

First identification of a trail pheromone of an army ant (*Aenictus* species)

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Abstract. Totally blind army ants carry out massive and highly organised foraging raids, apparently guided by chemical stimuli. Until now, this phenomenon has not been closely analysed. The existence of a trail pheromone in a postpygidial gland of an *Aenictus* species has been demonstrated and the substances identified as methyl anthranilate and methyl nicotinate. The pheromone consists of two parts: a primer effect, caused by methyl nicotinate, which prepares workers to follow trails, but is not itself followed, and a releaser effect, due to methyl anthranilate, which causes trail-following only in conjunction with the primer substance.

Key words. Formicidae; *Aenictus*; army ant; trail pheromone; methyl anthranilate; methyl nicotinate; primer pheromone; releaser pheromone.

The raiding behaviour of army ants has been widely described and extensively studied biologically¹⁻³. The Old World army ants (subfamily Dorylinae) are completely blind and must therefore rely on chemical or tactile stimuli to co-ordinate and give cohesion to their raiding columns. While there is recent evidence that New World army ants (Ecitoninae) use chemical substances to follow trails⁴, until now similar substances have not been described for the Dorylinae. We now report the discovery of a trail pheromone and its glandular source in the doryline army ant *Aenictus*. We have demonstrated that this pheromone consists of a primer and a releaser substance. These substances have been identified and the synthetic equivalents shown to be active in the laboratory.

The ability of ants to orient along chemical trails is a well documented phenomenon^{1,5}. Trail pheromones are normally laid by workers returning to the nest with food to direct other nestmates to the food source, and thus exploit the resource efficiently. Workers of *Aenictus* raid in columns which emanate from a temporary nest or bivouac. Each column contains tens of thousands of individuals although the pattern of raiding and bivouacking in *Aenictus*, at least compared with *Dorylus* or *Eciton*, can be considered as relatively simple^{1,3}. Because of the great difficulty of maintaining army ants in captivity, they have not been studied chemically until very recently^{6,7}. We have succeeded in keeping several hundred workers of *Aenictus* alive for a few weeks to carry out these studies.

Materials and methods

Aenictus workers were collected at Tai Po Kau in Hong Kong and transported immediately to Leuven and thence to Keele. They belong to the *Typhlatta* group, and are near to *A. laeviceps*, although they are probably

an as yet undescribed species (W. A. Gotwald, pers. commun.). The abdominal tergites were carefully removed in a solution of cold 2% glutaraldehyde in sodium cacodylate buffer at pH 7.3, so as to expose the abdominal tissues for observation with a Philips SEM 515 scanning electron microscope. For this purpose, the opened abdomens were critical point dried and coated with a 30 nm layer of gold.

For chemical analysis, various abdominal parts were dissected with fine forceps in distilled water under a binocular microscope. Trail tests were performed on various tissues and glands using the circular trail bioassay of Pasteels and Verhaeghe⁸. Tissues were ground in hexane (100 µl) and the solution applied to the circumference of a circle, radius 5 cm, drawn on plain white paper. The solution was placed in a Normagraph pen (Blundell Harling, Dorset, UK) and applied by hand to the circumference, which was marked off in 1 cm arcs. After allowing 2 min for the solvent to evaporate, the paper was placed in an arena where the ants were able to explore it. The activity of the trail could then be determined from the number of arcs followed by individual ants.

Glands prepared for chemical analysis were sealed in soft glass capillaries and chromatographed by the solvent-less technique of Morgan and Wadhams⁹. Gas chromatography-mass spectrometry was performed on a Hewlett Packard 5890 Gas Chromatograph with a 5970B Mass Selective Detector (quadrupole mass spectrometer using 70 eV electron impact ionization). The system was controlled by a Hewlett Packard series 300 computer with HP 59970C Chemstation.

Chromatography was carried out on a modified 5% phenyl 95% dimethylsiloxane phase (non-polar) in a fused silica capillary column (12 m × 0.22 mm × 0.25 µm film thickness, SGE, Milton Keynes, UK) linked directly to the mass spectrometer source with a

5 m length of deactivated fused silica capillary tubing (0.22 mm i.d. SGE). Helium was used as the carrier gas at 1 ml min⁻¹. The sample was heated in the injection port at 200 °C for 3 min before crushing the glass capillary. The oven was programmed to shift from 30 °C to 250 °C at 7° min⁻¹. The split vent was closed before crushing and reopened 30 s later.

Synthetic samples of methyl anthranilate and methyl nicotinate (Aldrich, Gillingham, UK) were used as external standards to confirm identification and allow quantification.

Results and discussion

Preliminary trail-following tests with hexane extracts of heads, thoraces and abdomens of *Aenictus* workers quickly indicated that only the abdomens induced trail-following. Further work was therefore directed to the abdominal organs. Dissection of worker abdomens of *Aenictus* revealed a pair of red ovoid bodies at the posterior end of the abdominal tip, below the 7th tergite (fig. 1) which correspond to the postpygidial glands described by Hölldobler and Engel in several species of *Rhytidoponera*, a ponerine genus¹⁰. In *Aenictus*, these postpygidial glands appear much larger than in other ants. The secretory cells occur mainly on the posterior side of the reservoirs, each of which has a diameter of approximately 60 µm. The reservoirs open into the cloacal chamber, from where the secretion can be deposited on the substratum by the tip of the abdomen or the sting. Bioassays using the artificial circular trails of Pasteels and Verhaeghe⁸ and extracts of various abdominal tissues and glands demonstrated that only the contents of the postpygidial glands induced trail-following in workers. This trail-following behaviour was intense even when an extract of one pair of glands was diluted 100-fold. Almost all workers (>90%), on reaching the trail, immediately began to follow it and

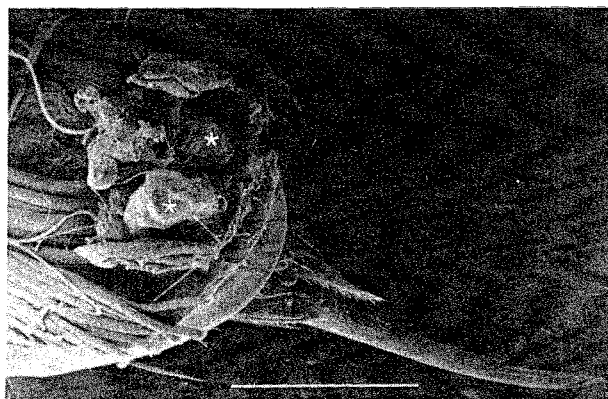
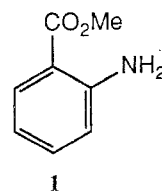


Figure 1. Scanning electron micrograph of the partially dissected abdominal tip of *Aenictus* (tergites removed) showing the postpygidial glands (*). Scale bar 100 µm.

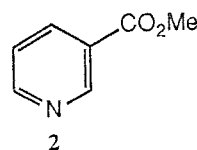
continued following for several hours, making quantification of the activity impossible.

Chemical analysis of the glandular secretion by linked gas chromatography-mass spectrometry using single glands revealed methyl anthranilate (methyl 2-amino-benzoate: structure 1), at a mean of 100 ng per ant as essentially the only component (fig. 2).



Trails of synthetic methyl anthranilate (100 ng per trail) induced little activity, with only a few ants travelling 1 or 2 cm along the trail. However, when workers which had recently been running on trails made from the glandular extract were transferred onto the artificial methyl anthranilate trails, they followed them as well as they followed the natural secretion, and this effect lasted for several hours after exposure to the glandular secretion. This suggested to us that the ants had been primed to follow the methyl anthranilate trails by some other component in the glandular extract.

Careful examination of the chromatogram from the glands of six workers (fig. 2) revealed a second component identified as methyl nicotinate (methyl pyridine-3-carboxylate: structure 2), at 1 ng per ant. This substance



alone at a concentration of 1 ng per trail (i.e. 30 pg per cm) induced no trail-following in workers, whether they had been previously running on a trail of glandular extract or not.

Trails made with a synthetic mixture of methyl nicotinate (1 ng) and methyl anthranilate (100 ng) induced activity comparable to that of the glandular extract, which persisted even when the concentration was reduced 100-fold. Furthermore, ants which had run on this trail for approximately 3 min would follow trails made with methyl anthranilate alone, even when placed on methyl anthranilate trails 6 h after their exposure to the two-component trail.

Since the exposure to methyl nicotinate potentiates the workers' ability to follow methyl anthranilate trails up to several hours later, we classify methyl nicotinate as a primer pheromone, that is a pheromone which 'alters a set of long-term physiological conditions so that the