A Caffeic Acid Ester Mediates Host Recognition by a Parasitic Wasp

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Kairomones are semiochemicals that benefit the receiver but are detrimental to the emitter [1]. One important class of kairomones are the semiochemicals utilized by parasitic Hymenoptera to identify their hosts [2]. For example, Luck and Uygur [3] reported that the parasitoid, *Aphids melinus* DeBach (Hymenoptera: Aphelinidae), utilized water- or ethanol-soluble chemicals from covers of the California red scale, *Aonidiella aurantii* (Maskell) (Homoptera: Diaspididae) and other scale species as oviposition-stimulating kairomones, but no compounds were identified. Here, we show (1) that a previously unknown ester of caffeic acid and tyrosine, O-caffeoyltyrosine, is a major component of the kairomone from California red scale, (2) that *A. melinus* responds quantitatively to variation in O-caffeoyltyrosine concentration, and (3) that the dose eliciting peak activity by the wasp corresponds with the amount observed in scale covers when scales are most suitable for parasitization by *A. melinus*.

Bioassays were developed based upon the characteristic “drumming and turning” behavior of wasps as they investigate scale covers [3] and the tendency of adult females to probe attractive disks with their ovipositors. Bioassays were carried out using measured quantities of extracts and test chemicals applied to filter-paper disks 2 mm in diameter. Treated disks plus a solvent-only control disk were placed in a Petri dish, and the solvent was allowed to evaporate. A female wasp, reared on oleander scale, *Aspidiotus nerii* Bouéch [3], was placed in the dish and observed under a dissecting microscope (6 ×) for 10 min. The number of drums and turns and the number and variety of oviposition probes made by each wasp were recorded.

Covers of virgin, third-instar scale, the stage most attractive to *A. melinus* [4–6], were collected by aspiration and placed in 20-ml vials filled with methanol/water (3:1). The vials were placed in an ultrasonic cleaning bath (34°C) and sonicated for 2 h. The extract was vacuum-filtered and washed with additional methanol/water. Particulates were removed by centrifugation (10,000 g for 20 min). The supernatant was partitioned three times against chloroform/hexane (3:1) or pure hexane. These nonpolar fractions were biologically inactive. The biologically active aqueous phase was concentrated by rotary evaporation (55°C) to bring the extract to a standardized concentration of 1 scale-cover equivalent per μl.

The aqueous fraction was acidified with 0.1 ml of 0.075 % trifluoroacetic acid (TFA) in HPLC-grade water and applied to a C18 solid-phase extraction (SPE) column (300 mg, Fisher Scientific) previously equilibrated with 0.075 % TFA. The SPE column was eluted by suction using mixtures of 0.075 % TFA and acetonitrile starting with 0 % acetonitrile and increasing to 50 % acetonitrile in 5 % increments. Fractions eluted with 20 and 25 % acetonitrile were biologically active. These fractions were combined and concentrated until material started to precipitate. Enough acetonitrile was added to keep all material in solution. The active fractions were purified further by preparative reverse-phase HPLC using a C18 column (Alltech “Econosil,” 10 μm particle size, 250 × 10 mm ID) with a Beckman “UltraspHERE ODS” guard column (45 × 4.6 mm ID, 5 μm particle size) monitoring the eluent at 325 nm. The kairomone, which eluted isocratically in 11 min with 25 % acetonitrile in 0.075 % aqueous TFA at 5 ml/min, was collected and concentrated to 1 scale-cover equivalent per μl.

Size exclusion chromatography on polycrylamide gels (Bio-Gel P4, Bio-Rad Laboratories) showed the molecular weight of the kairomone to be less than 500 Da. Hydrolysis under strongly acidic or weakly basic conditions showed that tyrosine was the only amino acid present in the hydrolysate, but the hydrolysate was biologically inactive. Proton NMR in deuterated methanol, UV spectrometry and mass spectrometry using fast-atom bombardment (FAB) and desorption ionization methods suggested that the kairomone was O-caffeoyltyrosine (Fig. 1). This structure was confirmed by synthesis from caffeic acid and L-tyrosine (Sigma Chemical Co.), and the synthetic material was as active biologically as the natural product (Millar and Hare, in prep.).

Although numerous caffeic acid esters occur in plants [7], O-caffeoyltyrosine has not been reported previously. N-Caffeoyltyrosine, the amide of caffeic acid and tyrosine, however, is found in some varieties of coffee beans [8].

Our second objective was to determine how *A. melinus* responded behaviorally.

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Fig. 1. Chemical structure of O-caffeoyltyrosine. Mass spectrometry (desorption electron impact, 50 eV), m/z (relative abundance): 326 (5, M–NH2), 279 (2), 263 (3), 220 (16), 189 (70), 163 (13), 136 (27), 120 (15), 107 (100), 89 (12), 77 (20). High-resolution FAB mass spectrometry, calculated for C19H16NO5 (M + H+): 344.1134; found: 344.1155. Proton NMR (CD3OD): δ 7.72 (d, 1H, J = 16 Hz, olefin H β to carbonyl), 7.35 (d, 2H, J = 8.45 Hz, aromatic H, tyrosine), 7.14 (d, 2H, J = 8.45 Hz, aromatic H, tyrosine), 7.12 (d, 1H, J = 2 Hz, ortho H, caffeic acid), 7.01 (dd, 1H, J = 8.4, 2 Hz, ortho H, caffeic acid), 6.79 (d, 1H, J = 8.4 Hz, meta H, caffeic acid), 6.64 (d, 1H, J = 16 Hz, olefinic H α to carbonyl), 3.76 (dd, 1H, J = 9.1, 4.2 Hz, CH2NH2), 3.34 (dd, 1H, J = 14.5, 4.2 Hz, benzyllic H), 3.01 (dd, 1H, J = 14.6, 9.1 Hz, benzyllic H)
to variation in kairomone concentration. Ten thousand scale covers from late third-instar female California red scale were collected, extracted, and purified as described above. The purified O-caffeoyltyrosine was reconstituted in acetonitrile/water (1:1) at 29.6 ng O-caffeoyltyrosine per µL (1 scale-cover equivalent per µL), and assayed at six concentrations: 0.0 (control), 2.9, 5.9, 14.8, 29.6, and 59.2 ng/disk. Two replicate trials were performed, and a fresh set of disks was prepared for each trial. Twenty-five wasps were bioassayed in each trial; each wasp was used only once. The proportion of the total of each type of behavior (drums and turns and oviposition probes) on each disk was calculated and subjected to ANOVA after applying the arcsine square root transformation [9].

The proportion of drums and turns differed significantly among treatments ($F_{4,6} = 8.03, P = 0.0124, Fig. 2$). The attractiveness of disks increased with increasing concentrations of O-caffeoyltyrosine to 29.6 ng/disk and then declined. The proportion of ovipositions also differed significantly among treatments ($F_{4,6} = 4.66, P = 0.044$) and followed the same pattern (Fig. 2). Additional assays at up to 148 ng/disk confirmed that peak activity occurred at 29.6 ng/disk.

The quantitative response of A. melinus to variation in O-caffeoyltyrosine concentration suggests that any factors causing variation in kairomone concentration among scale covers could contribute to the variation in susceptibility of scales to parasitization. In particular, scale susceptibility to A. melinus is known to vary with age. Mature adults and very young scales are unsuitable for A. melinus, while virgin third-instar females are more susceptible than second instars [4-6]. Therefore, we determined how the concentration of O-caffeoyltyrosine varied with scale age and scale cover growth. Groups of between 20 and 300 scale covers, depending upon life stage, from even-aged scale reared in a laboratory colony were removed manually and collected in preweighed glass centrifuge tubes. The length and width of 20 randomly selected scale covers were measured as they were collected, and the net weight of all scale covers was determined after all were collected. The total number of scale covers collected per life stage was varied to provide a total mass of at least 0.5 mg. Four replicate groups of covers per scale age class were collected and extracted as follows: 1 ml of extraction solvent, consisting of 750 µl methanol, 240 µl 0.075% aqueous TFA, and 10 µl of a stock internal standard solution of 3.7 mg ferulic acid (the 3-methoxy analog of caffeic acid) per 100 ml of 0.075% TFA was added to the centrifuge tube containing the scale covers, and the tube was placed in an ultrasonic cleaning bath and sonicated for 2 h. Extracts were concentrated by evaporation to ≤250 µl to remove the methanol and brought back to a volume of 1 ml with aqueous 0.075% TFA. A SPE column was prepared as described above, and the extract was loaded onto the column. The kairomone and internal standard were eluted by suction with 1 ml of 50% acetonitrile in 0.075% TFA.

The concentration of O-caffeoyltyrosine was determined by isocratic HPLC relative to the internal standard using a Phenomenex "Ultramex 5" C18 column (250×4.6 mm ID, 5 µm particle size, 25% acetonitrile in 0.075% aqueous TFA at 1.2 ml/min). Scale cover area was calculated using the formula for the area of an ellipse. The physiological age of scale at the time of collection (degree-days) was determined using a temperature-dependent development model of California red scale growth [10].

The concentration of O-caffeoyltyrosine in covers remained below 60 ng/mg until the beginning of the third instar. Concentrations then increased to 509 ± 92 (SE) ng/mg at the end of the third instar, when the scale covers had nearly reached their maximum size (Fig. 3). This peak concentration was equivalent to 21.3 ± 1.4 ng O-caffeoyltyrosine per cover, or slightly more than 70% of the concentration used in our dose-response studies. O-caffeoyltyrosine concentrations then declined as the scales matured further. We were unable to determine accurately the concentrations of O-caffeoyltyrosine in older scale covers (>550 degree-days) because the cover becomes attached to the bodies of the scale in the adult stage [11] and cannot be removed. Scale covers do not grow continuously [11, 12] (Fig. 3). Rather, new material, secreted by glands in the pygidium, is deposited at the cover margins during the first, second, and third instars, then the scale body grows beneath the enlarged cover [11]. Covers of California red scale have long been known to be composed of about half waxes and half "proteinaceous material" [11]. The latter material is a large (> 200,000 Da) tyrosine-rich, melanin-like compound [13]. We suggest that O-caffeoyltyrosine is a precursor of this melanin-like compound. The vicinal hydroxyl

![Fig. 2. Relative attractiveness of filter-paper disks treated with the indicated doses of O-caffeoyltyrosine. Bars indicate the mean (± standard error) percentage of drums and turns (filled bars) and oviposition probes (open bars) observed on disks at five dosages.](image-url)

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![Fig. 3. Concentration of O-caffeoyltyrosine in California red scale covers as a function of scale age and size. ○ Mean (± SE) O-caffeoyltyrosine concentration, ● mean (± SE) scale cover area. Scale cover growth occurs during the first instar (not shown), second instar (ca. 180-250 degree-days), and third instar (ca. 340-450 degree-days). No scale cover growth occurs during the intervening molt stages or after the scale reaches maturity (> 475 degree-days).](image-url)
groups on the caffeic acid moiety may become oxidized to quinones, which then react with other functional groups, as is the case with other dihydroxyphenols involved in melanin formation [14].

By utilizing a precursor of California red scale's cover, and responding in a dose-dependent manner, *A. melinus* may facilitate the identification of the most suitable scale life stages for parasitization. However, scale body and cover size vary with the citrus cultivar and substrate upon which scales grow, and scale cover size is more variable than body size [12]. We therefore predict that any environmental factors affecting scale cover growth will affect the concentration of O-caffeoyltyrosine in those covers, thereby altering the susceptibility of scales to attack by *A. melinus*.

Finally, other *Aphytis* species attacking other armored scale species may be expected to utilize similar chemicals for host recognition. However, a related species, *A. yanonensis* DeBach, utilizes components of the nonpolar wax from the cover of its host, the arrowhead scale, *Unaspis yanonensis* Kuwana (Homoptera: Diaspididae) as its host recognition kairomone [15]. Thus, the potential range of compounds and their chemical properties utilized for host recognition by related parasitoids may be surprisingly broad.

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