

## Reduction of Venom Alkaloids in *Solenopsis richteri* × *Solenopsis invicta* Hybrid: An Attempt To Identify New Alkaloidal Components

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The alkaloid chemistry of the venom of hybrid fire ant, *Solenopsis richteri* × *Solenopsis invicta*, was investigated using silica gel chromatography and GC–MS techniques. In addition to most *cis* alkaloids of parental species, *S. richteri* Forel and *S. invicta* Buren, the *cis* alkaloid fraction of the body extract of hybrid fire ants also contains five significant new alkaloids. Hydrogenation of the *cis* alkaloid fraction yielded only five piperidines, **4'**, **12'**, **12**, **20'**, and **20**. Sodium borohydride and lithium aluminum hydride selectively reduced C=N double bond in piperidine alkaloids to give a mixture of *cis* and *trans* piperidines. However, reduction of the five new components yielded several new peaks with much longer retention times and increasing molecular weights over 30. It is evident that the chemical identities of the five new peaks are quite different from those known piperidines or piperidine derivatives found in *Solenopsis* fire ants.

**KEYWORDS:** Fire ant; *Solenopsis richteri*; *Solenopsis invicta*; venom; hybrid

### INTRODUCTION

It is well-known that the imported fire ant, *Solenopsis invicta*, produces characteristic 2,6-dialkylpiperidines in the venom (1–4). The alkyl or alkenyl side chain on position 6 of the piperidine ring has 11, 13, 15, or 17 carbons. A total of eight pairs of *cis* and *trans* 2-methyl-6-alkylpiperidines (or 2-methyl-6-alkenylpiperidines) (Figure 1) have been reported in this species and in the closely related *geminata* and *saevissima* groups (1, 4–6). In previous studies (5, 6), we have reported the first separation of the *cis* and *trans* stereoisomers of venom alkaloids of *Solenopsis* fire ants by silica gel column chromatography, and further separately presented the GC traces of the *cis* and *trans* alkaloids isolated from fire ant whole body. We also reported the identification of six 2,6-dialkyl- $\Delta^{1,2}$ -piperidine and seven 2,6-dialkyl- $\Delta^{1,6}$ -piperidine. Almost at the same time, a paper published by another group reported six 2,6-dialkyl- $\Delta^{1,6}$ -piperidine from the poison gland of *S. invicta* workers (7). Furthermore, except for the *cis* and *trans* alkaloids from *S. invicta* fire ant whole body extract, 2-methyl-6-(6-pentadecenyl)- $\Delta^{1,6}$ -piperidine and 2-methyl-6-*n*-pentadecyl- $\Delta^{1,6}$ -piperidine proved to elicit significant GC–EAD responses in phorid fly *Pseudacteon tricuspis*, a parasitoid of *Solenopsis* fire ant (8). These alkaloids might serve as host location cues for *P. tricuspis*.

In the *saevissima* complex, both the black imported fire ants, *S. richteri* Forel, and the red imported fire ants, *S. invicta* Buren, were accidentally introduced into the United States from South

America through the Port of Mobile, Alabama, during the first half of the 20th century (9). The red imported fire ants are currently distributed across much of the southern United States and California, whereas the black imported fire ants are confined to a very limited area in northeastern Mississippi and southwestern Tennessee because of displacement from its original range by its subsequently introduced congener (10). Both imported fire ant species are known to hybridize in many parts of the region, but the *S. richteri* × *S. invicta* hybrids occur together with both parental species only in the Mississippi hybrid zone, which consists of areas in northern Mississippi and Alabama (11, 12). Vander Meer et al. (13) first presented chemical evidence of the natural occurrence of hybridization between *S. richteri* and *S. invicta* as hybrids showed a GC pattern of alkaloids intermediate to that of both parental species (12–14). The venom alkaloid compositions characterized by the presence of various 2,6-disubstituted piperidines in amounts distinctive of the *cis* and *trans* forms show considerable interspecific variations (1, 4, 15, 16). In a recent study, Chen et al. (17) reported interspecific variations of the  $\Delta^{1,6}$ -piperidine in the imported fire ants very similar to those of the *trans* 2,6-dialkylpiperidines.

In this study, we reported on the venom alkaloid chemistry of *S. richteri* × *S. invicta* hybrids as well as the reduction of the *cis* alkaloids from hybrid fire ants. We also attempted to identify some new alkaloid components from hybrid fire ants.

### MATERIALS AND METHODS

**Source of Colonies.** Three collection trips were made in September 2007, March 2008, and June 2008 which yielded 56 hybrid (*S. richteri* × *S. invicta*) fire ant colonies (northern Alabama and northeastern

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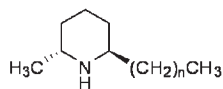
**trans 2-Methyl-6-alkylpiperidines**

Solenopsin A (4): n = 10

Solenopsin B (12): n = 12

Solenopsin C (20): n = 14

Solenopsin D (23): n = 16

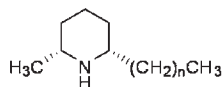
**cis 2-Methyl-6-alkenylpiperidines**

Isosolenopsin A (4'): n = 10

Isosolenopsin B (12'): n = 12

Isosolenopsin C (20'): n = 14

Isosolenopsin D: n = 16

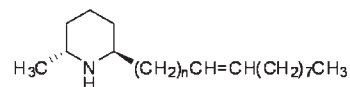
**trans 2-Methyl-6-alkylpiperidines**

Dehydrosolenopsin A (2): n = 1

Dehydrosolenopsin B (9): n = 3

Dehydrosolenopsin C (17): n = 5

Dehydrosolenopsin D (22): n = 7

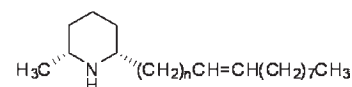
**cis 2-Methyl-6-alkenylpiperidines**

Dehydroisosolenopsin A: n = 1

Dehydroisosolenopsin B (9'): n = 3

Dehydroisosolenopsin C (17'): n = 5

Dehydroisosolenopsin D: n = 7



**Figure 1.** Chemical structures of piperidine alkaloids from *Solenopsis* fire ants.

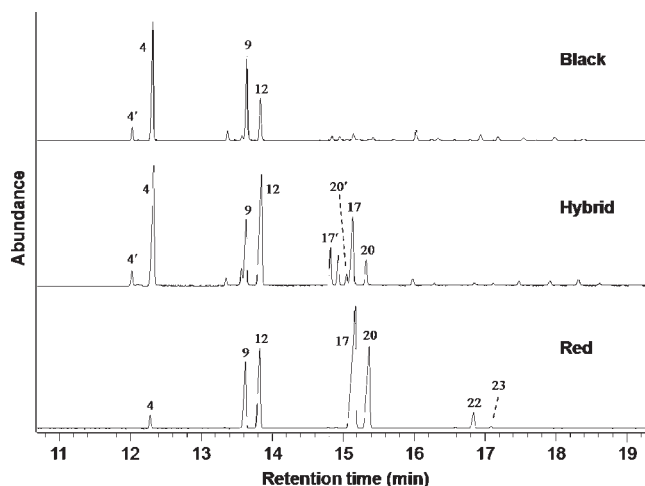
Mississippi where hybrids are known to occur almost exclusively). At the same time colonies of the black imported fire ant *S. richteri* (24 colonies from Tennessee) and the red imported fire ant *S. invicta* (12 colonies from Auburn University campus, Alabama) (shortened as “the black” and “the red”, respectively) were also collected for comparison with the hybrids. All colonies were maintained in 1-gallon plastic jars and were fed sugar water and crickets.

**Confirmation of Identity of Fire Ant Colonies.** About 50 worker ants from each colony were soaked in 1 mL of hexane for 2 h. One microliter of hexane extract was directly injected into a Shimadzu GC17A equipped with an Rtx-1MS column (30 m × 0.25 mm i.d., 0.25 μm, Restek, Bellefonte, PA). The GC oven was programmed at 15 °C/min from 90 to 270 °C with 2 min initial time and 16 min final holding time. Each colony was confirmed to be black, red or hybrid fire ants by using both alkaloid and hydrocarbon characters (13, 18).

**Isolation and Identification of Alkaloids.** Alkaloids were extracted and isolated from fire ant samples as previously described (5, 6). Briefly, the process involved extraction of ants by soaking them in hexane followed by silica gel chromatography to separate the extract into different fractions and thus to isolate the *cis* and *trans* venom alkaloids. Gas chromatography–mass spectrometry (GC–MS) analyses of alkaloid fractions were performed on an Agilent 7890A GC coupled to a 5975C mass selective detector, with a HP-5 ms capillary column (30 m × 0.25 mm i.d., 0.25 μm, Agilent, USA). The GC oven temperature was programmed from 90 °C (isothermal for 2 min) to 210 at 15 °C/min, then to 280 at 2 °C/min, and held for 10 min. The injection temperature was set at 270 °C, and the transfer line temperature was set at 280 °C. Alkaloids were identified by analysis of their mass spectra produced by EI (70 eV), as well as by comparison of characteristic peaks of the alkaloids in the black and the red fire ants (5, 6).

**Reduction of the *Cis* Alkaloid Fraction.** Several reduction methods, including hydrogenation catalyzed by 5% rhodium on charcoal or 10% palladium on charcoal, diisobutylaluminum hydride (DIBAH), sodium borohydride (NaBH<sub>4</sub>) and lithium aluminum hydride (LiAlH<sub>4</sub>), have been reported for the synthesis of *cis*- and *trans*-(±)-solenopsins (19). In a previous report, NaBH<sub>4</sub> in ethanol has been used in reduction of the C=N double bond in the piperidine ring to identify piperidine alkaloids from the *Solenopsis* fire ants (7). In this study, reduction of *cis* alkaloid fraction was achieved using hydrogenation, NaBH<sub>4</sub>, and LiAlH<sub>4</sub>.

The *cis* alkaloid fraction was divided into three parts for reduction reactions. After the solvent (hexane/acetone) of a portion of the *cis* alkaloid fraction was evaporated under a mild stream of nitrogen, 2 mL of anhydrous ethanol was immediately added to resolve *cis* alkaloids. Then Pd–C powder (10%, 10 mg) was added to the solution. The mixture was stirred under hydrogen atmosphere overnight. The ethanol was removed under nitrogen flow, and 2 mL of hexane was immediately added. The resulting mixture was loaded onto a glass pipet column (0.1 g silica gel, 300–400 mesh) eluting with 5.5 mL of hexane/acetone (10:1). The collected fractions were subjected to GC–MS analyses. A slightly different temperature increasing program was used. The GC oven temperature was programmed from 90 °C (isothermal for 1 min) to 190 at 10 °C/min, to 220 at 1 °C/min, then to 250 at 5 °C/min, and held for 3 min. The total run time was 50 min.



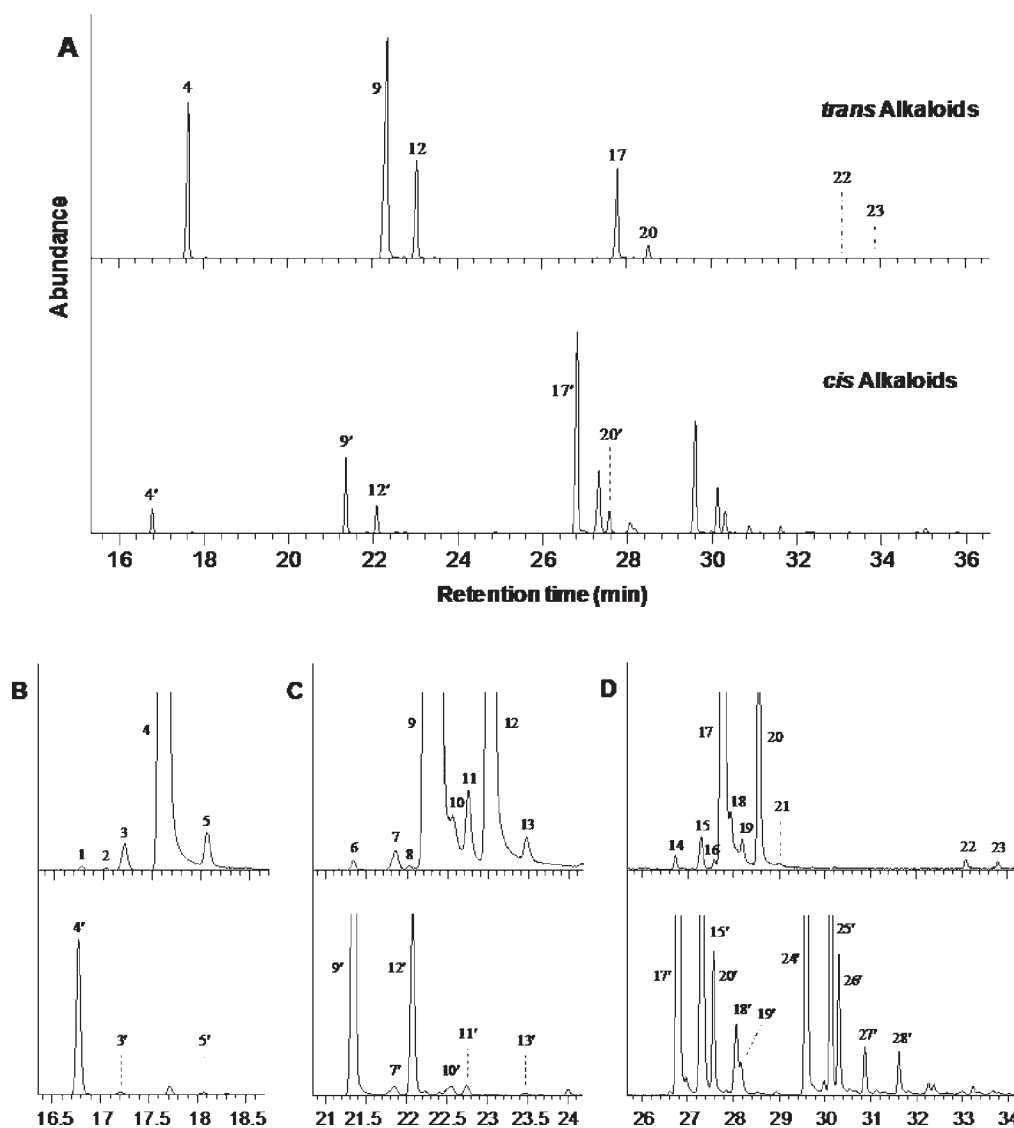
**Figure 2.** Typical GC traces for collected samples of black, red and hybrid fire ants.

In the same manner of hydrogenation, solvent of the second portion of the *cis* alkaloid fraction was removed under nitrogen flow prior to addition of 2 mL of anhydrous ethanol. About 6 mg of NaBH<sub>4</sub> was added. The reaction mixture was stirred at room temperature for 1 h. After workup with the same method as above, the resulting product was purified by column chromatography on silica gel and subjected to GC–MS analysis. The fraction containing reduced alkaloids was further subjected to workup by an acidification–basification method. The fraction was acidified by addition of two drops of 1 M HCl, and then the pH of the mixture was adjusted to 7–8 by addition of three drops of 1 N NaOH. The upper layer was dried over anhydrous MgSO<sub>4</sub>, purified by flash column chromatography as above, and subjected to GC–MS analysis again.

Solvent in the third portion of the *cis* alkaloid fraction was removed under nitrogen flow prior to reduction by LiAlH<sub>4</sub>, and dry THF (2 mL) was immediately added to resolve alkaloids. The *cis* alkaloid solution was added dropwise to a mixture of LiAlH<sub>4</sub> (8 mg) and dry THF (2 mL) at 0 °C. The reaction mixture was stirred overnight and quenched with two drops of 1 N NaOH. After removal of THF under nitrogen flow, 2 mL of hexane was immediately added. The resulting solution was dried over anhydrous MgSO<sub>4</sub>, purified by flash column chromatography as above, and then subjected to GC–MS analysis.

## RESULTS AND DISCUSSION

Typical GC traces of the collected fire ant samples are shown in **Figure 2**. Alkaloid peaks are significantly dominant in the hexane extracts of the fire ant colonies. The chemical identities of major peaks can be easily determined by comparing peak characteristics with previously published profiles of alkaloids of the two parental species since the chemical structures and GC profiles of piperidine



**Figure 3.** Typical GC traces of *cis* and *trans* alkaloid fractions from the hybrid: (A) visible peak area of GC chromatogram; (B–D) amplified GC peaks in (A).

and piperidine alkaloids in fire ant venom have been well-defined (1, 4–6, 14, 20). Dehydrosolenopsin B (9) and solenopsin B (12) are major peaks in all three fire ant species. In addition, the black fire ants contain solenopsin A (4) as major peak, and dehydrosolenopsin C (17), solenopsin C (20) as minor peaks, whereas the red fire ants contain 17 and 20 as major peaks, and 4 as minor peak. As compared to the black, peaks 17 and 20 of the hybrid are distinctive. The red fire ants also contain dehydrosolenopsin D (22) and solenopsin D (23) as minor peaks, which are detectable in some hybrid colonies. Clearly, the GC profile of the hybrid is intermediate to that of both parental species. The intermediate proportions of these *trans* alkaloids suggest additive inheritance or balanced ambidirectional dominance (21).

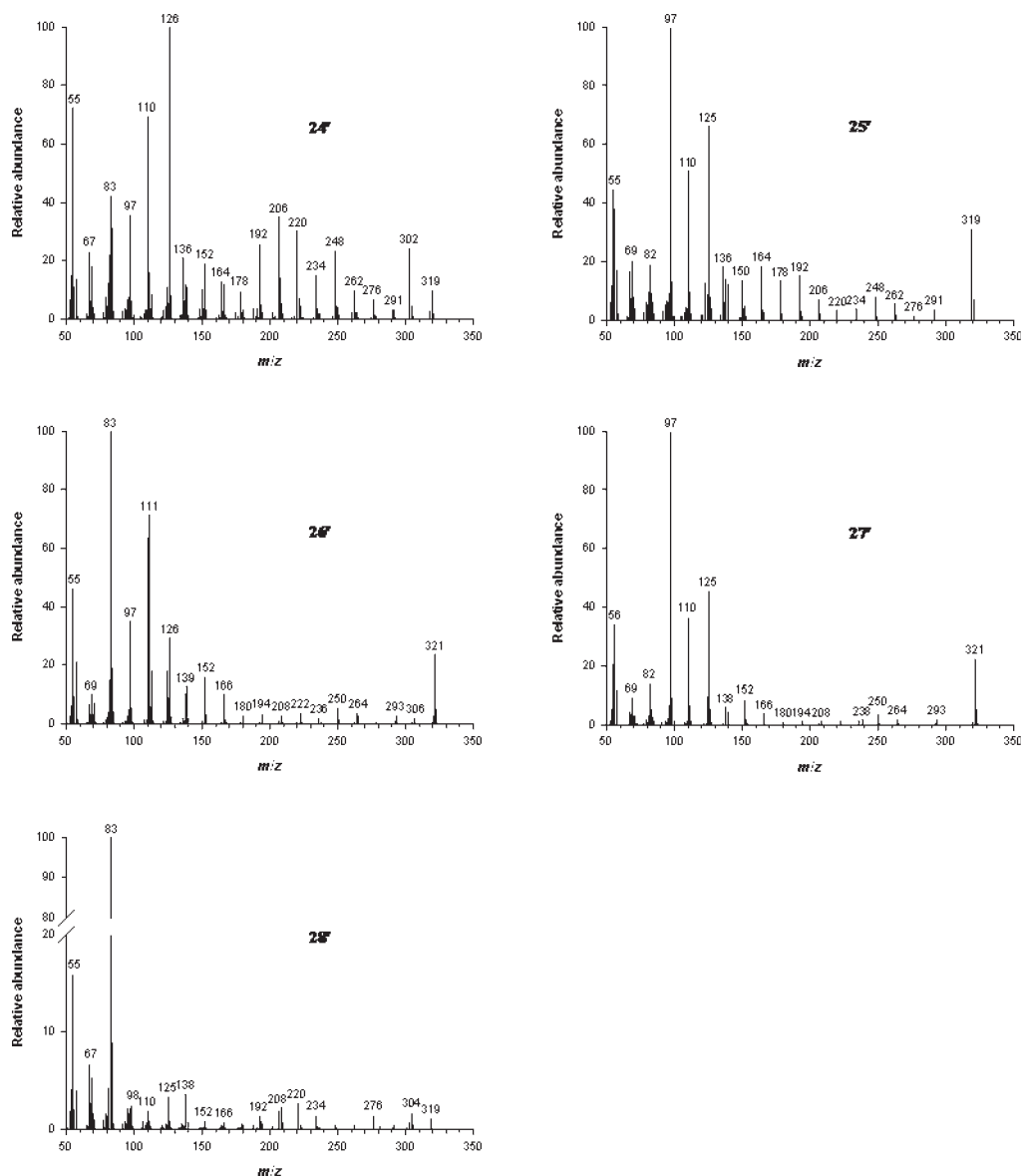
As previously reported (5, 6), silica gel chromatography allows separation of the two alkaloid fractions (*cis* and *trans* alkaloid fractions) from ant worker whole body extract. The chemical identities of major peaks in these two alkaloid fractions (Figure 3) can be easily determined by comparing with Figure 2. The chemical identities of the minor peaks in the *trans* alkaloid fraction of the hybrid are determined by comparing mass spectrum and retention time with those present in the black and the red (Figures 3B–D and Table 1). Peaks 3, 7, 10, 15, 18 are  $\Delta^{1,6}$ -2,6-dialkylpiperidines. Peaks 5, 11, 13, 19, 21 are  $\Delta^{1,2}$ -2,6-dialkylpiperidines. The mass spectra of some minor peaks, 1, 6, 8, 14,

and 16, which are also present in the black and the red have the same base peak  $m/z$  98. These peaks might be 2-methyl-6-alkyl- or -alkenyl-piperidines or -piperidines with minor differences in the side carbon chain. Some hybrid colonies contain peaks 22 and 23 in trace amount. Comparison of amplified GC profiles of venom alkaloids shows that the hybrid contains qualitatively similar minor peaks as both parental species. All the minor peaks match the peaks found in the *trans* alkaloid fraction of the red (6).

The GC profile of the *cis* alkaloid fraction of the hybrid shows similar intermediate characteristics to that of the *trans* alkaloid fraction. GC–MS analysis of the *cis* alkaloid fraction reveals that the hybrid has corresponding *cis* stereoisomers of major *trans* alkaloids (Figure 3 and Table 1). The *cis* alkaloid fraction of the hybrid has all the same peaks corresponding to 11-, 13-, 15-carbon-chain piperidines as of the red. The chemical identities of these components can be easily determined by comparing the mass spectrum and retention time with those in the two parental species. The mass spectra and retention times of *cis* alkaloids 4', 9', 12', 17', and 20' are identical to those in the black and the red. Dehydroisolenopsin D and isolenopsin D present in the red fire ants are not detectable in all analyzed hybrid colonies, and no other alkaloidal peaks with retention times corresponding to 17-carbon-chain piperidines are detected. Interestingly, there are five new peaks with retention times between those of the

Table 1. Chemical Identity of Known Alkaloids from *S. richteri* × *S. invicta*

<i>trans</i> Alkaloids			<i>cis</i> Alkaloids		
Peak	Configuration	Structure	Peak	Configuration	Structure
2	2 <i>R</i> ,6 <i>R</i>				
3	2 <i>R</i>		3'	2 <i>R</i>	
4	2 <i>R</i> ,6 <i>R</i>		4'	2 <i>R</i> ,6 <i>S</i>	
5	6 <i>R</i>		5'	6 <i>R</i>	
7	2 <i>R</i>		7'	2 <i>R</i>	
9	2 <i>R</i> ,6 <i>R</i>		9'	2 <i>R</i> ,6 <i>S</i>	
10	2 <i>R</i>		10'	2 <i>R</i>	
11	6 <i>R</i>		11'	6 <i>R</i>	
12	2 <i>R</i> ,6 <i>R</i>		12'	2 <i>R</i> ,6 <i>S</i>	
13	6 <i>R</i>		13'	6 <i>R</i>	
15	2 <i>R</i>		15'	2 <i>R</i>	
17	2 <i>R</i> ,6 <i>R</i>		17'	2 <i>R</i> ,6 <i>S</i>	
18	2 <i>R</i>		18'	2 <i>R</i>	
19	6 <i>R</i>		19'	6 <i>R</i>	
20	2 <i>R</i> ,6 <i>R</i>		20'	2 <i>R</i> ,6 <i>S</i>	
21	6 <i>R</i>				
22	2 <i>R</i> ,6 <i>R</i>				
23	2 <i>R</i> ,6 <i>R</i>				

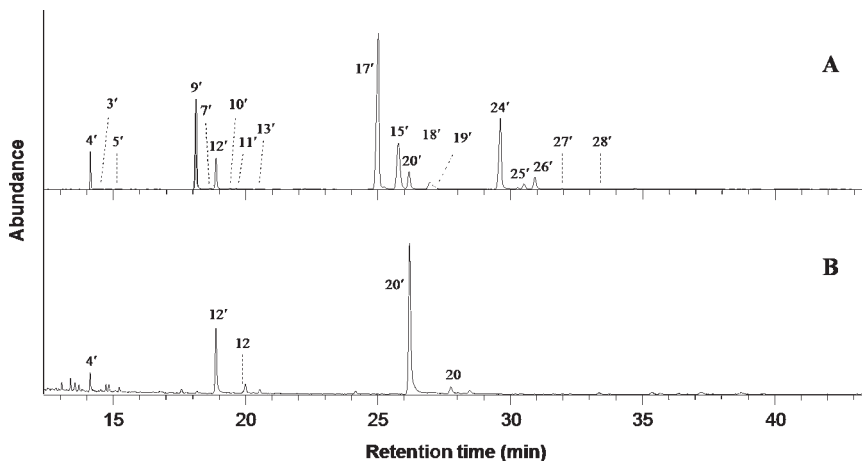


**Figure 4.** Mass spectra of the new alkaloids to be identified from the hybrid.

15-carbon-chain piperidines and the 17-carbon-chain piperidines present in the *cis* alkaloid fraction of the hybrid. All these new peaks (**24'**, **25'**, **26'**, **27'** and **28'**) have the same mass of either  $m/z$  319 or 321 (**Figure 4**). GC analyses of two additional hybrid colonies have confirmed that these peaks are exclusively present in the hybrid colonies, suggesting that the presence of these five new peaks can be used as a marker to differentiate hybrid from the two parental species. Previous reports have demonstrated that phorid fly *P. tricuspis* could distinguish among the black, red, and hybrid fire ants with greater preference for the red and hybrid (22), and that phorid fly proved to utilize fire ant venom alkaloids for host location (8). It is likely that these new alkaloids in hybrid fire ants might be used by parasitic phorid fly to differentiate hybrid from the two parental species for host preference. From the mass spectra and GC retention times of these five new peaks, we presume that they are 16-carbon-chain piperidines. As the GC behavior of **24'**, **25'**, **26'**, and **27'** is very similar to that of respectively **17'**, **15'**, **20'**, and **18'**, we initially considered them as corresponding homologues with only the difference of a  $\text{CH}_2$  in the side chain. However, none of the five new peaks has a similar mass spectrum to peaks of 17-carbon-chain piperidines. The mass spectrum of peak **24'** is quite special.

Two intense peaks,  $m/z$  110 and 126, exclude a saturated piperidine ring, and probably reveal an unsaturated piperidine ring with 2-ethyl substitution instead of 2-methyl substitution. The molecular ion of odd mass 319 of peak **24'** indicates a possible unsaturated piperidine ring with a monounsaturated side chain.

The spectra of **25'** and **27'** show three intense peaks:  $m/z$  97 (base peak), 110, 125 (**Figure 4**), which probably indicate a double bond on a 5- or 6-membered ring, i.e.,  $\Delta^{1,2}$ -2-ethyl-5-alkylpyrroline or  $\Delta^{1,2}$ -2-methyl-6-alkylpiperideine. The 5-membered ring structure is favored because the series of peaks at P-28 (291 for **25'** and 293 for **27'**) involves cleavage on the side of the 2-ethylpyrroline ring with loss of an ethyl group, whereas 2-methylpiperideine ring would undoubtedly lose a methyl group. It is evident that peaks at P-15 are absent for the mass spectra of **25'** and **27'**. The molecular ion of odd mass 319 of **25'** indicates a monounsaturated side chain. A similar structure, 2-ethyl-5-undecyl- $\Delta^{1,2}$ -pyrroline, which was identified from an undescribed Australian *Monomorium* species (23), has important mass peak ions at  $m/z$  96, 97 (base peak), 110, 111, and 124. The mass spectrometric data of this 2-ethyl- $\Delta^{1,2}$ -pyrroline from the Australian *Monomorium* ant may be helpful to some degree in our identification of **25'** and **27'**.



**Figure 5.** Characteristic GC traces of palladium-catalyzed hydrogenation of the *cis* alkaloid fraction: (A) the *cis* alkaloid fraction; (B) hydrogenolysis product.

The mass spectra of **26'** and **28'** are completely different from all alkaloids identified from *Solenopsis* fire ants (**Figure 4**). The base peak at  $m/z$  83 and two intense ions at  $m/z$  97, 111 in the spectra of **26'** indicate a favorable 5-membered pyrrolidine ring with a double bond, presumably a 2-methyl- $\Delta^{1,2}$ -pyrroline. Furthermore, base peak  $m/z$  83 of **26'** is in agreement with base peak  $m/z$  97 of 2-ethyl-5-undecyl- $\Delta^{1,2}$ -pyrroline with difference of a  $\text{CH}_2$ . 2-Ethyl-5-pentyl- $\Delta^{1,2}$ -pyrroline identified from South African fire ant (*Solenopsis punctaticeps*), however, has intense mass peaks  $m/z$  82, 96, 97, 110, 111 with  $m/z$  96 as base peak (**24**). Similarly, the base peak at  $m/z$  83 and two weak ions at  $m/z$  98, 110 in **28'** suggest the absence of a double bond in the pyrrolidine ring. The mass spectrum of **28'** rules out a double bond in the pyrrolidine ring as there is no intense mass peak other than base peak  $m/z$  83. In addition, a weak molecular ion at  $m/z$  319 in the spectrum of **28'** suggests the presence of two double bonds in the side carbon chain. We consider **28'** as a 2-methyl-5-alkylpyrrolidine instead of 2-alkylpiperidine (i.e., without 2-methyl) since an  $m/z$  84 base peak has been reported for 2-pentylpiperidine (**25**).

All double bonds in the piperidine ring and side carbon chain can be readily reduced by palladium catalyzed hydrogenation at room temperature. As shown in **Figure 5**, palladium-catalyzed hydrogenation of the *cis* alkaloid fraction generated five piperidine alkaloids found in the venom, **4'**, **12'**, **12**, **20'**, and **20**. All peaks with double bonds in the piperidine ring and/or in side carbon chain, **3'**, **5'**, **7'**, **9'**, **10'**, **11'**, **13'**, **15'**, **17'**, **18'**, and **19'**, disappeared, suggesting that all double bonds in the piperidine ring and side carbon chain were reduced to give corresponding saturated piperidine alkaloids. The presence of the two minor peaks, **12** and **20**, suggests that reduction of piperidine ring by hydrogenolysis gave a mixture of *cis* and *trans* piperidines. Hydrogenation of the *cis* alkaloid fraction caused disappearance of the five new peaks. However, we failed to detect any related peak in all fractions of the hydrogenolysis product. A reasonable interpretation is that the reduced products of these new alkaloids are somewhat more volatile and evaporate into air during the workup of the product before flash silica gel chromatography.

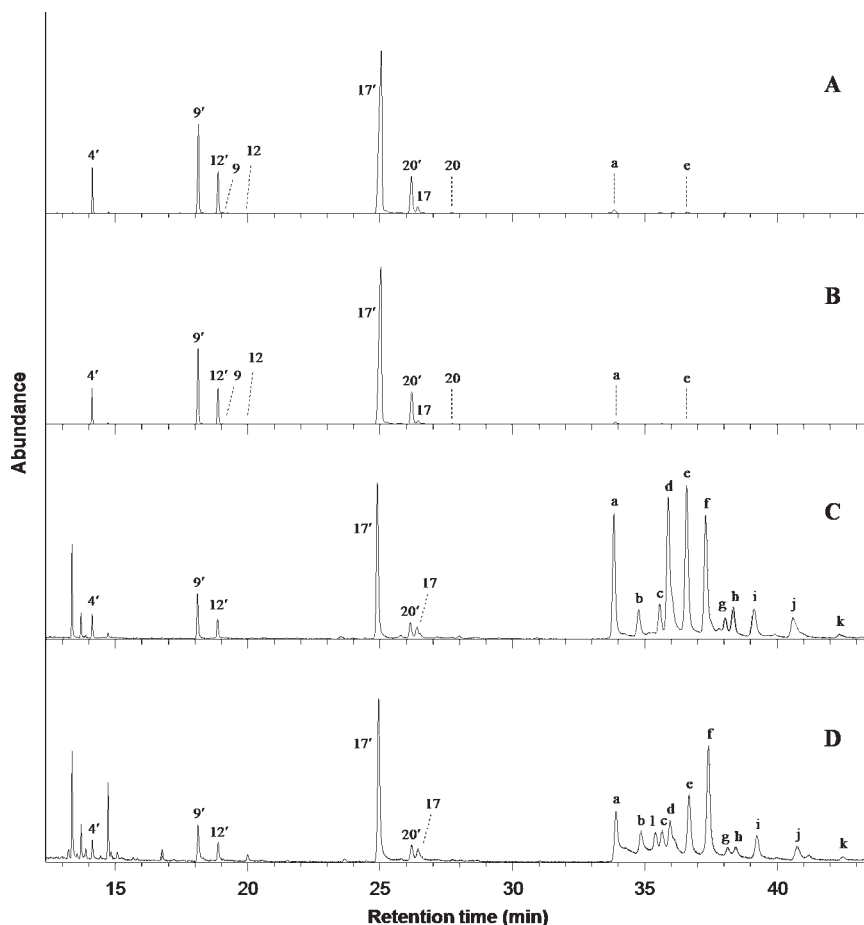
As different from the palladium catalyzed hydrogenation that reduce all double bonds,  $\text{NaBH}_4$  and  $\text{LiAlH}_4$  can selectively reduce  $\text{C}=\text{N}$  double bond without any influence on  $\text{C}=\text{C}$  double bond in the side carbon chain of fire ant venom alkaloids. Previous synthesis work has shown that reduction of piperidine with  $\text{NaBH}_4$  yielded an 80:20 mixture of *cis* and *trans* piperidine (**26**) and that reduction of piperidine with  $\text{LiAlH}_4$  yielded an 67:33 mixture of *cis* and *trans* piperidine (**27**). Two fractions containing piperidine alkaloids were obtained from the *cis*

alkaloid fraction treated with  $\text{NaBH}_4$  in ethanol. All peaks having  $\text{C}=\text{N}$  double bond in the piperidine ring, **3'**, **5'**, **7'**, **10'**, **11'**, **13'**, **15'**, **18'**, and **19'**, could be reduced by  $\text{NaBH}_4$  in ethanol to yield of a mixture of *cis* and *trans* piperidines (**Figure 6**). In comparison with corresponding *cis* stereoisomers, *trans* alkaloids **9**, **12**, **17**, and **20** were obtained in much less amounts, suggesting that peak **15'** was mainly reduced to *cis* isomer (peak **17'**). Treatment of the *cis* alkaloid fraction with  $\text{NaBH}_4$  caused disappearance of the five new peaks. It is very interesting that about eleven visible peaks appeared with retention times beyond the five new peaks. After treatment of the two fractions with HCl and then NaOH, no qualitative or proportional changes of known piperidine peaks were observed. However, acidification and then basification of the two fractions did cause a proportional decrease of peaks **a**, **d**, **e** and occurrence of a new peak **l**.

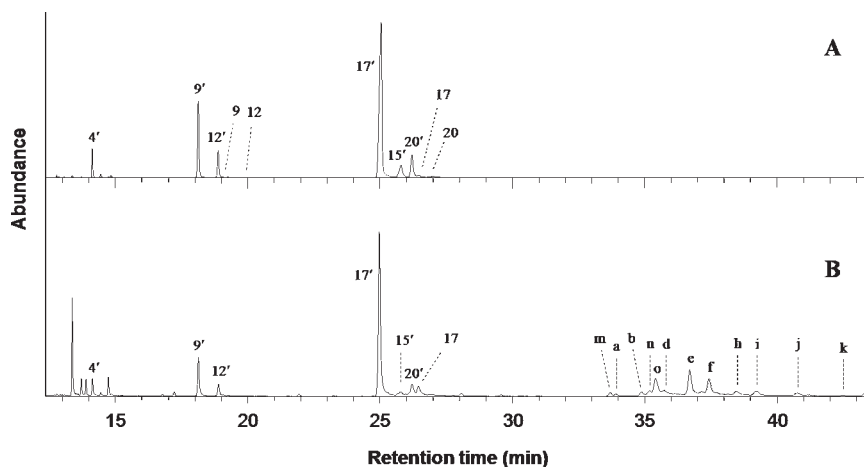
When reacted with  $\text{LiAlH}_4$ , all peaks with  $\text{C}=\text{N}$  double bond in the piperidine ring, **3'**, **5'**, **7'**, **10'**, **11'**, **13'**, **15'**, **18'**, and **19'**, in the *cis* alkaloid fraction were reduced to yield *cis* and *trans* piperidine alkaloids (**Figure 7**). Probably due to insufficient amount of  $\text{LiAlH}_4$  added to the *cis* alkaloid fraction, piperidine **15'** was partially reduced. We observed some new peaks the same as those found in the  $\text{NaBH}_4$  reduced product including peaks **a**, **b**, **d**, **e**, **f**, **h**, **i**, **j**, **k**. Three new peaks **m**, **n**, **o** were present, but peaks **c**, **g** were absent in the  $\text{LiAlH}_4$  reduced product.

Mass spectra of several unknown major peaks present in the  $\text{NaBH}_4$  and  $\text{LiAlH}_4$  reduced products were shown in **Figure 8**. Base peak ions at  $m/z$  84, 97, 98, 111, 125 in the mass spectra of these peaks are typical ions for 5- or 6-membered *N*-heterocyclic ring, which can be found in pyrrolidine and piperidine alkaloids. The relatively abundant peaks **a**, **d**, **e**, **f**, **l**, **o**, were presumably converted from the three major peaks (**24'**, **25'** and **26'**) in the *cis* alkaloid fraction.

Given that the five new peaks were  $\text{C}=\text{N}$  containing alkaloids, reduction of these components in the *cis* alkaloid fraction could provide additional information on their chemical identities. For instance, base peak ion at  $m/z$  84 might indicate a 2-methyl pyrrolidine ring in peak **o**, indirectly supporting a 2-methyl pyrroline ring in peak **26'**. Reduction of known alkaloidal components containing double bond(s) showed that the molecular weights of reduction product normally had an increase of 2 or 4. However, the molecular weights of new peaks obtained from the reduction by  $\text{NaBH}_4$  or  $\text{LiAlH}_4$  increased over 30. Furthermore, treatment of the two fractions obtained from  $\text{NaBH}_4$  reduction product with HCl and then NaOH did not cause either qualitative or proportional changes of known piperidine peaks. It is evident that the chemical identities of the five new peaks are



**Figure 6.** Characteristic GC traces of  $\text{NaBH}_4$  reduction products: (A) the first alkaloid-containing fraction; (B) the first alkaloid-containing fraction after acidification and then basification; (C) the second alkaloid-containing fraction; (D) the second alkaloid-containing fraction after acidification and then basification.



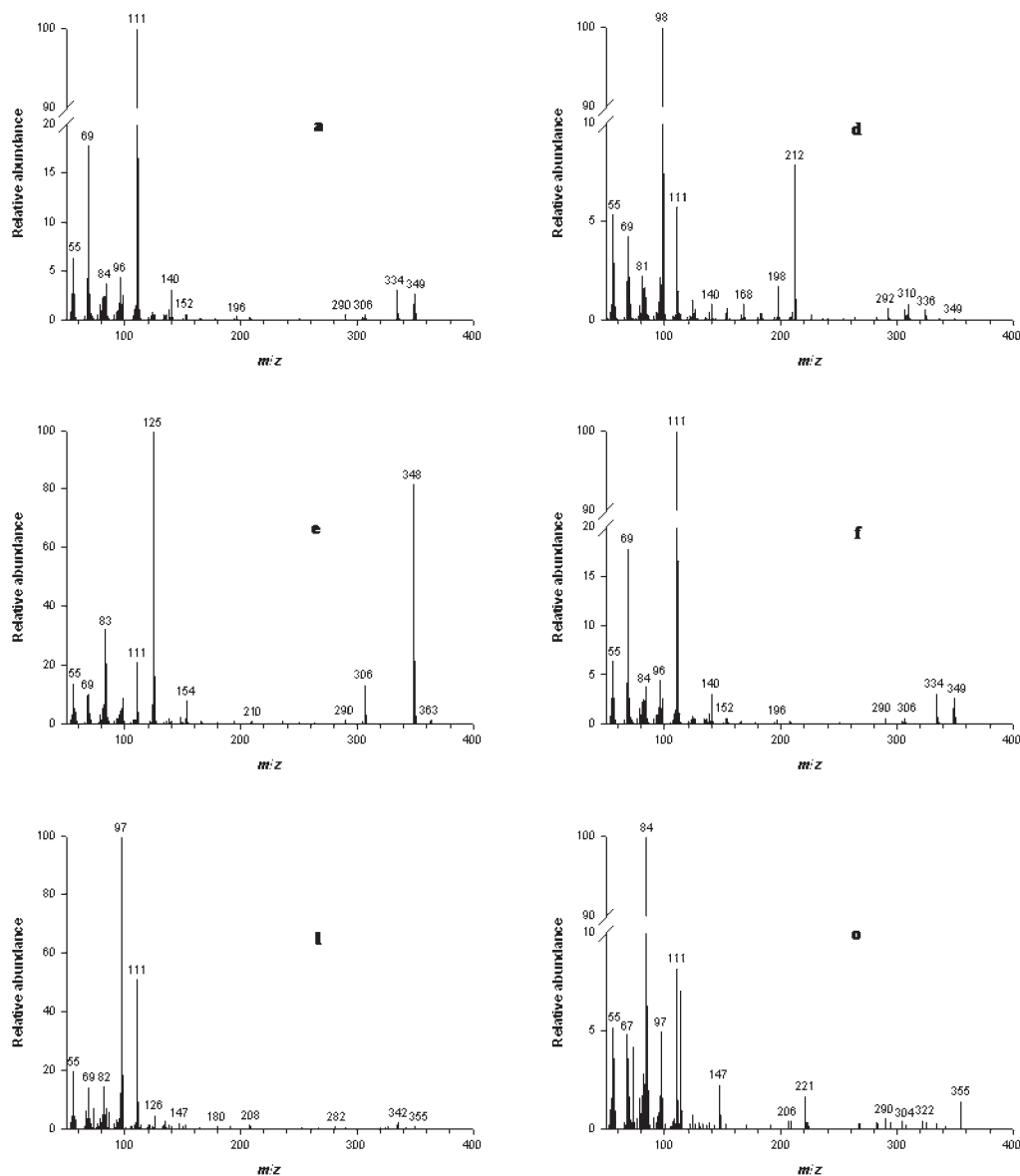
**Figure 7.** Characteristic GC traces of  $\text{LiAlH}_4$  reduction products: (A) the first alkaloid-containing fraction; (B) the second alkaloid-containing fraction.

quite different from those known piperidines or piperideines. Further studies on the identification of these new alkaloids and their biological significances in fire ant hybridization are needed.

We hypothesized in previous reports that  $\Delta^{1,2}$ -piperideines and  $\Delta^{1,6}$ -piperideines could be precursors of *cis* and *trans* alkaloids in fire ant venom (5, 6). As our data unambiguously showed the existence of these two types of piperideines, both parent species and their hybrid may utilize the same biosynthetic pathway to produce both *cis* and *trans* alkaloids and even maintain favorable stereochemical composition diverging rather markedly from the thermodynamic equilibrium mixture. Furthermore, the red fire

ants presumably have elaborated specific enzymes that could synthesize piperidines with longer and unsaturated side chains associated with greater toxicity and biological advantage. It seems likely that hybrid fire ants have inherited these features as evident by the increase in the proportions of dehydrosolenopsin C and solenopsin C as compared to the black fire ants. Thus, the hybrids could be considered as relatively more closely related to the red than to the black fire ants (4).

A diversity of pyrrolidines and pyrrolines have been identified as venom constituents of ant species in the genera *Solenopsis* and *Monomorium* (25). The *Solenopsis* species of which venom



**Figure 8.** Mass spectra of major peaks in  $\text{NaBH}_4$  and  $\text{LiAlH}_4$  reduction products.

contains pyrrolidines and pyrrolines are members of the subgenus *Diplorhoptrum*, and many species are known as thief ants. These *Solenopsis* species, such as South African fire ant *S. punctaticeps*, do not belong to the group of true fire ants that are primarily limited in their distributions to the New World tropics. As  $\Delta^{1,2}$ -piperidine and  $\Delta^{1,6}$ -piperidine are probable biosynthetic precursors of *cis* and *trans* alkaloids in fire ant venom (5, 6),  $\Delta^{1,2}$ -pyrrolines and  $\Delta^{1,5}$ -pyrrolines could be precursors of 2,5-dialkylpyrrolidines present in the venom of *S. punctaticeps*. We can further hypothesize that these pyrrolines and piperidines may originate from the same or homologous precursors by cyclization to different positions of a double bond. Since dialkylpiperidines are particularly characteristic of fire ant workers and queens in the genus *Solenopsis*, 2,5-dialkylpyrrolidines and -pyrrolines have not been reported in true fire ants to date. The possible presence of 2,5-dialkylpyrrolidines and -pyrrolines in hybrid fire ants (the five new peaks, **24'**, **25'**, **26'**, **27'** and **28'**) suggests that *Solenopsis* fire ants have evolved biosynthesis pathway of venom alkaloids from 5-membered-ring pyrrolidines to 6-membered-ring piperidines.

There are different castes in social insects. The secondary chemistry of hybrid individuals of social insects becomes

more complicated. The alkaloidal chemistry of hybrid fire ant individuals in the same caste may vary qualitatively and quantitatively from each other. It is necessary to define a hybrid population at the colony level. Based on alkaloidal chemistry, some hybrid colonies are closer to *S. richteri*, while others are closer to *S. invicta*. To define a hybrid colony correctly, the chemical character of cuticular hydrocarbons is needed in addition to that of venom alkaloids (12, 14).

By analyzing the chemistry of samples collected four decades ago, Vander Meer et al. (14) concluded that hybridization has been occurring for a long time wherever the two parental species met. The patterns of hybrid venom alkaloid chemistry observed in the present study are very similar to previous reports in the 1980s (11–14, 28), suggesting that hybridization of the two parental species and subsequent backcrossing with the parental sexuals may not have caused a change in alkaloid chemistry. The alkaloid chemistry of the hybrid might have been well maintained by hybrid viability (12), while the hybrid zone was moving northwestward to northern parts of Alabama and Mississippi. Alkaloid chemistry may be an important component of hybrid fitness and influence speciation and introgression. Future genetics studies on hybridization between the two parent fire ant species



and subsequent investigation of alkaloid chemistry may provide insights into the evolutionary nature of the hybridization between two parental species.

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