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TRAIL PHEROMONE OF THE ANT *Tetramorium impurum* AND MODEL COMPOUNDS: STRUCTURE–ACTIVITY COMPARISONS[1]

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Abstract—The major component of the trail pheromone of *T. impurum* is methyl 2-hydroxy-6-methylbenzoate (methyl 6-methyl salicylate). The poison reservoir of each worker contains about 1.0 ng of this substance in the venom. To ascertain the degree of specificity of the substance as pheromone and to determine any correlation between structure and biological activity, 38 synthetic analogs were tested in a bioassay. Sixteen compounds showed activity at 100 ng per artificial trail. The importance of the methyl group and methyl ester were demonstrated, while the hydroxyl group has a relatively small effect.

Key Words—*Tetramorium impurum*, Hymenoptera, Formicidae, trail pheromone, ant, venom, methyl 6-methylsalicylate, structure–activity correlation.

INTRODUCTION

Tetramorium impurum Foerster and *T. caespitum* L. are morphologically almost indistinguishable and said to be differentiated only by the male genitalia (B. Poldi, quoted in Pasteels et al., 1981). Chemically they are more easily distinguished by the contents of their Dufour glands (Billen et al., 1986), but a still more striking difference is that they do not use the same trail pheromone. Available information indicates that some trail pheromones are shared by many spe-

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cies of ant (Attygalle and Morgan, 1985), and the phenomenon of shared trail pheromones is not confined to ants (Morgan, 1990a). For example, 3-ethyl-2,5-dimethylpyrazine (EDMP) is used by all 13 species of *Myrmica* so far examined and also by a number of other species in other genera (Jackson et al., 1989).

The trail pheromone of *T. caespitum* is a 7:3 mixture of 2,5-dimethylpyrazine and EDMP (Attygalle and Morgan, 1983). We already have reported briefly the isolation of the trail pheromone of *T. impurum* and its identification as methyl 6-methylsalicylate (Morgan and Ollett, 1987). We now report the experimental evidence for the isolation and identification of this substance together with quantitative information on the extent of trail following evoked by a gland extract and the pure compound in a simple bioassay. Because of the simplicity of the compound, and its essentially two-dimensional shape, a number of simple analogs and derivatives were obtained or prepared to draw some conclusions about the necessary and sufficient structural features required for activity as this pheromone.

METHODS AND MATERIALS

T. impurum colonies were collected at various site in Belgium and France by R. and M.C. Cammaerts and sent live by post to Keele, where they were maintained in artificial nests in plastic bowls, lined with Fluon emulsion (ICI, Blackley) and fed on sugar water and dipteran larvae.

Bioassays were performed using the method of Pasteels and Verhaeghe (1974) in which a circle (radius 5 cm) was drawn on white paper and the circumference marked off into 1-cm arcs. The solution or extract to be tested was placed in a Standardgraph funnel pen (Blundell Harling, Weymouth) and the solution placed on the circumference of the circle. After 2 min to allow the solvent to evaporate, the sheet of paper was placed in the foraging area of the ant colony. Individuals walking onto the paper were observed for a period of 20 min. Ants approaching the circle either walked across it without detecting the odor (scored zero) or turned and followed the circumference for a time, when the number of centimeter arcs of trail followed without deviation were counted and their medians recorded in the tables; ants that ran a continuous path of 50 cm then were removed from the test. Each experimental solution was preceded by a control experiment in which the pen was filled with hexane alone, which was placed on the circle and observed in the same way. The pen was flushed thoroughly with hexane between each test solution.

Extracts of poison reservoirs or other glands in hexane were made in a small tissue grinder made from a glass rod and a small test tube roughened with carborundum paste. Solutions of pure chemicals of known concentration were prepared in hexane and diluted to the required concentration. All compounds

were examined by gas chromatography–mass spectrometry (GC-MS) to determine their purity. The compounds were all tested at 100 ng per trail initially and, if found active, were tested at 10-fold dilutions until activity ceased.

For thin-layer chromatography (TLC), a hexane extract of two poison glands was placed at the origin of a silica TLC plate (5 × 20 cm), which was then developed with hexane–acetone (3 : 2). The plate was dried and the silica cut into 10 equal bands, according to the R_f regions 0 to 1.0. The silica from each band was placed in a glass tube, plugged with glass wool, and any active compounds were eluted with acetone and the eluant tested in the bioassay.

Gas chromatography with trapping of the effluent was carried out as described by Evershed et al. (1982) using the splitter and trapping apparatus illustrated by Attygalle and Morgan (1988), with a packed column coated with OV-101, temperature program from 50 to 250°C at 10°C/min, and using the solventless solid injection method of Morgan and Wadhams (1972; see also Morgan, 1990b).

Identification of the pheromone compound was made by GC-MS. The poison glands of 10 workers were sealed in a glass capillary, which was introduced into the gas chromatograph by the method of Morgan and Wadhams (1972). After heating for 2 min to 140°C, the capillary was crushed and the volatile materials were flushed onto the chromatography column in a Hewlett Packard 5890 GC linked to a 5970B Mass Selective Detector with a HP 59970C ChemStation data processor. A fused silica capillary column (12 m × 0.2 mm) coated with HP-1 (cross-linked methylsilicone gum, equivalent to OV-1) of 0.33 μm film thickness was connected through a length of deactivated silica tubing (10 m × 0.32 mm) to the source of the mass spectrometer. The carrier gas was helium at 10 psi column head pressure. Injection was made in the splitless mode, and the injection port purge was turned on 30 sec after the injection. The oven temperature was initially at 30°C for 2 min, then increased to 250°C at 8°C/min. The Mass Selective Detector was set to monitor m/z 35–350 in the scan mode (~ 1.5 scans per second) under Autotune conditions using 70 eV ionization.

The quantity of methyl 6-methylsalicylate in the glands was determined by measuring the peak area obtained above and comparing it with the peak areas obtained by injecting known amounts of the synthetic compound at similar levels of dilution.

Twenty-six methyl esters were prepared from the corresponding acids by treatment with diazomethane in ether. Ethyl 6-methylsalicylate was prepared by bromination–dehydrobromination of ethyl 6-methyl-2-oxocyclohex-3-enecarboxylate, itself prepared by alkaline condensation of ethyl acetoacetate and crotonaldehyde by the method of Hauser and Pogany (1980). 2-Methoxy-6-methylbenzoic acid was prepared by the method of Anslow and Raistrick (1931), and then esterified with diazomethane. A number of aminoacids were the gift

of G.J. Thomas, others were gifts of C.J.W. Brooks or were available commercially. The purity of all synthetic compounds was checked by GC-MS.

RESULTS

Extracts made from the poison gland, Dufour gland, abdomen without the poison apparatus, head, and legs of *T. impurum* workers were all tested in the bioassay. Only the poison gland showed activity. The Dufour gland and other body parts were inactive. One poison gland had median activity of 8 cm in the bioassay, 0.1 poison glands had activity of 9.5 cm, 0.01 glands had activity of 3 cm, and at 0.001 glands, no activity was detectable. This test depended upon the age of the colony, and when the colony had been cultured in the laboratory for one year, the ability of workers to follow a glandular extract or pure methyl 6-methylsalicylate fell to zero. The test of ability of workers to follow an extract of one Dufour gland of a worker therefore was checked at frequent intervals, and most of the tests were carried out within four months of collection of the colony.

After thin-layer chromatography of a hexane extract of two poison glands, the band of R_f 0.6–0.7 showed strong activity in bioassay (median of 18 cm) while other R_f bands were inactive. Reaction of the gland extract with bromine, diazomethane, or conc. hydrochloric acid before chromatography had no effect on R_f value or activity.

Gas chromatography of 10 glands by the solid sampling method (Morgan and Wadhams, 1972), collecting three broad bands, gave one band eluting between 5 and 15 min, strongly active in bioassay (median value 22 cm). Repeating the experiment, but collecting the effluent in 1.5-min intervals gave only one strongly active band (Table 1), corresponding to a Kovats index between 1300 and 1400.

When 10 poison glands were subjected to coupled GC-MS, the only peak visible in the 1300–1400 Kovats index range was identified by its mass spectrum as either methyl 6-methylsalicylate or methyl 5-methylsalicylate [M^+ 166 (27%), *m/z* 134 (100), 106 (56), 105 (49), 78 (45), 77 (38), 63 (9), 51 (24)]. Both compounds gave the same mass spectrum but had different retention times. The mass spectrum and retention time corresponded to those of a synthetic sample of methyl 6-methylsalicylate. The synthetic compound had R_f 0.68 in the TLC system used and had a retention time of 6.5 min on the OV-101 column. In bioassay the synthetic methyl 6-methylsalicylate gave median values of 21 cm at 10 ng/trail and 5 cm at 1 ng/trail (Table 2). Methyl 5-methylsalicylate was inactive (Table 2).

By comparison of areas of the methyl 6-methylsalicylate peak from 10 poison glands with peak areas from similar known amounts of synthetic com-

TABLE 1. ACTIVITY OF VOLATILE FRACTIONS OF CONTENTS OF 10 POISON GLANDS OF *T. impurum*, SUBJECTED TO GAS CHROMATOGRAPHY, AND COLLECTING 95% OF MATERIAL FOR BIOASSAY[a]

Fraction	Elution time interval (min)	Activity (median cm)
1	4.5–6.0	5
2	6.0–7.5	19
3	7.5–9.0	3
4	9.0–10.5	0
5	10.5–12.0	0
6	12.0–13.5	1
7	13.5–15.0	0

[a] For column and temperature program, see Methods and Materials.

pounds, it was calculated that the mean amount per worker poison gland was 1.0 ng.

The activity in bioassay of all the synthetic compounds tested are listed in Table 2. The Wilcoxon-Mann-Whitney test (Siegel and Castellan, 1988) was applied to the results of the bioassays. All nonzero medians were significantly different ($P < 0.001$) from the controls.

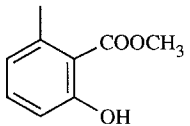
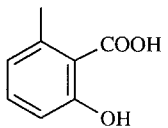
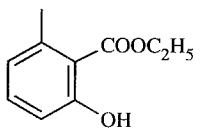
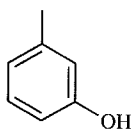
DISCUSSION

Preliminary experiments showed that the trail pheromone of *T. impurum* was contained in the venom of workers. TLC and GC experiments indicated that only one substance or, if more than one, a group of substances very closely related in polarity and mass was responsible for the trail-following behavior of workers. The only detectable peak in the active region of the gas chromatogram was methyl 6-methylsalicylate, and this compound was highly active in the bioassay. No dimethylpyrazine or ethyldimethylpyrazine was present in *T. impurum* venom, even at $\frac{1}{10}$ the amount present in *T. caespitum*. Similarly, *T. caespitum* venom glands provided no evidence for the presence of methyl 6-methylsalicylate. The later compound had no effect in inducing trail-following in *T. caespitum*.

The average amount of methyl 6-methylsalicylate per worker venom gland (1.0 ng) was the same as that reported in our preliminary communication (1 ng) (Morgan and Ollett, 1987). The two determininations were performed two years apart by different operators on different colonies from different locations.

Methyl 6-methylsalicylate has been identified in insect exocrine glands a

TABLE 2. ACTIVITY OF COMPOUNDS MEASURED BY MEDIAN NUMBER OF 1-cm ARCS RUN WITHOUT INTERRUPTION ON CIRCULAR TRAIL OF RADIUS 5 cm[a]

Name	Structure	Concentration (ng/trail)				
		100	10	1	0.1	0.01
1 Methyl 6-methylsalicylate		4(50+)	21(50+)	5(50+)	1(9)a	
2 6-Methylsalicylic acid		0(4)				
3 Ethyl 6-methylsalicylate		3(14)	0(2)			
4 *m*-Cresol		3(50+)a	0(29)a			

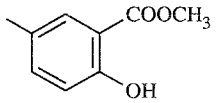
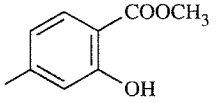
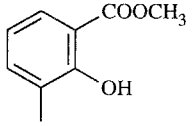
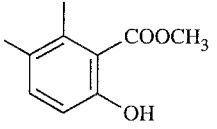
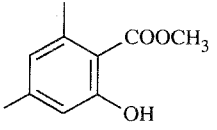
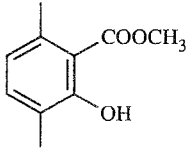
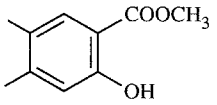
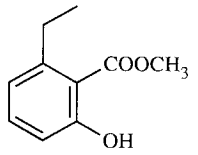
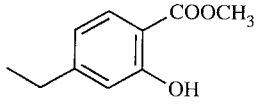
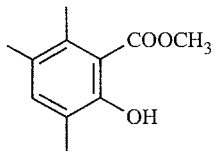
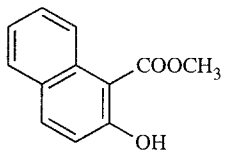
5 Methyl 5-methylsalicylate		0(44)	0(2)			
6 Methyl 4-methylsalicylate		0(25)	0(2)			
7 Methyl 3-methylsalicylate		0(13)	0(2)			
8 Methyl 5,6-dimethylsalicylate		5(46)	0(6)			
9 Methyl 4,6-dimethylsalicylate		2(50+)	4(50+)a	3(50+)b	4(50+)b	2(50+)a[b]
10 Methyl 3,6-dimethylsalicylate		3(50+)a	4(45+)b	7(50+)b	4(50+)b	2(50+)a[b]

TABLE 2. Continued

Name	Structure	Concentration (ng/trail) 100	10	1	0.1	0.01
11 Methyl 4,5-dimethylsalicylate		0(5)	0(2)			
12 Methyl 6-ethylsalicylate		0[c]				
13 Methyl 4-ethylsalicylate		0[c]				
14 Methyl 3,5,6-trimethylsalicylate		0(2)a				
15 Methyl 2-hydroxy-1-naphthoate		0(10)				

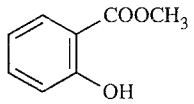
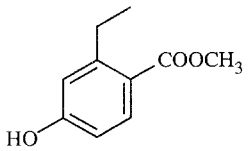
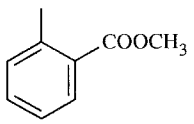
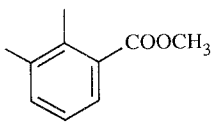
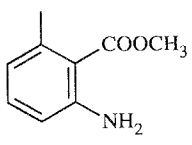
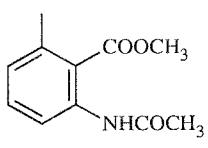
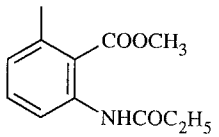
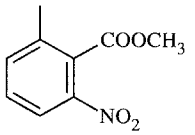
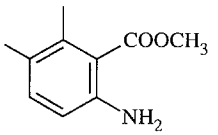
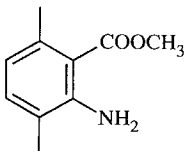
16 Methyl salicylate		1(50+)	0(1)		
17 Methyl 2-ethyl-4-hydroxybenzoate		0[c]	0[c]		
18 Methyl *o*-toluate		9(50+)	3(36)	0(5)	
19 Methyl 2,3-dimethylbenzoate		0(16)	0(4)		
20 Methyl 2-amino-6-methylbenzoate		7(48)	0(4)		
21 Methyl N-acetyl-2-amino-6-methylbenzoate		3(34)	5(50+)	3(50+)a	0(39)

TABLE 2. Continued

Name	Structure	Concentration (ng/trail) 100	10	1	0.1	0.01
22 Methyl N-propionyl-2-amino-6-methylbenzoate		0(50+)	0(1)			
23 Methyl 6-methyl-2-nitrobenzoate		0(2)				
24 Methyl 2-amino-5,6-dimethylbenzoate		0(3)				
25 Methyl 2-amino-3,6-dimethylbenzoate		0(10)				

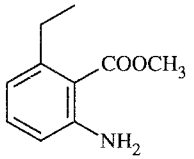
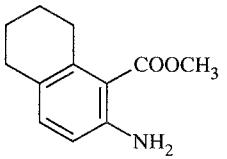
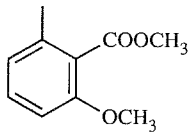
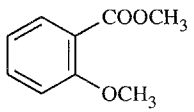
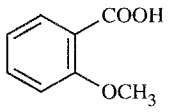
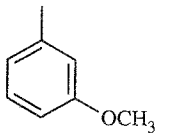
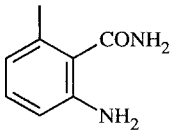
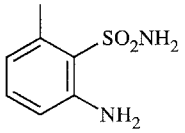
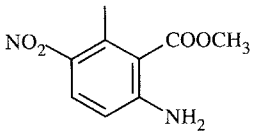
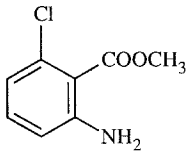
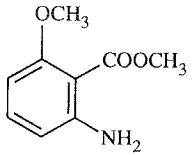
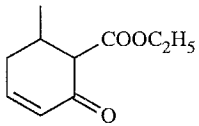
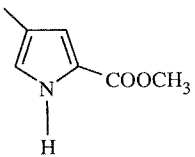
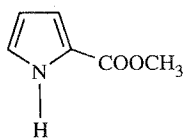
26 Methyl 2-amino-6-ethylbenzoate		20(50+)a	22(50+)	0(8)
27 Methyl 2-amino-5,6,7,8-tetrahydro-1-naphthoate		0(24)	0(1)	
28 Methyl 2-methoxy-6-methylbenzoate		2(12)	1(8)	0(5)
29 Methyl *o*-anisoate		4(29)a	1(50+)	1(34)
30 *o*-Anisic acid		3(50+)	0(18)	
31 *m*-Methoxytoluene		1(42)a	0(17)b	

TABLE 2. Continued

Name	Structure	Concentration (ng/trail) 100	10	1	0.1	0.01
32 2-Amino-6-methylbenzamide		0(5)				
33 2-Amino-6-methylbenzenesulfonamide		16(31)	7(32)	1(8)		
34 Methyl 6-amino-2-methyl-3-nitrobenzoate		0(50+)	0(2)			
35 Methyl 2-amino-6-chlorobenzoate		0(26)	0(2)			

36 Methyl 2-amino-6-methoxybenzoate		0(25)	0(2)
37 Ethyl 6-methyl-2-oxocyclohex-3-enecarboxylate		0(3)	
38 Methyl 4-methylpyrrole-2-carboxylate		0(18)	0(3)a
39 Methyl pyrrole-2-carboxylate		0(15)	

[a] According to the test of Pasteels and Verhaeghe (1974). All compounds were tested at 100 ng/trail and if active, tested at 10-fold dilutions until activity ceased. All tests were preceeded by a control test with hexane only which showed no activity (median = 0). The lower extreme value was zero for all tests, the upper extreme values are given in parentheses after each median; 50+ indicates that the ant followed for 50 continuous arcs, after that the ant was removed from the test. Workers were observed for 20 min, during that time between 30 and 135 observations were made, except (a) where 21–30 counts were made and (b) where 16–20 counts were made. All nonzero medians were significantly different from the control (Wilcoxon-Mann-Whitney test, $P < 0.001$).

[b] Activity was not reproducible at lower concentrations.

[c] Median values only recorded.

number of times before, but this is the first time it has been found in the venom of an ant or identified as a trail pheromone. It is also the first simple benzenoid compound found to serve as a trail pheromone. The compound is found in the mandibular glands of males of a number of *Camponotus* species, where it has been associated with the swarming of males and virgin females (Lloyd et al., 1984). For a summary of its occurrence also in ponerine ant workers' mandibular glands and the pygidial defensive secretion of a beetle, see Lloyd et al. (1984).

The activity of dimethylsalicylates (compounds 9 and 10, Table 2) in the bioassay at high dilution, higher than the identified natural pheromone was surprising, and caused us to look carefully to see if small amounts of such a compound were present in the poison reservoir. Using selected ion monitoring, a related compound with longer retention time and 14 units greater mass was discovered in the venom, at approximately 50 pg/insect. However, neither the retention time nor the mass spectrum corresponded to a methyl dimethylsalicylate. We expected, therefore, that methyl 6-ethylsalicylate, already identified in insects (Gnanasunderam et al., 1984), and highly probable on biosynthetic grounds, would be the substance. Methyl 6-ethylsalicylate was synthesized from 2-pentenal and methyl acetoactate by the general method of Hauser and Pogany (1980); however, the synthetic compound did not show much activity in the bioassay and possessed a different mass spectrum. The naturally occurring minor component was finally identified as methyl 2-methoxy-6-methyl benzoate by comparison with a synthetic specimen. The spectra of methyl 6-ethylsalicylate and methyl 2-methoxy-6-methylbenzoate are shown in Figure 1, since these compounds are likely to be confused. Compounds of this group with a free hydroxyl group show a strong M^+-32 ion in their mass spectra, due to loss of methanol. The methyl ether shows a strong M^+-31 ion through loss of a methoxy radical.

Model compounds. The effectiveness in the bioassay of a number of compounds of structure similar to methyl 6-methylsalicylate is given in Table 2. Each compound was tested initially at 100 ng/trail. At this concentration, workers show by their behavior that they can detect the compound, even if it is too concentrated for them to follow it well. If active, the compound was tested at successive 10-fold dilutions. Eleven compounds were detected by the foraging ants at 100 ng/trail, and some ants followed the artificial trail, although the median values were zero. These substances were tested again at 10 ng/trail. None were active.

That most of the compounds were inactive attests to the specificity of structure required for the antennal receptors to induce trail-following. The small number of model compounds that showed activity is still more interesting. Compounds 2–4 (Table 2) show the importance of the methyl ester. Conversion to the free acid, or to its ethyl ester, or the total absence of the group destroys

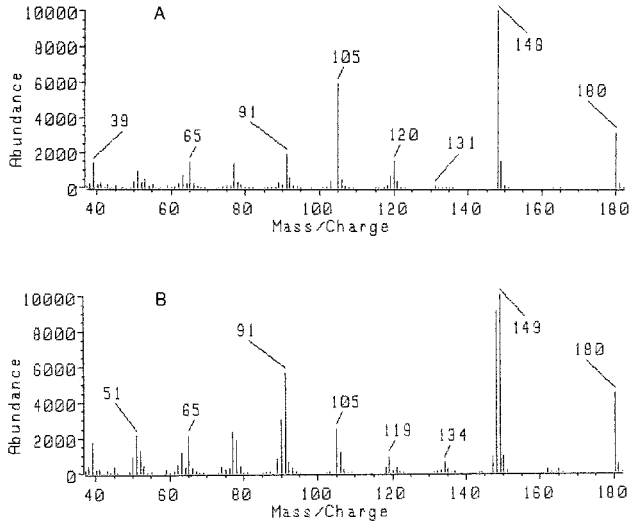


FIG. 1. Mass spectra of methyl 6-ethylsalicylate (A) and methyl 2-methoxy-6-methylbenzoate (B). The differences in the mass spectra, particularly the relative intensities at *m/z* 149, 120, 105, 91, and 78, are easily apparent. There was no evidence of (A) in *T. impurum* poison glands but (B) was identified in picogram quantities by selective ion monitoring.

activity. Compounds 5–15 explore the activity of the methyl group. Movement of the group to another position (compounds 5–7) or its total absence (16) completely removes activity, but an extra methyl group in addition to that at C-6 (compounds 8–10) gave activity at higher dilution beyond that of compound 1, but a third methyl (14) seems to destroy it again. 6-Ethylsalicylates are known in nature and several were tried (12, 13, 17), but they did not show high activity (but see 26). Another aromatic ring fused in the 5,6 positions (15) destroyed activity. Compounds 17–28 explored the effect of the hydroxyl group. Here the evidence is less clear, but the OH appears to be a less critical part for activity, for its total absence (18) does not destroy activity completely, but the loss of activity in the dimethyl benzoate (19) is confusing. Replacement of OH by NH_2 (20) still shows some activity, and the acetylation of this (21) enhanced its activity. Lengthening the chain by one carbon atom to an *N*-propionyl amide (22) again destroyed activity. Not surprisingly, replacing OH by the strongly electron-withdrawing nitrogroup gave an inactive compound (22). Since compound 20, in which NH_2 replaces OH, shows some activity, 25, by analogy with 10, might be expected to be active but was not. Compounds 24, 26, and 27 are further variations in this series.

The methyl ether of the parent pheromone, methyl 2-methoxy-6-methyl-

benzoate (28), present in the gland in picogram quantities, was also active but less than so its parent. It showed some slight synergistic effect when mixed with methyl 6-methylsalicylate, but this was difficult to quantify because the colony was aging when this test was made. Other methoxy derivatives (29–31 and 36) showed expected behavior, i.e., only methyl *o*-anisoate (29) was appreciably active. Methyl 6-methoxysalicylate was prepared too late to test. The group 32–36 are miscellaneous related structures. The comparatively high activity of the aminosulfonamide (32) was surprising, but recalls the way sulfanilamide (parent of the sulfa drugs) mimics the structure of *p*-aminobenzoate in inhibiting the synthesis of folic acid bacteria (Sexton, 1963). It is tempting to suggest that this substance is strongly adsorbed on the antennal receptors. Compound 37, an intermediate in the synthesis of 3, is, in its enol form, a dihydroderivative of 3. It was therefore interesting to examine it, but it had no activity. The superficial structural similarity between 1 and 38, the trail pheromone of *Atta texana* (Tumlinson et al., 1972) and *Atta cephalotes* (Riley et al., 1974) were the justification of including 38 and 39, but no activity was found.

Sonnet and Moser (1972, 1973) carried out similar structure–activity studies with *Atta texana* and compounds similar to methyl 4-methylpyrrole-2-carboxylate (37). Their work showed that some analogs of 38, with similar structure, retained activity for *Atta texana*, but too great a divergence from the true pheromone quickly destroyed it, while positional isomers of 38 were generally inactive.

The biosynthetic origin of methyl 6-methylsalicylate in the venom is unknown. The biosynthesis of 6-methylsalicylic acid from acetate-malonate has been studied in detail in microorganisms (Weiss and Edwards, 1980), and it has been reported to be synthesized in barley (Kannangara et al., 1971). In general, higher animals are not known to synthesize simple aromatic compounds, but Tecle et al. (1986) have shown positively that 2-hydroxy-6-methylacetophenone is biosynthesized from acetate via a C_{10} polyketide in a ponerine ant. Methyl 6-methylsalicylate could arise directly from this by a Baeyer–Villiger oxidation. On the other hand, mellein (3,4-dihydro-9-hydroxy-3-methylisocoumarin), normally regarded as a fungal metabolite, has been identified as the main component of the male wing gland pheromone of the bumble-bee wax moth *Aphomia sociella*, and *Aspergillus ochraceus*, a mellein-producing microorganism was detected in the gut of *A. sociella* larvae and bumble-bee nests (Kunesch et al. 1987). The biosynthetic relationship between mellein and 6-methylsalicylate and other compounds is discussed by Brand et al. (1973), Gnanasunderam et al. (1984), and Tecle et al. (1986). Brand et al. (1973) found methyl 6-methylsalicylate and mellein together in the mandibular gland secretion of males of three species of *Camponotus* ants (Subfamily Formicinae). It would have been interesting to test 2-hydroxy-6-methylacetophenone among our model compounds but it was not available in time.

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