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Piperideine Alkaloids from the Poison Gland of the Red Imported Fire Ant (Hymenoptera: Formicidae)

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The major chemical components in the venom of red imported fire ants, *Solenopsis invicta* Buren, are 2-methyl-6-alkyl or alkenyl piperidines. After isolating the extracts of poison glands and whole worker bodies with column chromatography, we obtained fractions containing a mixture of six piperideine alkaloids. Reduction of those samples using $NaBH_4$ in ethanol generated piperidine alkaloids found in the fire ant poison gland, resulting in both the *cis*- and the *trans*-piperidine alkaloids. The mass spectra and gas chromatographic behavior of most piperidine alkaloids from fire ant venom have been well-characterized, which significantly facilitated the identification of these piperdeine compounds. On the basis of the mass spectra and profiles of $NaBH_4$ reduction products, we identified these alkaloids as 2-methyl-6-tridecenyl-6-piperideine, 2-methyl-6-tridecyl-6-piperideine, 2-methyl-6-pentadecenyl-6-piperideine, 2-methyl-6-pentadecyl-6-piperideine, 2-methyl-6-heptadecenyl-6-piperideine, and 2-methyl-6-heptadecyl-6-piperideine. To our knowledge, this is the first time that these piperideine alkaloids have been reported in red imported fire ant poison glands.

KEYWORDS: ***Solenopsis invicta*** **Buren; venom; alkaloids; piperideine; piperidine; stings**

INTRODUCTION

The red imported fire ant, *Solenopsis invicta* Buren, is a well-known invasive ant species that was inadvertently introduced into the United States from South America in the 1930s. It is a serious pest affecting humans, wildlife, crops, and livestock. The current distribution range of the red imported fire ant in the United States covers all or part of many southern and western states and Puerto Rico (*1*). It has also been reported in Australia, New Zealand, Bahamas, British and U.S. Virgin Islands, Cayman Islands, mainland China, Hong Kong, Malaysia, Singapore, Taiwan, Trinidad and Tobago, Turks and Caicos Islands, and Mexico (*2, 3*). Because of its vicious stings, the red imported fire ant is a significant threat to public health. The painful sting may cause a persistent local reaction or anaphylaxis (*4*). As of 1989, 32 fire ant-related deaths had been reported in the United States (*5*).

In contrast to the venoms of other insects in Hymenoptera, such as bees, hornets, and wasps, which are usually aqueous solutions containing proteins, 95% of red imported fire ant venom consists of alkaloids with only a small fraction of proteins. The alkaloid component of fire ant venom contains predomonately 2-methyl-6-alkyl or alkenylpiperidines (*6*).

In addition to venom production, the poison gland has many other physiological and behavioral functions. For example, the poison sac of the red imported fire ant queen is a source of a pheromone attractant (*7, 8*) and a primer pheromone (*9*). Red imported fire ant queens deposit an antimicrobial agent on eggs (*10*), and workers disperse venom through the air using gaster flagging behavior to repel heterospecifics encountered in the foraging arena or to dispense to the brood surface as an antibiotic (*11*). Venom alkaloids were also found in red imported fire ant artificial nest material in a laboratory study focused on ant-derived compounds. In that study, moistened silica gel was used as the only nest-building material to reduce the interference from other soil-borne chemicals during chemical analysis (*12*).

The chemistry of the red imported fire ant alkaloids has been a subject of numerous investigations (*6*). The chemical structures of piperidine alkaloids in the red imported fire ant poison gland are well-defined. The mass spectrum of all 2-methyl-6-alkyl or alkenylpiperidines showed a strong ion at *m*/*z* 98 due to α cleavage of the alkyl group, an $[M - CH_3]^+$, and an $[M - 1]^+$ ion (*6*). The alkyl or alkenyl side chains have 11, 13, 15, and 17 carbons. The double bond isomer is always *cis*. Both 2,6-disubstituted piperidine ring configurational isomers are found in the venom with the *trans* isomer as the dominant one. It was found that the absolute configuration of the *trans* alkaloids in the fire ants is always (2*R*,6*R*), while that of the *cis*-alkaloids is (2*R*,6*S*) (*13*).

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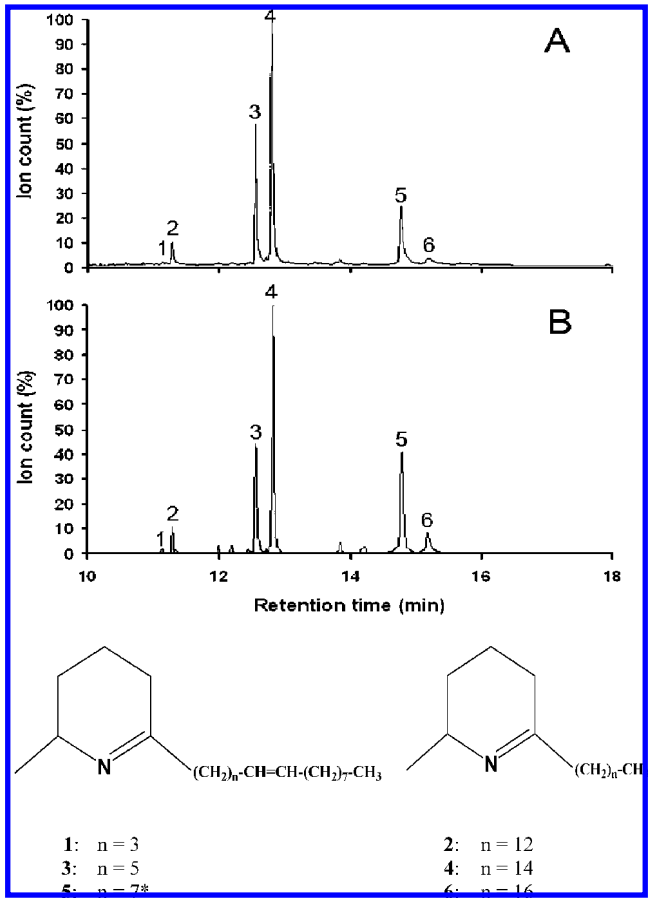

Figure 1. GC-MS-EI TICs of hexane extracts of fire ant poison glands (**A**) and worker whole bodies (**B**) after column chromatography and peak assignment. *The position of the double bond in the heptadecenyl side chain of 2-methyl-6-heptadecenyl-6-piperideine is unknown.

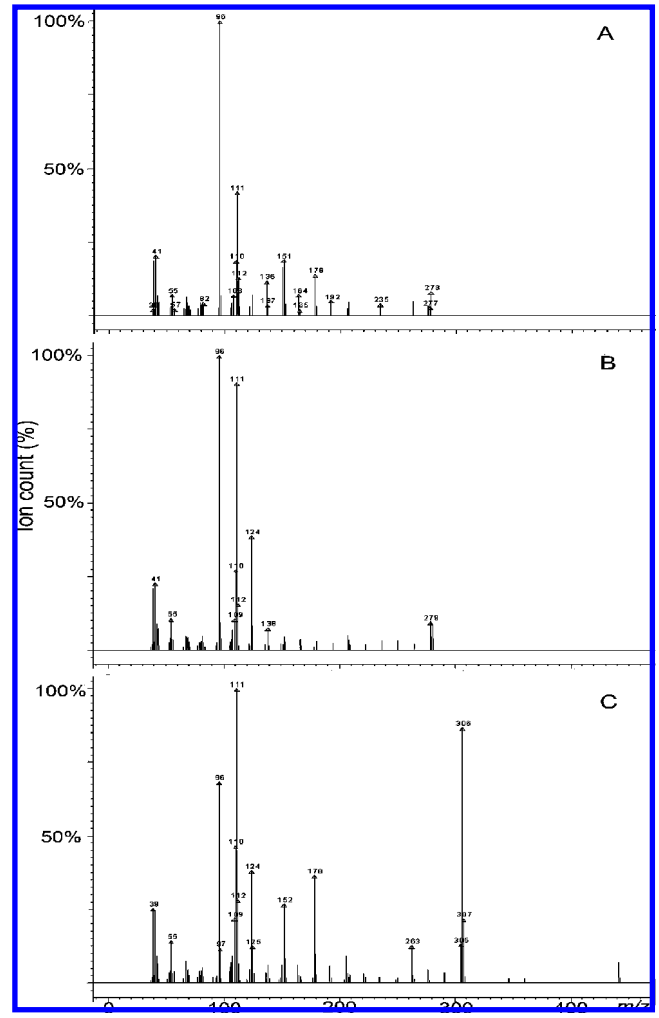

Figure 2. EI mass spectra of piperideine alkaloids. (**A**) 2-Methyl-6-tridecenyl-6-piperideine (**1**), (**B**) 2-methyl-6-tridecyl-6-piperideine (**2**), and (**C**) 2-methyl-6-pentadecenyl-6-piperideine (**3**).

These alkaloids are toxic to many biological systems (*6*). They inhibit Na^+ and K^+ ATPases, reduce mitochondrial respiration and uncouple oxidative phosphorylation at low concentrations, block neuromuscular junctions, and release histamine from mast cells. They are capable of producing toxic cardiovascular and central nervous system effects in mammals. (±)-Solenopsin A (*trans*-2-methyl-6-undecylpiperidine) and (±)-isosolenopsin A (*cis*-2-methyl-6-undecylpiperidine) have cardiorespiratory depressant activity and elicit seizures in rats, which may explain cardiorespiratory failure in some individuals who experience multiple fire ant stings (*14*). Isosolenopsin A is also a potent and selective inhibitor of neuronal nitric oxide synthase (*15*). Antibiotic and antifungal properties of fire ant venom alkaloids have also been documented (*16*−*18*).

So far, 2,6-disubstituted piperidines are the only alkaloids identified in the red imported fire ant poison gland. In an attempt to identify ant-derived compounds that may affect fire ant behavior, we found six new compounds in the red imported fire ant poison gland. Retention times (RTs) of gas chromatography and mass spectra of those compounds showed that they were not previously defined piperidine alkaloids. Identification of these compounds is important not only to our understanding of fire ant physiology but also to the study of the mechanism of human reactions to fire ant sting and the development of medical treatments. This research report describes the identity of these compounds and rapid methods to isolate these compounds from poison glands and worker whole body extracts.

MATERIALS AND METHODS

Ants. Six *S. invicta* colonies were collected in Sharkey County, Mississippi, including three polygyne colonies and three monogyne colonies. Ant mounds were shoveled and placed in a 19 L plastic bucket. The inside wall of the bucket was then coated with baby powder (Cumberland Swan Holdings, Inc., Smyrna, TN) to prevent ant escape. A modified water-drip method (*19*, *20*) was used to separate ants from soil. After they were separated from the soil, ants were placed in a 44.5 cm × 60.0 cm × 13.0 cm plastic tray with the inside walls coated with Fluon (Ag Fluoropolymers, Chadds Ford, PA). Distilled water and 10% (w/v) sucrose water solution were placed in separate test tubes (2 cm × 15 cm) with the opening plugged with cotton balls. Adult house crickets, *Acheta domestica* L., were used as additional food sources. One to three 14.0 cm × 2.0 cm Petri dishes with 1.0 cm of hardened dental plaster (Castone; Dentsply International Inc., York, PA) on the bottom were also placed inside each tray. The Petri dish also contained a 5.0 cm diameter brood chamber. Two 8 mm access holes were made on the wall of the Petri dish above the dental plaster. The Petri dish lid was painted with gloss black spray enamel to block light. All colonies were maintained at 22−25 °C, 12 h light, 12 h dark, and 60−70% relative humidity. The species identity was confirmed using the profiles of piperidine alkalids and cuticular hydrocarbons (*21*), and the social form was determined using polymerase chain reaction (*22*).

Chemicals. Synthetic fire ant piperidine alkaloids were provided by Dr. N. P. D. Nanayakkara at The University of Mississippi School of Pharmacy, including *cis*-2-methyl-6-undecylpiperidine, *trans*-2-methyl-6-undecylpiperidine, *cis*-2-methyl-6-tridecylpiperidine, *trans*-2-methyl-6-tridecylpiperidine, *cis*-2-methyl-6-pentadecylpiperidine, and *trans*-

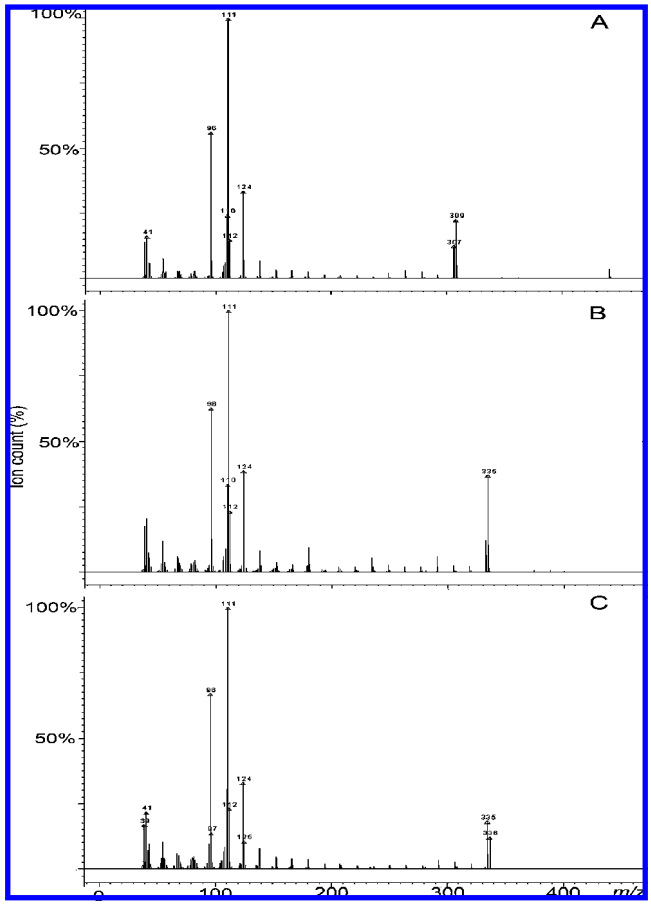

Figure 3. EI mass spectra of piperideine alkaloids. (**A**) 2-Methyl-6-pentadecyl-6-piperideine (**4**), (**B**) 2-methyl-6-heptadecenyl-6-piperideine (**5**), and (**C**) 2-methyl-6-heptadecyl-6-piperideine (**6**).

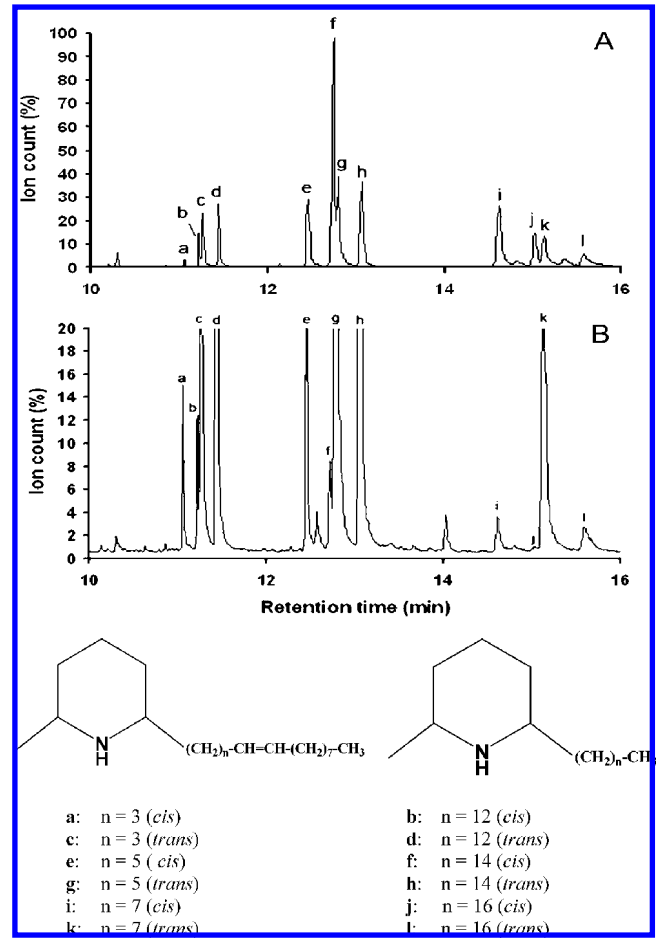

Figure 4. Typical chromatograms of the $NaBH_4$ reduction products of piperideine alkaloids (**A**) and fire ant piperidine alkaloids (**B**) and peak assignment.

2-methyl-6-pentadecylpiperidine. Hexane and acetone [99.9% purity, ACS high-performance liquid chromatography (HPLC) grade] used for extraction and purification of the piperideine alkaloids, reducing agent, $NaBH_4$ (≥98.5% purity, Reagent grade), and ethanol (200 proof, USP/NF grade) were purchased from Sigma-Aldrich (St. Louis, MO). Purified piperideine alkaloids were obtained by gravity column chromatography.

Venom Extraction. Six colonies were used in this study. Two methods of extraction were used. In the first method, the poison gland was dissected from the body, and its contents (venom) were then collected. This ensured that everything in the extract was from the venom. Workers were randomly sampled and killed at −20 °C in a refrigerator for about 30 min, and a single ant was dissected under a SZX16 stereo microscope (Olympus, Japan). After the cuticle was removed from the gaster, the poison gland and its reservoir were pulled and collected with a pair of microdissecting tweezers and then immediately transferred to a 100 μL conical glass insert with about 50 μL of hexane. Thirty ants were extracted for each colony. In the second method, whole worker bodies were used. Fire ant venom alkaloids can be easily extracted from whole fire ant worker bodies using hexane (*21*). This simple extraction method involved three steps: placing 2.5 g of workers in 15 mL of hexane in a 50 mL beaker at 20 °C for 10 min, transferring the hexane extract into a 20 mL vial, and concentrating the extract to 200 μL under air flow. All extracts were immediately used for further isolation and purification by gravity column chromatography.

Column Chromatography. Corresponding to the two methods used in extraction, two methods for gravity column chromatography were used. The first method was designed for the poison gland extract. A disposable borosilicate glass Pasteur pipet, approximately 14.6 cm long (Fisher Scientific, Pittsburgh, PA), was used as the column. The tip of the pipet was first blocked using a piece of glass wool, and 0.7 g of Davisil, grade 636, pore size 60 Å, 35−60 mesh silica gel (St. Louis, MO) was then filled into the pipet. After the column was washed with 5 mL of hexane, 25 μL of poison gland hexane extract was loaded on the column. A hexane/acetone mixture was used as the mobile phase. The procedure consisted of following steps: 20/0, 4 mL; 19/1, 2 mL; 18/2, 2 mL; 17/3, 4 mL; and 17/3, 6 mL. Only the 6 mL eluent of the last step was collected. The second method was designed for the whole worker body extract. A 1.5 cm × 15.0 cm column (Wilmad LabGlass, Buena, NJ) packed with 3.0 g of silica gel was used. The column was first washed with 8 mL of hexane, and then, 200 μL of whole body extract was loaded. Again, the hexane/acetone mixture was used as the mobile phase. The procedure consisted of the following steps: 20/0, 8 mL; 19/1, 4 mL; 18/2, 4 mL; 17/3, 8 mL; and then 17/3, 12 mL. As above, only the 12 mL eluent of the last step was collected. The purified 2-methyl-6-alkyl or alkenyl-piperdines were obtained by washing the column in the first method with an extra 8 mL of acetone, after the column was washed using hexane/acetone mixtures.

$NaBH_4$ Reduction. Reduction of the C═N double bond in the piperideine ring generates the corresponding piperidine alkaloids. Such reduction can be achieved using $NaBH_4$ in ethanol (*6, 23*). Because the chemistry of fire ant piperidine alkaloids was well-defined, we suspected that $NaBH_4$ reduction products of the samples would be helpful in identifying piperideine alkaloids. The concentrated sample (1 mL) was transferred to a 2 mL vial. About 1.0 mg of $NaBH_4$ was added to the vial; then, the original solvent (hexane/acetone) was removed under air flow, and 0.2 mL of ethanol was immediately added into the vial. The vial was tightly closed and kept in a dark oven at 100 °C for 20 min. The ethanol was removed under air flow, and 200 μL of hexane/acetone (17:3) was immediately added into the vial. Finally, the product was transferred into a new 2 mL vial for gas chromatography−mass spectrometry (GC-MS) analyses.

GC-MS Analyses. Three GC-MS conditions were used. In condition 1, a Varian GC-MS system was used. It consisted of a CP-3800 gas

Table 1. RTs and RPAs for Piperideine Alkaloids in the Fire Ant Samples[a]

peak	compound	RT (min)	RPAs (%, mean ± SE)			
			mean	SE	min	max
1	2-methyl-6-tridecenyl-6-piperideine	11.15	3.31	2.13	0.28	13.88
2	2-methyl-6-tridecyl-6-piperideine	11.31	2.87	0.69	0.60	4.75
3	2-methyl-6-pentadecenyl-6-piperideine	12.59	39.24	4.81	27.00	59.02
4	2-methyl-6-pentadecyl-6-piperideine	12.84	45.34	4.69	22.28	52.73
5	2-methyl-6-heptadecenyl-6-piperideine	14.83	8.51	2.75	0.07	17.63
6	2-methyl-6-heptadecyl-6-piperideine	15.26	0.71	0.425	0.00	2.17

[a] Data were obtained under GC-MS condition 1. The RPA was calculated based on the data from six colonies ($n = 6$).

Table 2. RTs of Piperidine Alkaloids after $NaBH_4$ Reduction of Samples[a]

peak	compound	RT (min)
a	*cis*-2-methyl-6-tridecenylpiperidine	11.08
b	*cis*-2-methyl-6-tridecylpiperidine	11.25
c	*trans*-2-methyl-6-tridecenylpiperidine	11.29
d	*trans*-2-methyl-6-tridecylpiperidine	11.47
e	*cis*-2-methyl-6-petadecenylpiperidine	12.48
f	*cis*-2-methyl-6-pentadecylpiperidine	12.75
g	*trans*-2-methyl-6-pentadecenylpiperidine	12.82
h	*trans*-2-methyl-6-pentadecylpiperidine	13.08
i	*cis*-2-methyl-6-heptadecenylpiperidine	14.66
j	*cis*-2-methyl-6-heptadecylpiperidine	15.06
k	*trans*-2-methyl-6-heptadecenylpiperidine	15.18
l	*trans*-2-methyl-6-heptadecylpiperidine	15.61

[a] Data were obtained under GC-MS condition 1.

chromatograph and a Saturn 2000 mass selective detector, controlled by a Mass Spectrometry WorkStation Version 6.4.1 (Varian, Walnut Creek, CA). A 30 m × 0.25 mm i.d., 0.25 μm film thickness, DB-1 capillary column was used (J & W Scientific, Folsom, CA). The GC temperature program was as follows: The initial temperature was set at 50 °C, held for 1 min and increased to 250 °C at a rate of 20 °C/min, and held for 40 min. A 2 μL sample was injected at 250 °C with 10:1 split ratio. Helium was used as the carrier gas at a flow rate of 1.0 mL/min, and the transfer line temperature was set at 200 °C. In condition 2, Varian CP-3800 GC coupled to a Varian Saturn 2000 MS/MS was used. The GC was equipped with a 30 m × 0.25 mm i.d., 0.25 μm film thickness, DB-5 capillary column operated with the following conditions: A 1 μL sample was automatically injected in splitless mode at an injector temperature of 240 °C with a gradient oven temperature of 60−240 at 3 °C/min, held at 240 °C for 5 min; a 1.0 mL/min helium flow was used as the carrier gas, with a transfer line temperature of 170 °C. The MS range was from *m*/*z* 40 to 650, with a filament delay of 3 min, a target total ion chromatogram (TIC) of 20000, a prescan ionization time of 100 μs, an ion trap temperature of 150 °C, and a manifold temperature of 60 °C. In condition 3, an Agilent 6890 GC coupled to a mass selective detector was used. The GC was equipped with a 30 m × 0.25 mm i.d., 0.25 μm film thickness, HP-5 ms fused capillary column, operated using the following conditions: A 1 μL sample was automatically injected in splitless mode at an injector temperature of 240 °C; the oven temperature went from 60 to 170 at 8 °C/min, then to 250 at 5 °C/min, and was then held for 5 min; helium at flow rate of 1.0 mL/min was used as the carrier gas. The mass spectrometer was operated at 70 eV in the electron impact mode.

High-Resolution LC-MS (HRMS) Analysis. High-resolution mass spectra were obtained using an Agilent 1100 HPLC coupled to a JEOL AccuTOF (JMS-T100LC) (Peabody, MA). All isolated compounds and fractions were prepared in MeOH and injected directly into a 0.3 mL/min stream of MeOH. Twenty microliters of sample (approximately 0.1 mg/mL) was manually injected at 0.5 min, while mass drift compensation standards [L-tryptophan (negative ion), PEG (positive ion)] were injected at 1.5 min over the course of a 2 min run.

RESULTS AND DISCUSSION

GC-MS chromatograms of the concentrated fractions from the gravity column chromatography of poison glands and body extracts are shown in **Figure 1**. Six peaks showed a characteristic mass spectrum of a 2-methyl-6-alkyl or alkenyl-6-piperideines (**Figures 2** and **3**). Each mass spectrum had significant ions at *m*/*z* 96, 111, and 124. Three parent ions $[M + H]^+$ were detected at *m*/*z* 306.3131, 308.3279, and 334.3440, respectively, in the HRMS analysis. On the basis of the GC-MS data, it was confirmed that three ions in HRMS analysis were from compounds **3**, **4**, and **5**, respectively. HRMS analysis showed that the molecular formulas for compounds **3**, **4**, and **5** were $C_{21}H_{39}N$, $C_{21}H_{41}N$, and $C_{23}H_{43}N$, respectively. We were not able to obtain the HRMS data on the other three compounds, most likely due to not having sufficient compounds in those particular samples to generate workable signals.

Reduction of these new compounds with $NaBH_4$ generated all piperidine alkaloids found in the venom (**Figure 4**), indicating that they are structurally related to piperidine alkaloids of the red imported fire ants. Because of numerous investigations on the chemistry of fire ant piperidine alkaloids, chemical structures of piperidine alkaloids in fire ant venom and their GC behavior were well-defined (*6*, *24*). This provides an excellent opportunity to identify these new piperideine alkaloids. The identification of piperidine alkaloids was achieved by comparing their retention and mass spectra to those of purified piperidine alkaloids from fire ant extracts or synthetic standards.

On the basis of the information from mass spectra, GC RTs, molecular weights obtained from high resolution mass analysis, and profiles of $NaBH_4$ reduction products, we identified these piperideine alkaloids as 2-methyl-6-tridecenyl-6-piperideine (**1**), 2-methyl-6-tridecyl-6-piperideine (**2**), 2-methyl-6-pentadecenyl-6-piperideine (**3**), 2-methyl-6-pentadecyl-6-piperideine (**4**), 2-methyl-6-heptadecenyl-6-piperideine (**5**), and 2-methyl-6-heptadecyl-6-piperideine (**6**). The position of the double bond in the heptadecenyl substituent of 2-methyl-6-heptadecenyl-6-piperideine is unknown because information on the corresponding piperidine alkaloid was not available. Because only the *cis* double bond isomer of alkenyl-substituted piperidine alkaloids exists in fire ant venom, their corresponding piperideine alkaloids most likely are also *cis* isomers.

The RTs and relative peak areas (RPAs) of six piperideine alkaloids under GC conditions 1 are summarized in **Table 1**. For all six tested colonies, the peak of 2-methyl-6-pentadecyl-6-piperideine (**4**) or 2-methyl-6-pentadecenyl-6-piperideine (**3**) was the greatest. The peak of 2-methyl-6-heptadecenyl-6-piperideine (**5**) was always more intensive than 2-methyl-6-heptadecyl-6-piperideine (**6**). Because chemical profiles of venom alkaloids have been used as a taxonomic tool to separate red imported fire ants from their close relatives, the black imported fire ant, *Solenopsis richteri* Forel, and their hybrids (*21*), whether the piperideine alkaloid profiles are correlated to genetic makeup of those species is an interesting research subject.

The RTs of $NaBH_4$ reduction products under GC condition 1 are summarized in **Table 2**. They matched their corresponding

piperidine alkaloids in the purified fire ant venom. On the basis of our data, peak k in **Figure 4** is t*rans*-2-methyl-6-heptadecenylpiperidine, which is the *trans* product of $NaBH_4$ reduction on 2-methyl-6-heptadecenylpiperideine (**5**). This is in agreement with the literature (*25*).

Although the peaks of 2-methyl-6-tridecyl-6-piperideine (**2**) and 2-methyl-6-pentadecyl-6-piperideine (**4**) are overlapped with those of *trans*-2-methyl-6-tridecenylpiperidine and *trans*-2-methyl-6-pentadecenylpiperidine, respectively, some of the piperideine peaks were observed in many whole body hexane extractions even without any clean up, such as 2-methy-6-pentadecenyl-6-piperideine (**3**). Why previous research did not reveal piperideine alkaloids is unknown. In hexane body extracts, the ratio of peak area of 2-methy-6-pentadecenyl-6-piperideine (**3**) to that of 2-methy-6-pentadecenylpiperidine was 0.015 ± 0.002 (mean $\pm$ SE, $n = 6$). As compared to piperidine alkaloids, these piperideine alkaloids are definitely minor components in the fire ant venom; however, from a biological standpoint, minor compounds do not necessarily have only lesser functions.

Leclerq and colleagues proposed that reduction of piperideine to piperidine is the final step of biosynthesis of piperidine alkaloids (*26*). The result of this study may serve as evidence to support this biosynthetic pathway.

Piperideine alkaloids have been found in other *Solenopsis* ants, including 2-methyl-6-undecyl-1-piperideine from *Solenopsis xyloni* (*27*) and 2-(4-penten-1-yl)-1-piperideine from a Puerto Rican ant species (*6*). In a comparative study on alkaloidal components among *S. invicta*, *S. richteri*, *S. geminata*, and *S. xyloni*. Brand et al. (*27*) found 2-methyl-6-undecyl-1-piperideine in *S. xyloni*; however, they did not detect piperideine alkaloids in *S. invicta*. Jones et al. (*6*) reviewed the chemistry of the ant venom alkaloids from the genera *Solenopsis* and *Monomorium* and described several new alkaloids, including 2-(4-penten-1-yl)-1-piperideine extracted from a Puerto Rican ant species. Again, they did not detect piperideine alkaloids in *S. invicta*. 2-Methyl-6-undecyl-1-piperideine described by Brand et al. (*27*) was structurally different to the 2-methyl-6-alkyl-6-piperideines reported here, due to the different positions of the double bond in the piperideine ring. To our knowledge, 2-methyl-6-alkyl or alkenyl-6-piperideine alkaloids have never been reported in *S. invicta*.

Piperideine alkaloids also occur in plants, such as 2-methyl-6-(2-oxopropyl)-1-piperideine, 2-methyl-6-(2-hydroxypropyl)-1-piperideine, and 2-methyl-6-(1-propenyl)-6-piperideine in several *Pinus* species (*28*) and γ-coniceine in the poison Hemlock (*Conium maculatum* L.) (*29*) and several *Aloe* species (*30*, *31*). The poison Hemlock was used to execute Socrates in 399 BC, and its toxicity is due to piperidine and piperideine alkaloids. Coniine (2-propylpiperidine) and γ-coniceine (2-propyl-1-piperideine) are two representative compounds in poison Hemlock, and the latter was found to be more toxic to mice (*29*).

This study is the first to identify piperideine alkaloids in the venom of red imported fire ants. Knowledge and further study of the chemistry of venom are important to our understanding of not only human reactions to fire ant stings but also the general biology and biochemistry of the red imported fire ant.

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