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Trail pheromone of the Argentine ant, Linepithema humile (Mayr) (Hymenoptera: Formicidae).

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Introduction

The ability of social insects to coordinate individual behaviors for colony-level tasks is central to their ecological dominance in most terrestrial ecosystems [1]. In the social insects, the intracolony communication mediated by semiochemicals plays an important role in organizing collective activities, such as defense, reproduction, foraging, and nest relocation [2,3]. The trail pheromones of ants, in particular, are known to play a critical role in foraging and nest relocation processes, by efficiently leading colony members to prospective food sources or nesting sites [4–7].

The Argentine ant, *Linepithema humile* (Mayr), has been spread by human commerce from its native South America to subtropical and Mediterranean-climate regions throughout the world [8,9], competitively displacing native ants in its introduced range [10,11]. In addition to its large colony size and occupation of spatially separated nests, the highly effective mass recruitment system of the Argentine ant has been recognized as one of the major mechanisms facilitating its interspecific competitive ability. For example, Argentine ant workers recruit their nestmates to food resources more quickly than do native competitors [12,13]. Furthermore, following environmental disturbances, such as flooding, Argentine ant workers relocate their entire colony to suitable nest sites via mass recruitment more quickly than other native ant species [14]. Finally, during intraspecific aggressive encounters between Argentine ant supercolonies, enormous numbers of workers can be recruited to conflict zones, resulting in considerable worker mortality [15].

The trail pheromone of the Argentine ant has been the focus of numerous studies because of its significance in the species’ mass recruitment behavior. Cavill et al. first isolated and characterized (Z)-9-hexadecenal from crude extracts of Argentine ants (whole body, dissected ventral gland) using a series of column chromatography and microchemical reactions [16,17]. Based on the evidence that (Z)-9-hexadecenal was a ventral gland secretion, and that it strongly attracted Argentine ant workers in a multi-choice olfactometer, Cavill et al. concluded that (Z)-9-hexadecenal might be a component of the trail pheromone complex of the Argentine ant, but conservatively referred to it as “a general aggregation factor” [16,17]. Since then, however, many researchers have primarily focused on (Z)-9-hexadecenal in studies of Argentine ant trail pheromones [18–22]. The concept of (Z)-9-hexadecenal as the key component of Argentine ant trails has become broadly adopted because the ants follow trails drawn with low concentra- tion of (Z)-9-hexadecenal did increase the efficacy of the trail-following behavior. In stark contrast with previous dogma, our study suggests that dolichodial and iridomyrmecin are major components of the Argentine ant trail pheromone. (Z)-9-hexadecenal may act in an additive manner with these iridoids, but it does not occur in detectable quantities in Argentine ant recruitment trails.
invasive Argentine ant populations. For example, one study showed that synthetic (Z)-9-hexadecenal increased the consumption of sugar-based liquid baits by Argentine ants when it was mixed with the baits [23]. Similarly, several field studies have been conducted in Hawaii and Japan to test if synthetic (Z)-9-hexadecenal disrupts trail formation and subsequent foraging activity of Argentine ants [24–26].

Trail pheromones of many species of ants are likely to have multiple chemical components [29–34]. Some evidence suggests that Argentine ants have other important chemicals in their natural recruitment trail. For example, Cavill et al. reported that the pure synthetic (Z)-9-hexadecenal was less effective in attracting Argentine ants than the entire lipid fraction isolated from the ants [17]. Moreover, a trail drawn with a gaster extract was effective for 4 h, while the activity of synthetic (Z)-9-hexadecenal trails only lasted for 1 h [18,20]. Van Vorhis Key and Baker reported that trail-following responses of Argentine ants to synthetic (Z)-9-hexadecenal were similar to their responses to the whole gaster extract trail, which contains ~100 times less (Z)-9-hexadecenal than the synthetic one [20]. The same authors also reported that a 0.2 ng cm⁻¹ rate of synthetic (Z)-9-hexadecenal was required to make artificial trail equally attractive with the whole gaster extract [21]. However, 0.2 ng cm⁻¹ of (Z)-9-hexadecenal far exceeds physiologically relevant concentrations because individual Argentine ant workers are known to have only a few nanograms of (Z)-9-hexadecenal in their bodies [16,20]. Furthermore, in recent attempts to disrupt the Argentine ant trail formation with synthetic (Z)-9-hexadecenal, researchers reported that some ants still followed their natural trails, even when high concentrations of synthetic (Z)-9-hexadecenal were released in the field [24,26,27].

Here, we examined the trail substances deposited by living Argentine ants to elucidate the chemical constituents of trail pheromone. To collect the trail substances, we provided Teflon coated wire as a bridge to large laboratory colonies of the Argentine ants during two different recruitment events induced in the laboratory: foraging and nest relocation (Figure 1A and B). We also collected trail chemicals by providing solid-phase microextraction (SPME) fibers as bridges during foraging (Figure 1C). Once an active recruitment trail was established on the bridges, we either extracted the chemicals from the wire and analyzed them with coupled gas chromatography-mass spectrometry (GC-MS), or directly analyzed the chemicals adsorbed on SPME fiber with GC-MS. After identifying putative trail chemicals, we conducted bioassays to test if the compounds induced trail-following behavior. Based on our chemical analyses and behavioral bioassays, here we report two major chemical constituents of Argentine ant trail pheromone.

**Results**

GC-MS analysis revealed that two characteristic compounds were consistently present in the chemical trails deposited by Argentine ants during both foraging and nest relocation events (Figure 2A). These compounds were not found in extracts from control wires. There was no apparent difference between the chemical trails obtained from foraging and nest relocation events. Based on comparison of retention times and mass spectra to those of authentic standards, we identified the two chemicals as trans,trans-dolichodial [referred to as “peruphasmal” in ref. 35] and cis,trans-iridomyrmecin (Figure 2B). We did not find a detectable quantity of (Z)-9-hexadecenal in the trail, even though the bridges (i.e., wires and SPME fibers) were being actively used by numerous ants immediately prior to extraction.

![Figure 1. Experimental set-up for collection of trail pheromones.](image)

(A) In the foraging scenario, a sucrose solution feeder on a foraging platform was connected to a starved colony using a Teflon coated wire bridge. (B) In the flooding-induced nest relocation scenario, a water container released water drops into a nest box to induce ants to abscond into a new nest box using a Teflon coated wire bridge. (C) A SPME fiber was used to collect trail chemicals produced by foraging ants by setting it up as a short bridge leading to a sucrose solution feeder. doi:10.1371/journal.pone.0045016.g001

For the colony fragments collected from Berkeley, California (Summer 2011), the concentrations of dolichodial and iridomyrmecin on the Teflon coated wire bridges (n = 7) were highly variable with average values of 0.1 ng cm⁻¹ (range 0.01–0.17 ng cm⁻¹) and 0.3 ng cm⁻¹ (range 0.07–0.66 ng cm⁻¹), respectively. The average amounts of dolichodial and iridomyrmecin adsorbed in 1-cm SPME fiber trail (n = 3) during foraging were 0.4 ng (range 0.08–0.66 ng) and 3.9 ng (range 0.43–9.1 ng), respectively. For the colony fragments collected from Riverside, California (Spring 2012), the average concentrations of dolichodial and iridomyrmecin on the wire bridges (n = 3) were 1.1 ng cm⁻¹ (range 0.57–1.52 ng cm⁻¹) and 3.1 ng cm⁻¹ (range 0.22–5.26 ng cm⁻¹), respectively. The quantitative analysis with worker ants collected from Berkeley showed that the total quantities of dolichodial, iridomyrmecin, and (Z)-9-hexadecenal in a single worker were 4.6 µg, 7.6 µg, and 13.2 µg, respectively. Based on another set of workers collected from Riverside, the total quantities of dolichodial, iridomyrmecin, and (Z)-9-hexadecenal per a single worker were estimated to be 3.8 µg, 11.7 µg, and 23.4 µg, respectively.

To test whether the mixture of dolichodial and iridomyrmecin (hereafter called MDI) attract Argentine ants, we performed Y-maze bioassays with an authentic standard of dolichodial (>99% pure) obtained from *Anisomorpha buprestoides* stick insect defensive secretion [35] and synthesized iridomyrmecin (94% pure). The compounds were dissolved in methylene chloride at a 1:1.87 (w/wt, dolichodial:iridomyrmecin) ratio to emulate the natural ratio of the two compounds found in the Argentine ants collected
Figure 2. Chemical trails left by recruiting and trail-following ants during foraging and nest relocation. (A) Chromatograms of methylene chloride extracts taken from a control wire (top) and from the wires used by ants during foraging (second) and nest relocation (third). A chromatogram obtained from a SPME fiber that was used as a foraging trail is also shown (bottom). Dolichodial (Dol) and iridomyrmecin (Ird) were consistently detected on these trails (arrows). (Z)-9-hexadecenal (not detected) would have eluted with retention time of 16.03 min. (B) Chemical structures of dolichodial and iridomyrmecin, with relative configurations as shown. The absolute configurations were not determined.

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from Berkeley (1:1:65, w/w/w). A slightly higher proportion of iridomyrmecin in the standard mixture was acceptable because the ratio between those two compounds in the trail was generally skewed toward iridomyrmecin (e.g., range 1:2–1:14), possibly because of the relatively higher volatility of dolichodial. In the Y-maze bioassay, the standards were effective in attracting worker ants at various concentrations. More than 70% of ants chose the arms treated with the four higher concentrations (Table 1). However, authentic standard MDI containing 6.2 pg cm\(^{-1}\) dolichodial (1.3 \(\times\) 10\(^{-6}\)-ant equivalent cm\(^{-1}\)) and 11.6 pg cm\(^{-1}\) iridomyrmecin (1.5 \(\times\) 10\(^{-6}\)-ant equivalent cm\(^{-1}\)), failed to attract worker ants (Table 1).

Y-maze bioassays also indicated that high concentrations of synthetic (Z)-9-hexadecenal were effective at attracting worker ants, as more than 90% of ants chose the arms treated with 200 pg cm\(^{-1}\) dolichodial (1.5 \(\times\) 10\(^{-2}\)-ant equivalent cm\(^{-1}\)) and 20 pg cm\(^{-1}\) (1.5 \(\times\) 10\(^{-3}\)-ant equivalent cm\(^{-1}\)) of (Z)-9-hexadecenal (Table 1). However, (Z)-9-hexadecenal did not attract worker ants at 0.2 pg cm\(^{-1}\) (1.5 \(\times\) 10\(^{-3}\)-ant equivalent cm\(^{-1}\)) (Table 1). The threshold of (Z)-9-hexadecenal for worker attraction was lower than the threshold of MDI, indicating the worker ants are more sensitive to (Z)-9-hexadecenal than MDI at the lower concentrations.

To determine the possible interaction between MDI and (Z)-9-hexadecenal in worker ant attraction, we performed Y-maze bioassays by simultaneously presenting various concentrations of MDI with subthreshold concentration of (Z)-9-hexadecenal (0.2 pg cm\(^{-1}\)). The subthreshold concentration was defined as the maximum concentration of the chemical(s) which failed to attract workers when presented alone. We found that 65–86% ants chose the treated arm when MDI was co-presented with subthreshold rate of (Z)-9-hexadecenal (Figure 3). Most interestingly, a similar pattern was observed for subthreshold concentration of MDI: 65% of ants tested chose the arm treated with a mixture of authentic standard MDI at a subthreshold level (6.2 pg cm\(^{-1}\) dolichodial and 11.6 pg cm\(^{-1}\) iridomyrmecin) and 0.2 pg cm\(^{-1}\) (Z)-9-hexadecenal (Figure 3, dark bar), while neither of MDI nor (Z)-9-hexadecenal attracted workers when tested individually at those concentrations (Table 1).

To test the effect of MDI and (Z)-9-hexadecenal in the trailing response (i.e., movement along a trail), we conducted bioassays with 17.3-cm long S-shaped trails drawn on rectangular filter paper pieces (Figure S1). We tested following concentrations: 1.3 ng cm\(^{-1}\) dolichodial, 4.1 ng cm\(^{-1}\) iridomyrmecin, and 0.7 pg cm\(^{-1}\) (Z)-9-hexadecenal. The concentration for MDI was considered “physiologically relevant” based on the quantification studies with the workers collected from Riverside. Based on the total numbers of ants successfully following the entire trail, we found that MDI+(Z)-9-hexadecenal trails elicited the highest trail-following responses (5.0 \(\pm\) 0.6 ants, mean \(\pm\) SEM) relative to MDI only (2.1 \(\pm\) 0.4 ants) and (Z)-9-hexadecenal only (0.2 \(\pm\) 0.1 ants) trails (ANOVA with Tukey HSD all-pairwise comparison test; \(F=37.7, df=2, P<0.0001\)) (Figure 4).

Discussion

Since the first identification of the trail chemicals in a leaf cutting ant, *Atta texana* [36], the study of trail pheromones has been carried out in numerous species of ants [3,37,38]. Because individual ants often secrete trail pheromones in subnanogram quantities [29,30], many studies have followed an indirect approach: examination of the chemicals stored in the internal glands. This approach typically includes following three steps: (1) identification of the putative glandular source by performing bioassays with extracts of whole body, gaster, and/or dissected glands, (2) purification and identification of active chemical constituents from the crude extracts, and (3) confirmation of the pheromone’s chemical identity by performing bioassays with synthesized standards and their mixtures [16,31,39]. Even when the glandular source of trail pheromone is unknown, this standard procedure allows researchers to quickly screen various kinds of exocrine gland products for their possible activity as trail substances. However, identifying the glandular source for trail-following activity and analyzing extracts of the dissected glands usually result in fewer bioactive compounds which are likely to contribute to trail-following. Once the investigator understands the chemical properties of the target compounds, the whole body or gaster can be extracted to obtain relatively large quantity of chemicals to work with, which is often necessary for detailed chemical identification processes (e.g., derivatization).

However, this approach has two limitations. First, one cannot assume that the chemicals in the extracts, either from whole gaster or dissected glands, are the ones that are produced and deposited on the substrate by recruiting ants. Second, when several unrelated chemicals from different glands are involved in the trail-following process, it is difficult to pinpoint the optimum ratio of the individual constituents, unless various permutations at different concentrations of chemicals are tested to achieve the behavioral responses elicited by natural trails.

We found that the recruitment trails of Argentine ants, during both foraging and nest relocation, contain dolichodial and iridomyrmecin. Bioassays with standard dolichodial and iridomyrmecin confirmed that this mixture attracted workers and induced trail-following behavior. Dolichodial and iridomyrmecin are produced and stored in the pygidial gland of Argentine ants
A nearly pure mixture of dolichodial and iridomymecin can be “milked” from the pygidial gland by gently pressing the gasters of chilled workers with fine forceps (D.-H. Choe, unpublished data). Historically, the pygidial glands of dolichoderine ants have been believed to produce defensive secretions that provoke the alarm response, or chemicals with antibiotic/insecticidal effects [39,41–48]. However, in some cases, the pygidial gland secretions of dolichoderine ants appear to play a role in other types of communication. For example, Tapinoma simrothi displayed a trail-following response to a pygidial gland secretion fraction that contained iridodials and iridomyrmecin [49]. Pygidial gland products of the Argentine ant, dolichodial and iridomymecin, are also known to play an important role in inhibiting necrophoresis in the workers [50].

The concentrations of dolichodial and iridomymecin on recruitment trails were variable, even though we collected and analyzed the chemicals while many ants were actively using the trails. This variation might be due to physical nature of dolichodial and iridomymecin: previous laboratory studies indicated that small amounts of the dolichodial/iridomymecin mixture (i.e., one ant-equivalent obtained via solvent extraction) dissipate from surfaces within 40 minutes under ambient conditions [50]. The ephemeral nature of dolichodial and iridomymecin deposits will produce trails that differ with age and length. This could provide trail-following ants with valuable information, and allow them to distinguish among different recruitment trails. In a complex network of foraging trails, for example, foragers might be able to distinguish the routes that lead to the most recently productive food sources [37].

### Table 1. Attraction of Argentine ants to different chemical trails.

| Chemical                  | Concentration (amount/cm) | No. of replicates | % of ants choosing the treated arm (mean ± SEM) | Number of ants choosing the treated arm/total |
|---------------------------|---------------------------|-------------------|-----------------------------------------------|---------------------------------------------|
| Dolichodial/Iridomymecin authentic standards | 6.2 ng/11.6 ng | 12 | 76.7±7.3* | 46/60 |
|                           | 3.1 ng/5.8 ng | 12 | 75±3.6* | 45/60 |
|                           | 0.62 ng/1.16 ng | 24 | 72.5±5.1* | 87/120 |
|                           | 62 pg/116 pg | 24 | 70±4.5* | 84/120 |
|                           | 6.2 pg/11.6 pg | 24 | 45±5.5 | 54/120 |
| (Z)-9-Hexadecenal          | 200 pg    | 12 | 93.3±2.8* | 56/60 |
|                           | 20 pg     | 12 | 93.3±2.8* | 56/60 |
|                           | 2 pg      | 12 | 70±6.7* | 42/60 |
|                           | 0.2 pg    | 12 | 53.3±7.1 | 32/60 |
| Control                   | Solvent only | 12 | 43.3±7.3 | 26/60 |

Asterisks indicate that significantly more ants chose the treated arm over the solvent control (Chi-squared test with one-sided Dunnett-type test: \( \alpha = 0.05 \)).

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Figure 3. Trail choice test using dolichodial/iridomymecin mixture (MDI) plus subthreshold concentration of (Z)-9-hexadecenal. The height of each bar indicates the mean percentage of workers choosing the treated arm (± SEM). Bars with asterisks are significantly different from the solvent-only control (Chi-squared test with one-sided Dunnett-type test: \( \alpha = 0.05 \)). Number within a bar indicates total number of ants choosing the treated arm/total number of ants tested. The concentration shown for the chemical is the amount per cm of trail. Black bar indicates that subthreshold levels of dolichodial/iridomymecin were used for the particular treatment.
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Figure 4. Trail-following response to dolichodial/iridomymecin mixture (MDI) plus (Z)-9-hexadecenal, MDI only, and (Z)-9-hexadecenal only. The height of each bar indicates the mean number of workers following the entire length of 17.3-cm trail drawn on a filter paper with the chemicals (± SEM). Concentrations of the test chemicals were 1.3 ng cm\(^{-1}\) dolichodial, 4.1 ng cm\(^{-1}\) iridomymecin, and 0.7 pg cm\(^{-1}\) (Z)-9-hexadecenal. Bars with different letters are significantly different (ANOVA with Tukey HSD all-pairwise comparison test: \( \alpha = 0.05 \)).
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We did not detect (Z)-9-hexadecenal in the recruitment trails of Argentine ants. However, we do not completely dismiss the notion that Argentine ants could also use (Z)-9-hexadecenal for recruitment for several reasons. First, it is possible that recruiting ants may deposit (Z)-9-hexadecenal in amounts below the detection limit of our current methods. A laboratory test with synthetic (Z)-9-hexadecenal indicated that ~0.1 ng was the limit of detection with the GC-MS (D.-H. Choe, unpublished data). Thus, (Z)-9-hexadecenal existing on the trail with a rate of 20 pg cm⁻² or less would not be detected with our current methods (i.e., 50 cm × 20 pg cm⁻² × 10⁻¹⁰ = 0.1 pg = 0.1 ng). However, we could not detect (Z)-9-hexadecenal even when we analyzed an entire extract obtained from a 350-cm foraging trail (Teflon coated wire), suggesting that concentration of the (Z)-9-hexadecenal in the trail (if it is present) could not exceed 0.3 pg cm⁻². We did not detect (Z)-9-hexadecenal even when we analyzed an extract of a 350-cm foraging trail (Teflon coated wire), suggesting that concentration of the (Z)-9-hexadecenal in the trail (if it is present) could not exceed 0.3 pg cm⁻² (i.e., 0.1 ng/350 cm = 0.0003 ng cm⁻² = 0.3 pg cm⁻²). Second, it is possible that (Z)-9-hexadecenal is not deposited on the substrate, but instead, is released by recruiting ants into the headspace, as a volatile. Because Argentine ants exhibit positive anemotaxis to airborne (Z)-9-hexadecenal [19], it is possible that they can follow an “airborne trail” of (Z)-9-hexadecenal. In a similar way, *Pachycondyla analis* (Latreille) [= *Mayaponera foetens* (Fabr.)] was suspected to release a recruitment pheromone from its pygidial gland into the air, stimulating other nestmates to follow behind the odor plume, which emanates from recruiting ants running in front [51]. Third, it is possible that Argentine ant workers utilize (Z)-9-hexadecenal only inside or around the nest to stimulate recruitment, but not on the recruitment trail. Similar examples can be found in ponerine ants, in which poison gland secretions contain longer-lasting orientation cues, while pygidial gland secretions have strong stimulatory effects on workers in the nest to lead them out [33,51]. This “recruitment activation” process appears to exist in Argentine ants because, after encountering fed workers, previously inactive ants at the nest entrance are likely to follow trails more continuously than ants lacking such encounters [52].

We found that mixtures of dolichodial, iridomyrmecin, and (Z)-9-hexadecenal induced the maximum trail-following behavior. This combined effect might explain, at least in part, why whole gaster extracts and synthetic (Z)-9-hexadecenal were different in their efficacies as a trail. Because the total amounts of dolichodial and iridomyrmecin stored in a single worker are several hundred times higher than that of (Z)-9-hexadecenal, the extraction of whole ants or gasters with an organic solvent will likely yield a large amount of dolichodial and iridomyrmecin, along with a trace amount of (Z)-9-hexadecenal. We speculate that the presence of dolichodial and iridomyrmecin in gaster extracts was responsible for the superior efficacy and longevity of gaster extract trails relative to synthetic (Z)-9-hexadecenal trails, which have been previously reported. Our results also suggest that Argentine ant workers might be able to distinguish their natural recruitment trails, even with an excessive amount of synthetic (Z)-9-hexadecenal in the environment. The unique presence of dolichodial and iridomyrmecin in their natural trails might be at least partially responsible for some level of continued trail formation and recruitment activity reported in several trail disruption trials using synthetic (Z)-9-hexadecenal. The possibility of incorporating the dolichodial and iridomyrmecin in the ant control programs as trail disruptors warrants further study.

The mechanism of secretion and deposition of dolichodial and iridomyrmecin by Argentine ant workers also requires further investigation. The opening of the pygidial gland in Argentine ant worker is located on the posterior end of the gaster between the 6th and 7th abdominal tergites [2,53]. During recruitment, Argentine ant workers typically dab their gasters on the substrate periodically [52]. During this action, a small amount of dolichodial and iridomyrmecin might be released from the pygidial gland and deposited on the substrate as a form of discontinuous line or a series of spots. Another possibility is a "passive" deposition of dolichodial and iridomyrmecin. The chemicals might be spread to other parts of their body (e.g., legs) by self grooming and adsorbed into the cuticular lipids, and subsequently transferred onto the trail when the ants walk on a substrate. This possibility is supported by the fact that trace amounts of iridomyrmecin have been detected in the head and thorax, even though these chemicals are exclusively produced in the gaster [41].

In conclusion, our study provides the first evidence that two pygidial gland chemicals are important constituents of recruitment trails of Argentine ant for foraging and nest relocation, and also validates a direct approach for the study of ant trail pheromones generally. The compositional variations of two known components of trail pheromone of pharaoh ant (*Monomorium pharaonis*) were recently investigated by extracting the substrate on which the ants had walked [57]. However, to our knowledge, no ant species has ever been examined with this approach to discover unknown constituents of the trail pheromone. Given the novelty and importance of the findings that we report here, we anticipate that the same approach will yield new insights into the chemical and behavioral ecology of other ant species.

**Materials and Methods**

**Ants**

Argentine ant colony fragments were collected in Berkeley (2011 Summer) and Riverside (2012 Spring), California, United States, by digging up the nest from the ground. No specific permits were required for the described studies. In the laboratory, the colony was divided into several smaller experimental colonies, and each was placed in a plastic box (20x33x11.5 cm) with soil. The inner walls of the boxes were lined with Fluon to prevent ants from escaping. The experimental colonies contained queens, brood, and workers at the time of the experiments. All colonies were maintained at 21–25°C on a natural light cycle, and provided with 25% (wt/vol) sucrose solution, scrambled eggs supplemented with yeast powder and freeze-killed crickets and cockroaches (*Periplaneta americana*) two or three times a week.

**Collection of chemical trail**

For the Argentine ant colony fragments collected in Berkeley, we used Teflon coated wire (50 cm × 1.5 mm outer diameter Teflon tubing with metal wire inserted in it) as a substrate to collect trail chemicals in two different recruitment scenarios: foraging (*n* = 2) and nest relocation upon flooding (*n* = 5). We also collected foraging trail chemicals (*n* = 3) with solid-phase micro-extraction (SPME) sampling (*Supelco* Inc., 100-µm polydimethylsiloxane (PDMS) or 65-µm polydimethylsiloxane/divinylbenzene (PDMS/DVB)) by allowing ants to recruit their nestmates via an exposed SPME fiber bridge (1 cm in length). For the Argentine ant colonies collected in Riverside, we used Teflon coated wire (70 cm × 2.2 mm outer diameter) as a substrate to collect trail chemicals in foraging scenario only (*n* = 3). For the foraging scenario, we starved a colony for at least three days, then workers were allowed to forage to a sucrose solution feeder by temporarily connecting the colony box with the foraging platform with the Teflon coated wire. For the SPME fiber method, we provided the test colony a glass bridge attached to the bottom of colony box, allowing ants to climb up to the tip, and the tip of the glass bridge was temporarily connected to the foraging platform with a bridge...
of SPME fiber. For the nest relocation scenario, we slowly flooded
a colony by dripping water into the colony box at a rate of one
drop per second. Once evacuation began, we provided a Teflon
coated wire bridge to permit access to a new nest box with dry soil.
In both scenarios, the ants showed active recruitment on the wire
within 30 min. The wire was carefully removed from the setting
while many ants were still using it, and the entire surface of the
wire was extracted with 0.5 ml of methylene chloride. (trail extract).
In the SPME experiment, the SPME sampler was
carefully removed from the apparatus and directly analyzed by
coupled gas chromatography-mass spectrometry (GC-MS). As a
control, we extracted a clean Teflon coated wire that was prepared
in the identical way, but not used by ants (n = 1).

Chemical analysis
The trail extract was concentrated down to ~10 μl under N2
flow, and 1 μl was analyzed by GC-MS. For GC-MS, electron
impact mass spectra (70 eV) were taken with an Agilent 5975C
mass selective detector interfaced to a Agilent 7890A gas
chromatograph fitted with an DB-5 column (30 m × 0.25 mm
inner diameter, Agilent Technologies). Extracts were injected in
splitless mode, with a temperature program of 50°C for 1 min and
then 10°C min⁻¹ to 300°C with 15-min hold. The temperature of
injector and transfer line was 280°C. For the SPME, we set
the GC injector temperature as 250°C, but otherwise the GC setting
was identical. Some quantitative analyses were also conducted
with a Agilent 7890 gas chromatograph equipped with a DB-5
column (30 m × 0.25 mm inner diameter, Agilent Technologies)
and a FID. For both GC-MS and GC analyses, helium was used as
the carrier gas. Compounds in the extracts were identified by
comparison of retention times and mass spectra with those of
authentic standards of natural or synthetic origin. The relative
configurations of dolichodial [40] and iridomyrmecin [54]
produced by Argentine ants have been unequivocally determined
by previous researchers, but the absolute configurations remain
unknown.

We estimated the concentrations of dolichodial and iridomyrmecin
on the trails using external standards of known concentrations.
We also estimated the total quantities of dolichodial, iridomyrmecin, and (Z)-9-hexadecenal in a single worker ant by
extracting 10 workers homogenized in 200-μl methylene chloride.
One or 0.5 μl (0.05- or 0.025-ant equivalent) of the crude extract
was analyzed by GC or GC-MS, and the quantities of chemicals
were determined using external standards.

Behavioral bioassays
A Y-maze bioassay was developed to determine orientation
choice of ants in response to candidate trailpheromone chemicals.
For this study, colony fragments collected in Berkeley were used.
The Y-maze (with three arms 120° apart, 4.5 cm in length, and
5 mm in width) was cut from a filter paper disk (90 mm in
diameter). One arm served as the point of introduction, and the
other two served as treatment and control arms. The treatment
arm was treated with test chemicals dissolved in methylene chloride (3 μl) by evenly spotting the preparation over the arm
with disposable glass pipettes. The control arm received an equal
amount of solvent only. The introduction arm was treated with
two test chemicals and solvent. For the control, all arms were
treated with solvent only. To minimize the possible loss of trail
chemicals due to volatilization during the solvent evaporation, we
always treated control arm side first before treating the treatment
arm side. We allowed the solvent to evaporate for 30 sec, and
placed one treated Y-maze in the ant colony box by leaning it
against a wall. Because the introduction arm was the only part
contacting the soil surface, ants typically traveled upward upon
encountering the introduction arm, and subsequently made a
choice at the junction of the three arms. As soon as the ants
reached the top of either choice arm, we removed them to prevent
recruitment of other ants. We recorded the choices of five ants per
Y-maze, and an individual ant was used only once. We repeated
this bioassay 12 or 24 times for each chemical trail treatment (total
60 or 120 ants) in a blind manner (i.e., the examiner did not know
which arm was treated with test chemicals). The data (i.e.,
proportion of ants choosing the treatment arm) were analyzed by a
Chi-squared test. If the null hypothesis was rejected, the
comparison of a control to each other treatment were conducted
with one-sided Dunnett-type test [55].

To determine the effect of candidate chemicals on continuous
trail following, we conducted a trail-following bioassay with an S-shaped
artificial trail (17.3 cm in length) drawn on a rectangular piece
of filter paper (9×17 cm). For this study, a colony fragment collected
from Riverside was used. The test chemicals dissolved in
methylene chloride (total 12 μl) were applied by evenly spotting
the preparation over a pencil-drawn guideline with disposable
glass pipettes. Based on our preliminary observations, the pencil
lines alone did not induce trail-following. After allowing 30 sec for
the solvent to evaporate, the filter paper with artificial trail was
placed in the foraging area of an large Argentine ant colony box
(86×42×14 cm; containing 30–50 queens, brood, and 3,000–
5,000 workers nesting in soil). Foraging workers on the soil surface
readily discovered the filter paper, climbed on it, and encountered
the trail. Once an ant reached the trail, we started videotaping the
filter paper for 2 min until the trial was terminated. Based on the
video images taken, the number of ants following the entire length
of chemical trail was counted. Different chemicals were alternated
between trials to insure all treatments were tested under similar
activity level of the test colony. Each filter paper with trail was
used only once, and we replicated this bioassay 11 times for each
candidate trail treatment. The data (i.e., number of ants that
followed the entire trail during the first 2 min) were square root
transformed [55], and tested using ANOVA followed by Tukey
HSD all-pairwise comparison test.

Chemicals
Authentic standards of trans, trans-dolichodial (from Anisomorpha
buprestoides) stick insect defensive secretion, referred to as “per-
uphalasmal” in ref. 33) and synthetic cis-trans-iridomyrmecin were
obtained from A.T. Dossey (Gainesville, Florida) and K.R.
Chauhan (USDA-ARS, Beltsville, Maryland), respectively. (Z)-9-
hexadecenal was obtained from Bedoukian Research Inc. (Dan-
bury, Connecticut). These authentic standards of dolichodial,
iridomyrmecin, and (Z)-9-hexadecenal were examined with GC-
MS in prior to bioassays, showing purities of >99, 94, and 97%,
respectively.

Supporting Information
Figure S1 Trail-following bioassay setup to test the
continuous trailing response of workers. Dolichodial/
iridomyrmecin mixture (MDI) plus (Z)-9-hexadecenal, MDI only,
and (Z)-9-hexadecenal only were tested on the trail. The S-shaped
curve was drawn on a rectangular filter paper (9×17 cm) with a
circular template of 5.3-cm diameter. The side of the filter paper
was cut in a triangular shape, so that the encounter of ants with the
chemical trails was fasciliated. The total number of workers which
successfuuly followed the entire trail (red arrows) were recorded for
2 min.
(TIF)
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Author Contributions
Conceived and designed the experiments: DC DBV NDT. Performed the experiments: DC DBV NDT. Analyzed the data: DC. Contributed reagents/materials/analysis tools: NDT. Wrote the paper: DC NDT.