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THE MAJOR COMPONENT OF THE TRAIL PHEROMONE OF THE LEAF-CUTTING ANT, *Atta sexdens rubropilosa* FOREL

3-Ethyl-2,5-Dimethylpyrazine

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Abstract—The major component of the trail pheromone of the South American leaf-cutting ant, *Atta sexdens rubropilosa* Forel, is 3-ethyl-2,5-dimethylpyrazine (II). Methyl and ethyl phenylacetate and methyl 4-methylpyrrole-2-carboxylate (I), which is the major component of the trail pheromone of *A. texana* (Buckley) and *A. cephalotes* (L.), were also identified and may be minor components. The pheromone is stored in the poison gland. *Atta sexdens sexdens* (L.) also responds strongly to the pyrazine, which in large amounts evokes a weak response from *A. texana*, *A. cephalotes*, and *Acromyrmex octospinosus* (Reich). Foraging workers of *Atta sexdens rubropilosa* did not preferentially pick up baits impregnated with the pyrazine. The pyrazine was puffed into the nests of *A. cephalotes*, and a particular response called "milling" was noted.

Key Words—Trail pheromone, 3-ethyl-2,5-dimethylpyrazine, leaf-cutting ants, *Atta sexdens*, *Atta cephalotes*, foraging, bait pickup.

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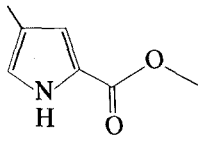
INTRODUCTION

The leaf-cutting ants, typified by the genera *Atta* and *Acromyrmex*, forage along trails defined at least in part by trail pheromones. The trails lead to sources of vegetation that the ants cut and use to cultivate the fungus they eat. In 1964, Blum et al. identified the poison gland as the source of the trail pheromone in some members of the tribe Attini, and later a highly active component, methyl 4-methylpyrrole-2-carboxylate (I) was identified from crushed abdomens of *Atta texana* (Buckley) (Tumlinson et al., 1971) and synthesized (Sonnet, 1972). The same component is also utilized by *A. cephalotes* (L.) (Riley et al., 1974). Robinson et al. (1974) found that *Acromyrmex octospinosus* (Reich)[7] would also follow a trail of I, but *Atta sexdens* (L.) would not. "Lost" workers of *A. sexdens* (ants that had fallen off the nest table and were thus highly motivated to follow a trail) followed trails made from the crushed poison glands of *A. cephalotes* and *A. texana* but not of *Acromyrmex*



I

octospinosus. Given a choice of two such trails, *Atta sexdens*, *A. cephalotes*, and *Acromyrmex octospinosus* generally preferred their own, although *Atta cephalotes* did not discriminate between its trail pheromone and that of *A. sexdens* (Robinson et al., 1974). In the field, ants usually follow trails of their own species, even when those of another species intersect them. For example, workers of *Acromyrmex octospinosus* placed on an *Atta cephalotes* trail invariably left the trail and resumed trail following only after crossing an *Acromyrmex octospinosus* trail (Blum et al., 1964). These results indicate that although similarities exist in the poison sac contents and trails of several species (e.g., the pyrrole), the ants can normally discriminate between trails. To gain a clearer understanding of the similarities and differences between attine trail pheromones, we chose to study the pheromone of *Atta sexdens rubropilosa* Forel.

Atta sexdens is the third most widely distributed attine species (Cherrett and Peregrine, 1976) and is often an agricultural pest. Control is best achieved by a slow-acting pesticide formulated in a bait that foraging ants will pick up and carry into the nest; the ants' activities eventually spread the pesticide throughout the colony.

Cherrett and Lewis (1974) have given a brief history of the development of toxic baits to control leaf-cutting ants. Practical baiting schemes are in op-

[7]Pyrrole I has since been identified in this species (J.H. Cross, R.M. Silverstein, and J.M. Cherrett, unpublished).

eration (Robinson and Aranda, 1975), and Lewis (1973) has shown that blanket spreading of toxic baits is the most effective way to control certain attine species. However, the wisdom of large scale baiting has been questioned (Anon., 1970; Edwards, 1970; Ehrlich and Ehrlich, 1970), since blanket spreading of insecticides can lead to undesirable side effects. Any improvements in the bait that increased bait pickup would make possible the use of smaller quantities of insecticide, thus reducing the amount spread into the environment, the danger to wildlife, and the cost.

Robinson and Cherrett (1973) found that, in the laboratory, citrus pulp baits and sugar impregnated filter paper disks containing the synthetic pyrrole were more readily picked up by *A. sexdens rubropilosa* than controls without the pyrrole, but later field tests were disappointing (unpublished). We reasoned that the authentic *A. sexdens rubropilosa* trail pheromone components might enhance bait pickup by this species.

METHODS AND MATERIALS

The *A. sexdens rubropilosa* ants were obtained from two locations, Rio de Janeiro, Brazil, and Asunción, Paraguay. Although morphological differences were noted between the two populations, Dr. C.F. Gonçalves, the taxonomist who last revised the genus *Acromyrmex*, considered them to be within the same subspecies (Gonçalves, personal communication to S.W.R.). The *A. sexdens sexdens*, *A. cephalotes*, *Acromyrmex octospinosus*, and *A. subterraneus* ants were from colonies maintained in the Bangor laboratory. The *Atta texana* ants were from laboratory colonies of Dr. John C. Moser, USDA-FS, Pineville, Louisiana.

The abdomens were removed from workers of *A. sexdens rubropilosa* and were macerated in methylene chloride (CH_2Cl_2). The supernatant was concentrated and sealed in glass ampoules, and these or the abdomens preserved in CH_2Cl_2 were sent to the Syracuse laboratory. The heads and thoraces of the Brazilian ants were analyzed for the alarm pheromones, which are produced in the mandibular glands (de Oliveira, 1975).

The remaining CH_2Cl_2 was removed in a short-path still and the viscous extract was distilled onto a dry-ice-cooled cold-finger condenser for 3 hr at 70°/0.5 mm Hg. The distillate was washed from the condenser with hexane, and this solution was fractionated on the following GLC columns: column A, 5% SE-30 on Chromosorb G 60/80 mesh, 2.5 m × 4 mm (ID) glass, 50 ml/min He flow rate at 75°C initial temperature for 6 min then programed at 4°/min to 250°C (ethyl phenylacetate retention time = 28 min); column B, 5% Carbowax 20 M on Chromosorb G 60/80 mesh, 5 m × 4 mm (ID) glass, 50 ml/min He flow rate at 170° isothermal (ethyl phenylacetate retention time = 36 min); column C, 5% diethylene glycol succinate (DEGS) on Chromosorb G 60/80 mesh, 5 m × 4 mm (ID) glass, 70 ml/min He flow at 150°C isothermal (methyl phenylacetate retention time = 47 min); column D, 5% Apiezon L on

Chromosorb G 60/80 mesh, 5 m × 4 mm (ID) glass, 50 ml/min He flow at 165°C isothermal (retention time of II = 14.5 min). All glass columns and solid supports were acid washed and treated with dichlorodimethylsilane. Fractions from the Varian model 204B gas chromatograph were collected in glass capillary tubes (30 cm × 2 mm OD) in a thermal gradient collector (Brownlee and Silverstein, 1968).

Nuclear magnetic resonance spectra were obtained ($CDCl_3$, TMS) on a Varian XL 100 (Fourier transform) spectrometer. Infrared spectra were obtained from samples dissolved in spectrograde carbon tetrachloride or carbon disulfide on a Perkin Elmer model 621 double-beam, grating spectrometer equipped with beam condensers and Barnes Engineering 4-μl cavity cells. Mass spectra were obtained on an Hitachi RMU-6 electron impact (70 eV) mass spectrometer, a modification of which allowed introduction of the glass capillaries used with the gas chromatograph.

Commercial methyl and ethyl phenylacetate were washed with $NaHCO_3$ to remove phenylacetic acid and distilled under vacuum. Methyl 4-methylpyrrole-2-carboxylate(I), synthesized by Sonnet (1972), had a melting point of 70–71°C and gave a single peak when chromatographed on column A. A 1:1 mixture of 3-ethyl-2,5-dimethylpyrazine(II) and 2-ethyl-3,5-dimethylpyrazine(III) was a gift of Pyrazine Specialties, Atlanta, Georgia, and was used as received, or the isomers were separated preparatively on column D.

The isolation of the pheromone components was monitored by bioassays based on the ability of the ants to follow a circular trail. Partially purified fractions were diluted with hexane to concentrations of 10, 1, and 0.1 mg whole ant equivalents/μl, and 10, 15, or 20 μl of each concentration was streaked with a microsyringe around a circle of 40–50 cm circumference penciled on white paper. Hexane controls were employed. The paper was then introduced to the ants and their responses were noted (Riley et al., 1974; Moser and Blum, 1963); trail-following behavior was distinct from alarm or food-investigating behavior. Some variations in bioassay practice occurred between laboratories, especially in respect to the origin and numbers of the ants and the manner in which they were introduced to the trail. Strongly active fractions and pyrazine II always elicited a marked effect that was reproducible when tests were repeated by more than one laboratory.

RESULTS

Isolation and Identification of the Pheromone Components

Isolation was started on a crude extract obtained from 4.2 kg (≃280,000) of whole ants from the Brazilian population. Bioassays of this extract and the short-path distillate (one unknown concentration of each) gave results recorded as "positive." A bioassay of one concentration of the distillation resi-

due gave a result recorded as "weakly positive"; considering our later results, we cannot state with certainty whether a nonvolatile component remained in the distillation flask (see Discussion). A single concentration of the distilled CH_2Cl_2 had no activity, and the wash of a liquid-nitrogen-cooled trap from the short-path distillation was inactive in all three standard concentrations (10, 1, and 0.1 mg whole ant equivalents/μl); it seems unlikely that an important component of the pheromone was highly volatile.

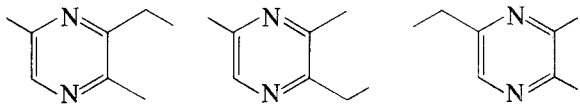
An aliquot of the short-path distillate was collected from column A as a single fraction, which was strongly active in all three standard concentrations. Thus, the pheromone was not thermally labile. The remainder of the short-path distillate was collected in five fractions. The first and fifth fractions were inactive in all three concentrations. The second and fourth displayed weak activity in the two higher concentrations but were inactive in the lowest. The third fraction was "strongly positive" in all three concentrations and was "still weakly positive" after dilution to 0.0001 mg whole ant equivalents/μl. This fraction was then collected in 10 fractions (3-1 through 3-10) from column B. Bioassays (two replicates, but at the same concentration) showed that fractions 3-3 and 3-7 were the most active. Fractions 3-6 and 3-8 also gave good responses; fractions 3-4, 3-5, and 3-9 were weakly active and were not examined further. Fraction 3-3 was fractionated into ten parts (3-3-1 through 3-3-10) on column C. Fraction 3-3-5 was the most active, but an insufficient amount was available for identification. Further fractionation (column C) of fraction 3-6 yielded three compounds, none of which was present in sufficient quantity to identify. Fractions 3-7 and 3-8 were chromatographed on column C; both produced single peaks, which were identified as methyl and ethyl phenylacetate by their NMR and mass spectra and by comparison with authentic samples. The pyrrole I was eluted from column B in fraction 3-10 (retention time = 81 min). The mass spectrum was congruent with that of the synthetic pyrrole. We estimated that 75 μg of methyl phenylacetate (MPA), 150 μg of ethyl phenylacetate (EPA), and 19 μg of pyrrole I were present in the 4.2 kg of whole ants. The synthetic compounds elicited weak to medium trail following from the Brazilian population but only investigatory behavior from the Paraguayan population.

Work was then continued on a second crude extract (4.8 kg of whole ants) of the Brazilian population. Fraction 3 from column A was again considerably more active at all three concentrations than the second and fourth fractions, and it was divided on column B, as reproducibly as possible, into the 10 fractions collected earlier. These fractions (three concentrations) were sent for bioassay against both the Brazilian and Paraguayan populations; both populations responded strongly to a single fraction, 3-2, which had been inactive in the earlier bioassays. The Brazilian population did show a "slight interest" in the fraction that should have contained methyl and ethyl phenylacetate; fractions 3-7 and 3-8 were collected together, because fraction 3-7 (MPA) was

much smaller than fraction 3-8 (EPA) and otherwise much of it would have been lost. Fractions 3-3 and 3-6 were completely inactive.

When refractionated on column D, fraction 3-2 proved to consist mostly of one compound, labeled fraction 3-2-2. The smaller peaks eluting before and after were collected and labeled fractions 3-2-1 and 3-2-3. All three fractions elicited strong trail-following from both the Brazilian and Paraguayan populations, although 3-2-1 and 3-2-3 were tested at much higher concentrations (300 mg/ml vs. 10 mg/ml). Chromatography on a very polar cyanosilicone column, Apolar 10C, failed to split fraction 3-2-2 further. Fractions 3-2-2 from the first and second shipments were identical and were combined to obtain the spectroscopic data.

That fraction 3-2-2 was an isomer of ethyldimethylpyrazine was readily deduced from its NMR and mass spectra. The NMR spectrum (Figure 1, top) showed two methyl groups on an aromatic ring (2.48δ, s, 3H and 2.52δ, s, 3H), an ethyl group on an aromatic ring (2.8δ, q, 2H and 1.27δ, t, 3H), and a single aromatic proton at 8.1δ. The mass spectrum (Figure 1, bottom) agreed with those published for 3-ethyl-2,5-dimethylpyrazine(II) and 2-ethyl-3,5-dimethylpyrazine(III) (Stenhagen et al., 1969; Goldman et al., 1967; and Friedel et al., 1971).



II III IV

The substitution pattern of the aromatic ring was determined from the fingerprint region of the IR spectrum by comparison with authentic samples of II and III. The IR spectrum of authentic II agreed well with that of fraction 3-2-2 (Figure 2), while that of authentic III was noticeably different. The published absorptions of IV are much different (Gelas and Rambaud, 1968). Coinjection of fraction 3-2-2 and authentic II on columns B and D produced on each column a single peak (retention times: column B, 9.7 min and column D, 14.5 min), but coinjection with III gave two peaks on each column (retention times: column B, 9.7 min and 10.3 min; column D, 16.5 min and 17.3 min; II eluted first). Column A (SE-30, 135° isothermal) would not separate isomers II and III, although Friedel et al. (1971) have reported the separation of III and IV on SE-30 and Carbowax 20 M packed columns; IV eluted after III. We concluded that fraction 3-2-2 was II. The quantity of II in the original 4.2-kg sample of ants was about 100 μg.

We collected the compounds in fractions 3-2-1 and 3-2-3 individually from column D. Two were present in quantities estimated to be 10% of pyrazine II, and the other seven were estimated to be between 3 and 7%. Although

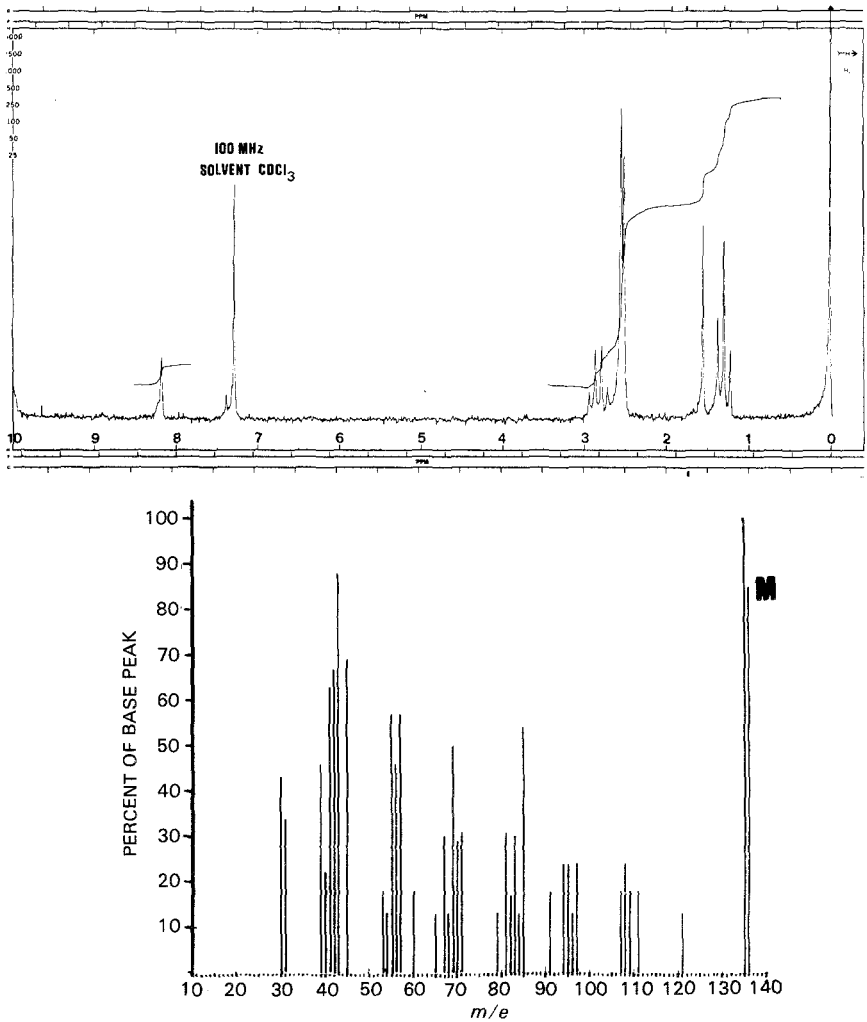


FIG. 1. NMR and mass spectra of fraction 3-2-2 (3-ethyl-2,5-dimethylpyrazine). H_2O peak at 1.56δ.

one appeared to be a methyl ester, none was identified. We were unable to confirm or refute the presence of other pyrazines.

Two samples of the Paraguayan population of *A. sexdens rubropilosa* were worked up as described and examined for the trail pheromone components. Pyrazine II and ethyl phenylacetate were found, but methyl phenylacetate and pyrrole I were not detected.

To test whether or not the main trail pheromone components were now isolated, we prepared from the Paraguayan population a bioassay set consist-

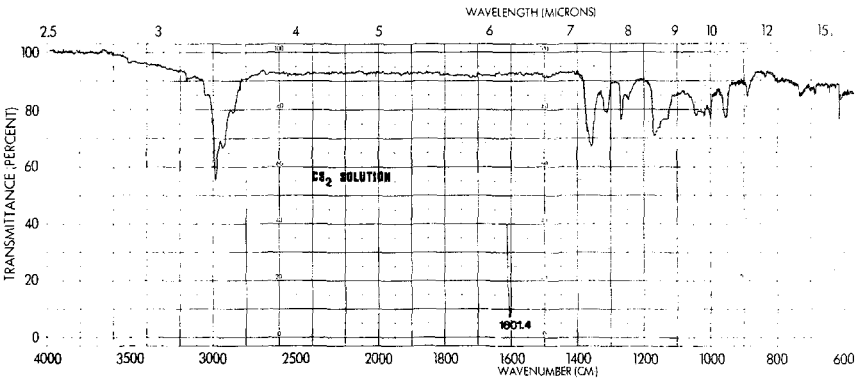


FIG. 2. Infrared spectrum of fraction 3-2-2 (3-ethyl-2,5-dimethylpyrazine).

ing of three samples: the cold-finger condensate, the pyrazine region from column D, and the total material eluted from column D minus the pyrazine region. Surprisingly the cold-finger condensate proved to be inactive. The pyrazine region from column D was moderately active at 10 mg/μl and weakly active at 1 mg/μl in both populations. The Paraguayan population also responded weakly at 0.1 mg/μl. The remainder of the effluent from column D elicited no response from the Paraguayan population and only a weak response at 10 mg/μl from the Brazilian population. These results indicated that no other compound had an activity equal to that of the pyrazine. The question of synergistic relationships was not resolved because of the inactivity of the cold-finger condensate (see Discussion).

Bioassays of the Synthetic Compounds

Synthetic pyrazines II and III were bioassayed separately, and II was obviously the more active (Tables 1 and 2, columns 6 and 7; Table 3, columns 2 and 3). Since pyrazine IV was neither found in the ant nor readily available, it was not tested. The commercially available 1:1 mixture of II and III is as active as II alone within limits of the bioassays; therefore III is not an inhibitor of the natural isomer (Tables 1 and 2, columns 6 and 8). MPA, EPA, and pyrrole I, singly or in combination, elicited weak and often variable responses. One solution (1:1:1), tested in Paraguay and Brazil, gave positive results (Tables 1 and 2, columns 5), but was much less active than the pyrazine. This solution and the pyrazine II did not interact synergistically when tested against the Paraguayan or the Brazilian populations (Tables 1 and 2, columns 5 and 6 or 8 vs. 9). Additive effects would probably not be detected with this bioassay. Other, independently prepared solutions of MPA, EPA, and pyrrole I were completely inactive in numerous bioassays conducted in Paraguay. Solutions prepared in the ratio found in the short-path distillate, I = 1, MPA = 4 and

TABLE 1. LABORATORY BIOASSAYS OF *Atta sexdens rubropilosa* COMPOUNDS AGAINST THE BRAZILIAN FIELD POPULATION

Quantity[a,b] (ng)	Compounds tested[c]								
	MPA	EPA	I	MPA + EPA + I (1:1:1)	II	III	II + III (1:1)	MPA + EPA + I + (II + III) (1:1:1:1)	I + MPA + EPA (1:4:7.5)
10^4	0	0	0	0	0	0	0	W	0
10^3	0	0	0	0	0	0	M	M	0
10^2	0	I	0	W	W	0	S	S	0
10^1	I	I	0	M	S	0	M	S	I
10^0	0	I	0	M	S	0	I	M	0
10^{-1}	0	0	0	W	M	0	0	W	0
10^{-2}	0	0	0	0	W	0	0	I	0
10^{-3}	—	—	—	—	0	0	—	0	—
10^{-4}	—	—	—	—	—	—	—	0	—

[a]Total quantity of chemical used to make a 50-cm-circumference circle.
[b]In mixtures, the quantity is that of each compound.
[c]Fifteen ants/test. 0 = no response; I = investigation; W = weak following (a few ants); M = medium following (<50%); S = strong following (>50%).

TABLE 2. LABORATORY BIOASSAYS OF *Atta sexdens rubropilosa* COMPOUNDS AGAINST THE PARAGUAYAN FIELD POPULATION

Quantity[a,b] (ng)	Compounds tested[c]							
	I	I + MPA (1:1)	I + MPA + EPA 1:4:7.5	I + MPA + EPA 1:1:1	II	III	II + III (1:1)	I + II + EPA + MPA (1:1:1:1)
0, control	0	0	0	0	0	0	0	0
10^5	0	—	R	—	—	—	—	—
10^4	0	W	R	W	0	0	W	0
10^3	0	W	0	M	M	0	M	M
10^2	0	W	W	M	S	0	S	S
10^1	0	W	0	M	S	0	S	S
10^0	0	W	0	0	S	0	W	S
10^{-1}	—	—	0	0	W	0	0	W
10^{-2}	—	—	—	0	0	0	0	0

[a] Total quantity used to make 50-cm-circumference circle.
[b] The quantity is that of each compound.
[c] Responses scored as in Table 1. R = repellent.

TABLE 3. LABORATORY BIOASSAYS OF *Atta sexdens rubropilosa* COMPOUNDS AGAINST LABORATORY COLONIES OF THE PARAGUAYAN FIELD POPULATION

Quantity[a] (ng)	Compounds tested		
	II	III	I + II + MPA + EPA (1:1:1:1)
10^2	9/40[b]	5/60	—
10^0	34/40	10/40	37/60
10^{-2}	29/40	5/60	49/60

[a]Total quantity of each compound used to make a 40-cm-circumference circle.
[b]Number of ants following trail/number of ants tested.

EPA = 7.5, were less active in both populations than the 1:1:1 mixture (Table 1, column 10; Table 2, column 4). They did not elicit trail-following, but the largest quantities tested repelled the Paraguayan population.

Pretreatment of Ants

For the bioassays conducted in Paraguay, ants were normally taken from the field and kept for 2–8 hr with nothing but water before any experiments were run. The ants were then calm and relatively easy to handle during the experiments. If left for a longer period, they became quite clearly agitated and anxious to follow a trail. In these circumstances the ants seemed to be willing to follow a trail of anything that was not actually repellent or to follow trails of the trail pheromone component II at much lower concentrations than they normally would (Table 4).

Source of Pheromone

Poison sacs and Dufours glands from *A. sexdens rubropilosa* were dissected out, separated, placed on the tips of sharpened match sticks and drawn along the 40-cm circumference of a circle (1 gland/circle). Four replicates with 10 ants each were made with Dufour's glands and three replicates were made with poison sacs. No ant followed the Dufour's gland trail for 90°, but 73% of them followed the poison sac trail for at least 90°.

Responses of Other Species in the Laboratory Bioassay

The responses of several attine species and subspecies to the commercial pyrazine mixture (1:1 ratio of II and III) are shown in Table 5. Table 6 shows responses of *A. texana* to the *A. sexdens rubropilosa* compounds. In addition, *Acromyrmex landolti* collected and tested in Paraguay responded strongly to a 1:1:1:1 solution of MPA, EPA, I, and the commercial pyrazine mixture; we did not bioassay I and II separately for this species.

TABLE 4. EFFECT OF LENGTH OF PRETREATMENT ON RESPONSES OF *Atta sexdens rubropilosa*, PARAGUAYAN FIELD POPULATION

Quantity[a] (ng)	Compounds tested[b]					
	II + III (1:1)			I + MPA + EPA (1:1:1)	EPA	I
0, control	0	0	0	0	0	0
10^5	—	—	—	—	R	—
10^4	W	—	—	—	WR	M
10^3	W	—	0	—	S	—
10^2	M	0	0	W	S	S
10^1	M	W	W	M	M	—
10^0	W	S	S	S	M	S
10^{-1}	0	M	S	S	M	—
10^{-2}	0	S	S	M	W	S
10^{-3}	0	S	S	W	—	—
10^{-4}	0	S	M	—	—	—
Length of pretreatment (hr)	2[c]	36[c,d]	36[d]	20	30	30

[a] Total quantity of each component used to make a 50-cm-circumference circle.
[b] Responses scored as in Table 1.
[c] Same solutions.
[d] Same ants.

TABLE 5. EFFECT OF COMMERCIAL PYRAZINE MIXTURE (II AND III) ON TRAIL-FOLLOWING OF SEVERAL ATTINE SPECIES IN THE LABORATORY

Quantity of each compound	% of ants following through 90°[a]				
	Atta sexdens rubropilosa	*Atta sexdens sexdens*	*Atta cephalotes*	*Acromyrmex octospinosus*	*Acromyrmex subterraneus*
50 ng	100.0	95.0	93.3	93.3	100.0
0.5 ng	100.0	95.0	0	0	Not measured
5 pg	33.3	15.0	0	0	Not measured

[a] Six replicates of 10 ants at each point, except for the grass-cutter, *Acromyrmex subterraneus*—1 test of 10 ants.

TABLE 6. LABORATORY BIOASSAYS OF *Atta sexdens rubropilosa* COMPOUNDS ON *Atta texana*[a]

Component	Concentration (ng/μl)[b]		
	0.04	0.4	4.0
Methyl phenylacetate	0	0	0
Ethyl phenylacetate	0	0	0
3-Ethyl-2,5-dimethylpyrazine (II)	++	+	+R
2-Ethyl-3,5-dimethylpyrazine (III)	0	—	0
Methyl 4-methylpyrrole-2-carboxylate (I)[c]	++++	—	—

[a]Bioassays by John Moser, USDA-FS, Pineville, Louisiana. The modified "lost ant" procedure was described by P.E. Sonnet and J.C. Moser, 1973 (*Environ. Entomol.* 2:851-854).
[b]++++ = very strong; ++ = weak but definite; + = weak; 0 = no response; R = repellent; — = not tested.
[c]The major component of the trail pheromone of this species.

Response of Field Nests of Atta cephalotes to Pyrazine Vapor

When the vapor of pyrrole I was blown into nest entrances of *Atta cephalotes* just before they were to begin their daily period of foraging, it triggered a foraging response (Robinson and Cherrett, 1975). This test was repeated using pyrazine vapor. One mg of the commercial pyrazine mixture was placed on a small wad of cotton wool in a glass container, and air was passed over this and into an entrance of a nest of *A. cephalotes* for 15 sec. Behavior was assessed after a further 300 sec. Control experiments were performed by passing air into an entrance for 15 sec. The pyrazines caused the ants to leave the nest entrance holes and to mill around on the nest surface. The response was statistically significant (Table 7).

Bait Pickup

Soybean baits, to which soybean oil with and without the commercial pyrazine mixture had been added, were dyed for identification and the baits

TABLE 7. FIELD RESPONSES OF *Atta cephalotes* TO VAPOR OF II AND III BLOWN INTO THE NEST ENTRANCE[a]

Test	Number of nests "milling"	Number of nests not "milling"
II + III	22	17
Air (control)	4	32

[a]$\chi^2 = 15.02$ with 1 *df*, $P < 0.001$

TABLE 8. PICKUP OF PYRAZINE-IMPREGNATED BAIT BY *Atta sexdens rubropilosa* IN THE FIELD IN PARAGUAY

Concentration of mixture of II + III (ppm)	Number of trials	Total number of bait pieces carried into nest (%)		P^a
		Tests with II + III	Control	
1	11	146 (26)	416 (74)	<0.001
10^{-2}	7	113 (49)	117 (51)	—
10^{-4}	11	175 (50)	174 (50)	—

[a] χ^2 test with 1 *df*.

were scattered together in areas where *Atta sexdens* ants were foraging. Individual bait pieces were counted as they were carried into the nest (Table 8). The pyrazine was repellent at a high concentration and is not shown to enhance pickup at lower concentrations.

DISCUSSION

Pyrazine II consistently elicits trail following activity. The Brazilian and Paraguayan populations of *A. sexdens rubropilosa* and a laboratory colony of *A. sexdens sexdens* are capable of detecting very small quantities of it (Tables 1 through 5). No other component or mixture is as active. This species is also more sensitive to it than are several closely related species (Tables 5 and 6). These results are good evidence that II is a major component of the pheromone.

It should be emphasized that a considerable quantity of II was present in the first shipment of Brazilian ants, although the fraction that contained it was inactive in the bioassay. Six other fractions, including those with MPA and EPA, were active. An explanation that fits these results is that these samples were made with relatively large quantities of material. Tables 1, 2, and 3 show that in large quantities the pyrazine is inactive but the minor components are active. By this reasoning, the bioassay samples made from the second shipment were made with smaller amounts of material and only the pyrazine was active. In retrospect, it is apparent that trail substances should be tested over a wide range of concentrations.

The results in Table 4 show that the ants' responses could be influenced by the time for which they were held before bioassay. When the time was long, the ants responded strongly to compounds that wider experience showed to be less important. This factor, and perhaps other undiscovered ones, caused some variability in our results. For an example, one may compare two bioas-

says of the commercial pyrazine mixture (Table 2, column 8 with Table 4, column 2).

The inactivity of the cold-finger condensate resulted from the preparation of the samples, since it was inactive in two independent bioassays. It may have been due to the concentration phenomenon discussed above, although three concentrations were tested. Nevertheless, the pyrazine-containing fraction isolated from this condensate was active. This fraction and the total-minus-pyrazine fraction were collected from the same chromatographic injection; their relative activities were probably correct.

As is the case with the other attine species examined (Blum et al., 1964), the source of the trail marking pheromone is the poison sac. Blum (1974) has postulated that the trail pheromones of myrmicine ants were originally trace constituents of the venom that were exploited as the function of the gland changed. The poison sac contents are rich in amino acids, which probably can react enzymatically to form pyrazines (cf. Maga and Sizer, 1973). Pyrazines have been identified previously in ants. They function as alarm pheromones in certain species of ponerine ants (Wheeler and Blum, 1973) and have been identified in the heads of the Argentine ant, *Iridomyrmex humilis* Mayr (Dolichodinae) (Cavill and Houghton, 1974).

The role of MPA, EPA, pyrrole I, and the compounds in the other less active fractions is not clear. They are active only in relatively large quantities and usually elicit weak or medium responses. Until the identification of the pyrazine, which consistently elicits strong responses, we thought we might be dealing with two subspecies of *A. sexdens*. The compounds may be minor constituents of the trail pheromone. These eusocial ants rely heavily on pheromones to regulate their colonies and may be able to take advantage of the increased information available from multicomponent pheromones (Silverstein and Young, 1976). The observation (Blum et al., 1964) that *A. cephalotes* and *Acromyrmex octospinosus* avoided each other's trails suggests that these species use multicomponent pheromones, because for both species pyrrole I is a major component (Riley et al., 1974; J.H. Cross, R.M. Silverstein, and J.M. Cherrett, unpublished). The work of Robinson et al. (1974), described in the introduction, supports this hypothesis also.

On the other hand, these compounds may simply be recognized by the ants as being from their species; the parent acid of MPA and EPA, phenylacetic acid, has been identified in *Atta sexdens* (Schildknecht and Koob, 1971) and is apparently associated with anabiosis.

We conducted two behavioral studies with the pyrazine. *A. cephalotes* uses pyrrole I as the major component of its trail pheromone (Riley et al., 1974). Robinson and Cherrett (1975) showed that I also releases foraging activity in a nest whose workers are preparing to forage. Since *A. cephalotes* can detect the pyrazine (Table 5), we repeated the experiment with the commercial pyrazine mixture. The ants responded to it by leaving the nest entrance

holes and actively milling around on the nest surface. Foraging behavior, however, was not observed. We cannot reach a firm conclusion about this response, because the pyrazine has not been sought in this species.

In the second study, bait pickup with and without the pyrazine was measured. Robinson and Cherrett (1973) had found that *A. sexdens* in the laboratory would more readily pick up baits that contained pyrrole I. In the field, however, Robinson (unpublished) observed no difference. Since discrepancies between laboratory and field results are often due to the use of imperfect pheromone compositions, we repeated the bait pickup experiment after confirming the activities of pyrazine II and the commercially available mixture of II and III, which was used for the experiment. The results in Table 8 demonstrate that the pyrazines did not enhance bait pickup, and in fact, larger quantities caused decreased pickup. This may be related to the fact that the ants do not follow trails made with large quantities of II. It seems unlikely that the results would differ if the experiment were repeated with pure II.

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REFERENCES

ANON. 1970. Fighting the fire ant; toxic side effects from mirex. Time 96(2 Nov), p. 40.

BLUM, M.S., MOSER, J.C., and CODERO, A.D. 1964. Chemical releasers of social behavior. II. Source and specificity of the odor trail substances in four attine genera (Hymenoptera: Formicidae). *Psyche J. Entomol.* 71:1-7.

BLUM, M.S. 1974. Pheromonal sociality in the Hymenoptera, pp. 222-247, *in* M.S. Birch (ed.). Pheromones. American Elsevier, New York.

BROWNLEE, R.G., and SILVERSTEIN, R.M. 1968. A micro-preparative gas chromatograph and a modified carbon skeleton determinator. *Anal. Chem.* 40:2077-2079.

CAVILL, G.W.K., and HOUGHTON, E. 1974. Volatile constituents of the Argentine ant, *Iridomyrmex humilis*, *J. Insect Physiol.* 20:2049-2059.

CHERRETT, J.M., and LEWIS, T. 1974. Control of insects by exploiting their behavior. pp. 130-146, *in* D. Price-Jones and M.E. Solomon (eds.). Biology in Pest and Disease Control. Blackwell Scientific Publications, Oxford.

CHERRETT, J.M., and PEREGRINE, D.J. 1976. A review of the status of leaf-cutting ants and their control. *Ann. Appl. Biol.* 84:124-128.

EDWARDS, C.A. 1970. Persistent pesticides in the environment. *Crit. Rev. Environ. Contr.* 1:7-67.

EHRLICH, P.R. and EHRLICH, A.H. 1970. Population, Resources, Environment: Issues in Human Ecology. W.H. Freeman, San Francisco, 383 pp.

FRIEDEL, P., KRAMPL, V., RADFORD, T., RENNER, J.A., SHEPHARD, F.W., and GIANTURCO, M.A. 1971. Some constituents of the aroma complex of coffee. *J. Agric. Food Chem.* 19: 530-532.

GELAS, J., and RAMBAUD, R. 1968. Action de l'ethyl-lithium sur les dimethyl-pyrazines. *C.R. Hebd. Seances Acad. Sci. Ser. C* 266:625-627.

GOLDMAN, I.M., SEIBL, J., FLAMENT, I., GAUTSCHI, F., WINTER, M., WILLHALM, B., and STOLL, M. 1967. 69. Recherches sur les aromes: Sur l'arome de cafe, II pyrazines et pyridines, *Helv. Chem. Acta* 50:694-705.

LEWIS, T. 1973. Aerial baiting to control leaf-cutting ants (Formicidae: Attini) in Trinidad. II. Field application, nest mortality and the effect on other animals. *Bull. Entomol. Res.* 63: 275-287.

MAGA, J.A., and SIZER, C.E. 1973. Pyrazines in foods. *Crit. Rev. Food Technol.* 4(1):39-115.

MOSER, J.C., and BLUM, M.S. 1963. Trail marking substance of the Texas leaf-cutting ant: Source and potency. *Science* 140:1228.

OLIVEIRA, JOÃO SABINO DE. 1975. Estudo dos Feromonios de *Atta sexdens rubropilosa Atta robusta.* MS thesis, Universidade Federal do Rio de Janeiro.

RILEY, R.G., SILVERSTEIN, R.M., CARROLL, B., and CARROLL, R. 1974. Methyl 4-methylpyrrole-2-carboxylate: A volatile trail pheromone from the leaf-cutting ant, *Atta cephalotes. J Insect Physiol.* 20:651-654.

ROBINSON, S.W., and CHERRETT, J.M. 1973. Studies on the use of leaf-cutting and scent trail pheromones as attractants in baits. Proc. VII Congr. I.U.S.S.I., London, pp. 332-338.

ROBINSON, S.W., and ARANDA, A. 1975. A proposito del estado de plaga de las hormigas cortadoras y un esquema de su control en el Paraguay. *Rev. Soc. Cien. Paraguay* 15:61-63.

ROBINSON, S.W., and CHERRETT, J.M. 1975. Some reactions of leaf-cutting ants (Attini: Formicidae) to a synthetic scent-trail pheromone. pp. 91-97, *in* Ch. Noirot, P.F. Howse, and G. LeMasne (eds.). Pheromones and Defensive Secretions in Social Insects. Imprimerie de l'Université de Dijon, Dijon.

ROBINSON, S.W., MOSER, J.C., BLUM, M.S., and AMANTE, E. 1974. Laboratory investigations of the trail-following responses of four species of leaf-cutting ants with notes on the specificity of a trail pheromone of *Atta texana* (Buckley). *Insectes Soc.* 21:87-94.

SCHILDKNECHT, H., and KOOB, K. 1971. Myrmicacin, the first insect herbicide. *Angew. Chem. Int. Ed. Engl.* 10:124-125.

SILVERSTEIN, R.M., and YOUNG, J.C. 1976. Insects generally use multicomponent pheromones. pp. 19-29, *in* M. Beroza (ed.). Pest Management with Insect Sex Attractants. ACS Symposium Series 23. American Chemical Society, Washington, D.C.

SONNET, P.E. 1972. Synthesis of the trail marker of the Texas leaf-cutting ant, *Atta texana* (Buckley). *J. Med. Chem.* 15:97-98.

STENHAGEN, E., ABRAHAMSSON, S., and MCLAFFERTY, F.W. (eds.). 1969. Atlas of Mass Spectral Data. Vol. 1, p. 701. John Wiley and Sons, New York.

TUMLINSON, J.H., SILVERSTEIN, R.M., MOSER, J.C., BROWNLEE, R.G., and RUTH, J.M. 1971. Identification of the trail pheromone of a leaf-cutting ant, *Atta texana. Nature* (*London*) 234:348-349.

WHEELER, J.W., and BLUM, M.S. 1973. Alkylpyrazine alarm pheromones in ponerine ants. *Science* 182:501-502.