaber* defense spray is the 2(S)-(-) enantiomer (Figure 4). Both peaks at 12.28 minutes and all three peaks at 12.39 minutes have identical EI mass spectra (Supporting Information Figure 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 (Supporting Information Figure S1) along with those of a series of dilutions of synthetic spiroketal **1** were compared via standard plot (Supporting Information Figures S12–13) to calculate the concentrations of that compound in *A. glaber* chemical defense spray reported in Table 1 (see also Supporting Information Figures S12–12 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 ¹H as well as 2D ¹H TOCSY) of natural and synthetic material both dissolved in D₂O, as shown in Figure 5 and Supporting Information Figure 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 D₂O (Figure 5 and Supporting Information Figures S14–17). In addition to spiroketal **1**, spectra for the natural sample dissolved in D₂O revealed the presence of sugar-like resonances and correlations (Figure 5 and Supporting Information S14–S15). Since glucose has been reported from several phasid chemical defense sprays to date (see Discussion section

below), it was chosen as the most likely candidate for the identity of the *A. glaber* defense spray sugar. Indeed, as shown in Figure 5, overlaying spectra of pure authentic commercially obtained glucose show an exact match for glucose (also see Supporting Information Figures S14–S17; S17 contains NMR spectra of an authentic sample of D-Glucose). Thus, *A. glaber* defense spray contains both spiroketal **1** and glucose.

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 versus the solvent control in the other choice arm of the olfactometer. The positive control, queen recognition pheromone, was always highly attractive (mean $\pm$ SE = $77.5 \pm 1.4\%$; $X^2 = 12.1$, 1df, two-tailed $P = 0.0005$, $n=3$). The results are shown in Figure 6A as the percent worker ants responding to the spiroketal. Significant repellent activity was only found in the 3.3% spiroketal concentration (Figure 6A, mean $\pm$ SE = $29.2 \pm 3.6\%$, $n=3$; $X^2 = 6.4$, 1df, two-tailed $P = 0.0114$). In previous work investigating possible repellents against fire ants the initial concentration of test compounds was 1.0 percent. 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 (Figure 6B) clearly 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 highly unlikely. In addition, careful observation showed that the ants approached and touched the test material before moving away. All evaluated concentrations (1.0% through 0.01% were significantly repellent to fire ant workers (above 0.067% not shown), except the 0.01% concentration (Figure 6B). Therefore, by inference 1% through 0.033% activities were also 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 in this study on fire ants, their effects on mosquitoes (*A. aegypti*) were also studied. The repellency of the 2(*R*)-(+)- and 2(*S*)-(–)-spiroketal were nearly identical at about 0.500 mg/cm². 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 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 (2*S*,6*R*)-(–)(*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 presented 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 exhibits a 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 other potential predators such as ants, frogs, and birds and thus are assumed to be used for defense. However, it is unknown how effective spiroketal **1** is at protecting *A. glaber* in the wild.

Given the biological assay results described above and previous literature, several conclusions can be made about the mechanism by which the chemical defenses of stick insects, particularly *A. glaber*, function. First, this defense 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. glaber* defense spray is a very weak. 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 personal experiences of the corresponding author of this article) 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 present and produce a generally irritating response in model predators tested as well as humans and dogs (Brutlag et al. 2011; Dossey 2010; Dziezyc 1992; Eisner et al. 1997; Paysse et al. 2001). Thus, stick insect defense sprays such as this appear to function primarily as contact repellents which are generally irritating to predators.

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 of 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*)-(-)-enantiomers are approximately 0.500 mg/cm², the amount of DEET required to repel these mosquitoes is 0.005 mg/cm² (100 times lower) (Table 2). However, DEET is known to be quite potent at low concentrations, typically ranging from 0.011 – 0.094 mg/cm² 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 target Dipterans as well as other arthropods posing a threat to stick insects. Of course 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 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 than DEET, there 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 (Booth et al. 2009; Francke et al. 1977; Francke and Kitching 2001; Ghosh et al. 2006; Tengö et al. 1982). 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*, *Megacrana* and others.

In fact, it has been postulated that phasmids may also utilize their chemical defense systems for other purposes such as pheromonal communication (Dossey et al. 2008; Tilgner 2002). In general, several other arthropods are known to utilize their various chemical production systems for multiple purposes, a concept described by Murray S. 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. However, these hypotheses are beyond the scope of this study and require further exploration for validation.

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 mocquersyi* (Dossey et al. 2007), *Phyllium westwoodii* (Dossey et al. 2009), *Megacrania nigrosulfurea* (Prescott et al. 2009), and *Asceles glaber*. It has also been shown that the phasmid *A. buprestoides* can biosynthesize its defensive spray monoterpene *de novo* from ¹³C-labeled glucose (Dossey et al. 2008). Additionally, beetles (Order Coleoptera) of the family Chrysomelidae (the leaf beetles) have been shown to 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 strongly suggests that glucose plays a critical role in their chemical defense system. By analogy to the pathways elucidated in Chrysomelid beetles so far, their biosynthetic systems, 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, of course, require further investigation.

The prothoracic exocrine glands of the Phasmatodea represent one key synapomorphy which defines this insect group (Bradler 2009; Dossey et al. 2009; Tilgner 2002). Chemical analyses have shown that the composition of their secretory product differs significantly among the phasmid lineages studied to date (see the introduction section above), suggesting a potential chemotaxonomic value of the semiochemicals expressed. *A. glaber* is a typical representative of the Subfamily Necrosiinae which includes also *S. sipylus*. This latter species is, according to Bouchard et al. (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 and others in the field believe that diethyl ether may have been an artifact in the report about *S. sipylus* secretion, since it is both very unusual to find it in an animal and it is also a very common laboratory solvent. At least, the spray of that species likely merits re-investigation, using also other analytical methods. At any rate, the composition of defensive secretions is notably different in the two Necrosiinae species analyzed so far. This underlines the heterogeneous nature of this phasmid assemblage, which is also evidenced by the analysis of some morphological characters (Bradler 2009; Sellick 1997).

In conclusion, 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 have also 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 further support to the

demonstration of the chemical biodiversity which exists in insect chemical defense systems as well as the utility of stick insects as models of studying those systems.

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. We also thank David Milne and Michele Hosack for conducting fire ant bioassays. A.T.D. thanks Dr. Spencer S. Walse (USDA-ARS) for lending the chiral GC column used in this study. NMR data were collected in the AMRIS facility in the McKnight Brain Institute of the University of Florida. Funding was generously provided by the NSF-supported National High Magnetic Field Laboratory (University of Florida) and NIH Grant GM026782 (WRR, Scripps Florida).

References

- BARNARD, DR.; BERNIER, UR.; XUE, R-D.; DEBBOUN, M. Chapter 5: Standard methods for testing mosquito repellents. In: Debboun, M.; Frances, SP.; Strickman, D., editors. Insect repellents: principles, methods and uses. Boca Raton, FL: CRC Press; 2007. p. 101-108.
- BEDFORD GO. Biology and Ecology of the Phasmatodea. *Ann Rev Entomol.* 1978; 23:125–149.
- BERNIER UR, KLINE DL, ALLAN SA, BARNARD DR. Laboratory comparison of *Aedes Aegypti* attraction to human odors and to synthetic human odor compounds and blends. *Journal of the American Mosq Contr Assoc.* 2007a; 23:288–293.
- BERNIER, UR.; KLINE, DL.; POSEY, KH. Natural compounds that inhibit mosquito host-finding abilities. In: Debboun, M.; Frances, SP.; Strickman, D., editors. Insect repellents: principles, methods, and uses. Boca Raton, FL: CRC Press; 2007b. p. 77-100.
- BLUM, MS. Chemical defenses of arthropods. New York: Academic Press; 1981. p. 562
- BLUM MS. Semiochemical parsimony in the Arthropoda. *Annu Rev of Entomol.* 1996; 41:353–374. [PubMed: 15012333]
- BOOTH YK, KITCHING W, DE VOSS JJ. Biosynthesis of insect spiroacetals. *Nat Prod Rep.* 2009; 26:490–525. [PubMed: 19642419]
- BOUCHARD P, HSIUNG CC, YAYLAYAN VA. Chemical analysis of defense secretions of *Sipyloidea sipyilus* and their potential use as repellents against rats. *J Chem Ecol.* 1997; 23:2049–2057.
- BRADLER S. Die Phylogenie der Stab- und Gespentschrecken (Insecta: Phasmatodea). *Species Phylog Evol.* 2009; 2:3–139.
- BRAGG, PE. Phasmids of Borneo. Kota Kinabalu: Natural History Publications (Borneo); 2001. p. 772
- BROCK, PD. The amazing world of stick and leaf-insects. Feltham (United Kingdom): Amateur Entomologists Society; 1999. p. 165
- BROCK, PD. Phasmida species file online. The Orthopterists' Society; London, UK: <http://Phasmida.SpeciesFile.org> of August 11, 2009
- BRUTLAG AG, HOVDA LR, DELLA RIPA MA. Corneal ulceration in a dog following exposure to the defensive spray of a walkingstick insect (*Anisomorpha* spp). *J Vet Emerg Crit Care.* 2011; 21:382–386.
- CHOW YS, LIN YM. Actinidine, a Defensive Secretion of Stick Insect, *Megacrana alpheus* Westwood (Orthoptera, Phasmatidae). *J Entomol Sci.* 1986; 21:97–101.
- DETTNER K, FETTKOTHER R, ANSTEEG O, DEML R, LIEPERT C, PETERSEN B, HASLINGER E, FRANCKE W. Insecticidal Fumigants from Defensive Glands of Insects - a Fumigant Test with Adults of *Drosophila melanogaster*. *J Appl Ent-Z Angewandte Entomologie.* 1992; 113:128–137.
- DOSSEY AT. Insects and their chemical weaponry: New potential for drug discovery. *Nat Prod Rep.* 2010; 27:1737–1757. [PubMed: 20957283]
- DOSSEY, AT. Chemical Defenses of Insects: A Rich Resource for Chemical Biology in the Tropics. In: Vivanco, JM.; Weir, T., editors. Chemical Biology of the Tropics: An Interdisciplinary Approach. Heidelberg, Dordrecht, London, New York: Springer; 2011. p. 27-57.

- DOSSEY AT, GOTTARDO M, WHITAKER JM, ROUSH WR, EDISON AS.
Alkyldimethylpyrazines in the defensive spray of *Phyllium westwoodii*: a first for order Phasmatodea. J Chem Ecol. 2009; 35:861–870. [PubMed: 19685263]
- DOSSEY AT, WALSE SS, CONLE OV, EDISON AS. Parectadial, a Monoterpenoid from the Defensive Spray of *Parectatosoma mocquersyi*. 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]
- DZIEZYC J. Insect defensive spray-induced keratitis in a dog. J Am Vet Med Assoc. 1992; 200:1969. [PubMed: 1639705]
- 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.; EISNER, M.; SIEGLER, M. Secret weapons: Defenses of insects, spiders, scorpions, and other many-legged creatures. Cambridge, Mass: Belknap Press of Harvard University Press; 2005. p. 372
- FRANCKE W, HEEMANN V, GERKEN B, RENWICK JAA, VITE JP. 2-Ethyl-1,6-Dioxaspiro[4.4]Nonane, Principal Aggregation Pheromone of *Pityogenes chalcographus* (L). Naturwissenschaften. 1977; 64:590–591.
- FRANCKE W, KITCHING W. Spiroacetals in insects. Curr Org Chem. 2001; 5:233–251.
- GHOSH SK, KO C, LIU J, WANG J, HSUNG RP. A ketal-tethered RCM strategy toward the synthesis of spiroketal related natural products. Tetrahedron. 2006; 62:10485–10496.
- GOUBAULT M, BATCHELOR TP, ROMANI R, LINFORTH RST, FRITZSCHE M, FRANCKE W, HARDY ICW. Volatile chemical release by bethylid wasps: identity, phylogeny, anatomy and behaviour. Biol J Linn Soc. 2008; 94:837–852.
- GÜNTHER K. Neue und wenig bekannte Phasmoiden aus dem Indian Museum, Calcutta. Records of the Indian Museum. 1938; 40:123–141.
- HO HY, CHOW YS. Chemical-Identification of Defensive Secretion of Stick Insect, *Megacrania tsudai* Shiraki. J Chem Ecol. 1993; 19:39–46.
- KATRITZKY AR, WANG ZQ, SLAVON S, DOBCHEV DA, HALL CD, TSIKOLIA M, BERNIER UR, ELEJALDE NM, CLARK GG, LINTHICUM KJ. Novel Carboxamides as Potential Mosquito Repellents. J Med Ent. 2010; 47:924–938.
- 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, S. Insect chemical defense. In: Schulz, S., editor. Chemistry of Pheromones and Other Semiochemicals II. Verlag, Berlin, Heidelberg: Springer; 2005. p. 167-229.
- 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.
- MOORE CJ, HUBENER A, TU YQ, KITCHING W, ALDRICH JR, WAITE GK, SCHULZ S, FRANCKE W. A New Spiroketal Type from the Insect Kingdom. J Org Chem. 1994; 59:6136–6138.
- NEFF SE, EISNER T. Note on two tachinid parasites of the walking stick, *Anisomorpha buprestoides* (Stoll). Bull Brooklyn Ent Soc. 1960; 55:101–103.
- PAYSSE EA, HOLDER S, COATS DK. Ocular injury from the venom of the Southern walkingstick. Ophthalmology. 2001; 108:190–1. [PubMed: 11150287]
- POSEY KH, BARNARD DR, SCHRECK CE. Triple cage olfactometer for evaluating mosquito (Diptera: Culicidae) attraction responses. J Med Ent. 1998; 35:330–334.

- POSEY KH, SCHRECK CE. An Air-Flow Apparatus for Selecting Female Mosquitos for Use in Repellent and Attraction Studies. *Mosquito News*. 1981; 41:566–568.
- PRESCOTT TAK, BRAMHAM J, ZOMPRO O, MACIVER SK. Actinidine and glucose from the defensive secretion of the stick insect *Megacrania nigrosulfurea*. *Biochem Syst Ecol*. 2009; 37:759–760.
- 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.
- SCHWARTZ BD, MOORE CJ, RAHM F, HAYES PY, KITCHING W, DE VOSS JJ. Spiroacetal biosynthesis in insects from Diptera to Hymenoptera: the Giant Ichneumon wasp *Megarhyssa nortoni nortoni* Cresson. *J Am Chem Soc*. 2008; 130:14853–60. [PubMed: 18841963]
- SCUDDER SH. Odoriferous glands in Phasmidae. *Psyche*. 1876; 1:137–140.
- SELICK J. The range of egg capsule morphology within the Phasmatodea and its relevance to the taxonomy of the order. *Ital J Zool*. 1997; 64:97–104.
- 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.
- TENGÖ J, BERGSTRÖM G, BORGKARLSON A-K, GROTH I, FRANCKE W. Volatile Compounds from Cephalic Secretions of Females in 2 Cleptoparasite Bee Genera, *Epeolus* (Hym, Anthophoridae) and *Coelioxys* (Hym, Megachilidae). *Z Natur C-a J of Biosci*. 1982; 37:376–380.
- TILGNER, EH. PhD Dissertation. University of Georgia; Athens: 2002. Systematics of Phasmida.
- TILGNER EH, MCHUGH JV. First record of parasitism of *Manomera tenuescens* (Phasmida: Heteroemiidae) by *Phasmophaga antennalis* (Diptera: Tachinidae). *Entomological News*. 1999; 110:151–152.
- 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]
- VANDER MEER RK, ALVAREZ F, LOFGREN CS. Isolation of the Trail Recruitment Pheromone of *Solenopsis invicta*. *J Chem Ecol*. 1988; 14:825–838.
- VANDER MEER, RK.; BANKS, WA.; LOFGREN, CS. Repellents for ants. 5,587,401. Dec 24. 1996
- VANDER MEER RK, GLANCEY BM, LOFGREN CS, GLOVER A, TUMLINSON JH, ROCCA J. The Poison Sac of Red Imported Fire Ant Queens - Source of a Pheromone Attractant Hymenoptera, Formicidae. *Ann Ent Soc Am*. 1980; 73:609–612.
- WELDON PJ, CARROLL JF, KRAMER M, BEDOUKIAN RH, COLEMAN RE, BERNIER UR. Anointing Chemicals and Hematophagous Arthropods: Responses by Ticks and Mosquitoes to Citrus (Rutaceae) Peel Exudates and Monoterpene Components. *J Chem Ecol*. 2011; 37:348–359. [PubMed: 21409496]
- WESTON RJ, WOOLHOUSE AD, SPURR EB, HARRIS RJ, SUCKLING DM. Spiroacetals and other venom constituents as potential wasp attractants. *J Chem Ecol*. 1997; 23:553–568.
- ZHANG HS, FLETCHER MT, DETTNER K, FRANCKE W, KITCHING W. Synthesis and absolute stereochemistry of spiroacetals in rove beetles (Coleoptera: Staphylinidae). *Tetrahedron Letters*. 1999; 40:7851–7854.

Figure 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 black bordered yellow arrows showing the position of the openings of its defensive glands. Photographs by Marco Gottardo.

Figure 2.

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

Figure 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 ^1H - ^1H 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 ^1H - ^{13}C HSQC spectra of *Asceles glaber* defense spray extracted with benzene- d_6 (black and red).

Figure 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.

Figure 5.

Identification of Glucose in *Asceles glaber* defense spray: Two dimensional (2D) ^1H - ^1H 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 .

A.

B.

Figure 6.

A) The Y-tube olfactometer response of fire ant workers to the spiroketal at the percent concentration indicated (pentane, W/V, mean $\pm$ 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 $\pm$ 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).

Table 1

GCMS quantification – Cris Dancel

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

Concentration of Compound 1 ($\mu\text{g}/\mu\text{L}$) in various *Asceles* samples (n=3)

Table 2

Minimum effective doses (MED)^a of 2(*R*)-(+)- and 2(*S*)-(-)-spiroketal and DEET tested on human volunteers against *A. aegypti* mosquitoes.

Volunteer No.	2(<i>R</i>)-(+)-spiroketal	2(<i>S</i>)-(-)-spiroketal	DEET
1	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)

^aMED in mg/cm²; 5 bites in 1 min exposure to 500 mosquitoes is considered a failure at that compound concentration.

^bVolunteer 1 was a female, the other three volunteers were males.

Table 3

Attraction of female *A. aegypti* mosquitoes to a synthetic blend compared to the blend with each spiroketal in a dual-port olfactometer.^a

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)

^aAttraction is presented as a percentage $\pm$ (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).