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Origin of *Epilachna paenulata* defensive alkaloids: Incorporation of [1-^{13}C]-sodium acetate and [methyl-^{2}H3]-stearic acid

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This paper is dedicated to the memory of Prof. Thomas Eisner (1929–2011), one of the most inspiring scientists in the field of insect biochemistry and chemical ecology.

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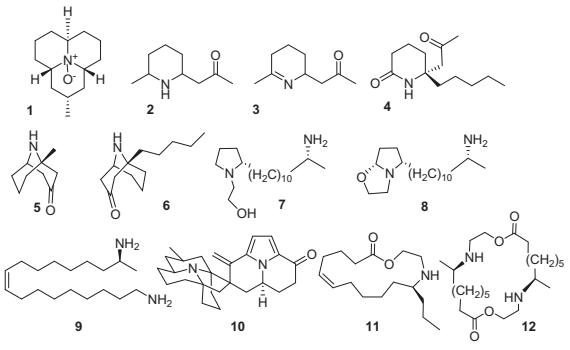
ABSTRACT

Ladybird beetles produce a large number of defensive alkaloids. Previous studies suggest that the structural diversity of these endogenous alkaloids can be traced to a common biosynthetic route based on the condensation of several acetate units. In this study, adults of *Epilachna paenulata*, a phytophagous neotropical species, were fed on diet enriched with potential precursors (sodium acetate, fatty acids and the amino acids lysine and ornithine) labeled with stable isotopes (^{13}C, ^{2}H and ^{15}N). Labeled acetate was incorporated into the structurally related homotropane and piperidine alkaloids. The later also showed incorporation of [methyl-^{2}H3] stearic acid. Our results hence support a fatty acid pathway for the biosynthesis of *E. paenulata* alkaloids. To our knowledge, this is the first report on the incorporation of a labeled fatty acid into a defensive piperidine alkaloid in insects.

1. Introduction

Insects as a group are able to produce many structurally different secondary metabolites for their defense (Laurent et al., 2005). Despite the large number of studies focusing on the chemical and biological characterization of these allomones, fewer investigations have addressed their biosynthetic origin. A review on the subject has been published recently (Laurent et al., 2003). Coccinellid beetles produce a large number of defensive alkaloids, including perhydroazaphenalenes (**1**), piperidines (**2**–**4**), homotropanes (**5** and **6**), pyrrolidines (**7** and **8**), linear amines (**9**), dimeric alkaloids such as chilocorine (**10**), and azamacrolides (**11** and **12**) (King and Meinwald, 1996). Although structurally diverse, ladybird alkaloids appear to share a common biosynthetic route, comprising the condensation of several acetate units, possibly through a fatty acid pathway. Indeed, experiments with stable isotopes showed that the azamacrolid epilachnene (**11**), from the pupal secretion of the Mexican bean beetle, *Epilachna varivestis*, is biosynthesized from oleic acid and L-serine (Attygalle et al., 1994). In *Adalia 2-punctata*, the biosynthesis of alkaloids adaline (**6**) and adalinine (**4**) follows a common pathway as incorporation of [^{2}H11] adaline in adalinine was proved when beetles were fed on labeled adaline (Laurent et al., 2001). These two alkaloids incorporated 1-^{14}C and 2-^{14}C acetate, in a proportion that indicated the incorporation of seven acetate units (Laurent et al., 2002, 2001). These observations support a polyacetate origin for these alkaloids, as previously suggested by Tursch et al. (1975) for coccinelline (**1**), a related perhydroazaphenalene. Moreover, adaline was found to be produced in the fat body of *Adalia 2-punctata*, and its biosynthesis was inhibited by 2-octynoic acid, an inhibitor of the fatty acid pathway (Laurent et al., 2002). Further, an experiment in absence of molecular oxygen, which is required if the fatty acid pathway is operating, showed a much slower incorporation of the radioactive acetate than when oxygen was present (Laurent et al., 2002). The evidence hence suggests that these alkaloids are produced through a fatty acid pathway, although in the case of adaline and adalinine, experiments involving the incorporation of labeled fatty acids per se have not been reported, and therefore there is no clear distinction between the fatty acid and polyacetate pathways.



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Epilachna paenulata Germar 1824 (Coleoptera: Coccinellidae: Epilachninae) is a phytophagous South American ladybird beetle that feeds on the leaves of cucurbits. The adults exhibit defensive capacity against generalist predators, due to the presence of endogenous systemic alkaloids (Camarano et al., 2009) that have been characterized as a mixture of piperidine (**2** and **3**), homotropane (**5**), and pyrrolidine alkaloids (**7** and **8**) (Camarano et al., 2006 and unpublished results). These alkaloids are present in all life stages of the insect, albeit showing qualitative and quantitative variations among developmental stages (Camarano et al., 2006).

Our previous studies on the biosynthesis of the defensive alkaloids in *E. paenulata* indicated that the homotropane **5** and the piperidines **2** and **3** incorporated up to five units of 2-^{13}C acetate (Camarano et al., 2009). Here we report further incorporation experiments that demonstrate that the piperidine **3**, and hence most likely all defensive alkaloids in *E. paenulata*, are biosynthesized by the fatty acid pathway (Fig. 1).

2. Material and methods

2.1. Insects

E. paenulata adults were obtained from a laboratory colony regularly maintained on squash plants (*Cucurbita* sp.), under controlled conditions of temperature (23 ± 2) °C and photoperiod (L:D, 16:8). For the initial settlement of the colony, individuals were collected on squash plants at organic farms near Montevideo, and new field-collected individuals were added every year. Individual life stages were maintained separately in screened cages (30 × 30 × 30 cm), where plants (9–12, 3 weeks old) were renewed every 3–4 days.

2.2. Labeled chemicals

All labeled precursors were from Cambridge Isotope Laboratories, Inc., with the following isotopic purities: [1-^{13}C]-sodium acetate (99%), [^{15}N2] L-ornithine (98%), [^{15}N2] L-lysine (98%), [9,10-^{2}H2] oleic acid (98%), [^{2}H35] stearic acid (98%) and [methyl-^{2}H3] stearic acid (98%).

2.3. Alkaloid labeling

Adult beetles were individually fed on squash leaf disks (2.5 cm diam.) in Petri dishes (4.5 cm diam.) coated with a layer of 2% agar, and kept at (23 ± 2) °C and 16:8, L:D. The leaf disks were treated topically with 80 μl of labeled precursor solution, and renewed every day. Control beetles were similarly fed with solvent-treated leaf disks. The labeled precursors were applied as follows: [1-^{13}C]-sodium acetate at 40 mg/ml in MeOH; [^{2}H35] stearic acid at 10 mg/mL in diethyl ether or methylene chloride; [methyl-^{2}H3] stearic acid at 40 mg/mL in methylene chloride; [9,10-^{2}H2] oleic acid at 10 mg/mL in ethyl ether; and the amino acids at 20 mg/mL in water. All incorporation experiments were done with newly emerged adults.

Labeled acetates and amino acids were fed to the beetles for 4 days, while fatty acids were offered for 14 days. The day following the feeding period, either hemolymph samples were obtained by reflex bleeding (Camarano et al., 2009) or the beetles were individually frozen (−20 °C) until whole-body extractions were performed. Samples from labeled acetate feeding experiments were analyzed by NMR and GC/MS. All other samples (labeled fatty acid and amino acids) were analyzed by GC/MS. A summary of the methodology is shown in Table 1.

2.4. Alkaloid extraction

Three procedures were carried out, depending on the matrix and sample size: (1) pooled frozen adults were extracted under stirring with methanol (3 h, 25 mL); the extracts were then filtered, dried under vaccum, re-suspended in HCl (0.2 M, 25 mL) and washed with hexane (3 × 15 mL). The pH of the remaining aqueous phase was increased to 10 (NaOH, 1 M) and the alkaloids were extracted with methylene chloride (3 × 15 mL). The final extract was concentrated under vacuum at room temperature; (2) individual whole body extracts were obtained under stirring with methanol (24 h, 1 mL), centrifuged and purified on ionic exchange SPE columns (Sulphonic) in three steps [(i). 1 mL MeOH: HCl 2 N (98:2); (ii). 1 mL MeOH and (iii). 2 mL MeOH: NH4OH (91:9)]; and (3) hemolymph samples were extracted by suspending freshly collected hemolymph in 200 μL ammonium chloride (pH 8) and extracting the basic compounds with methylene chloride (3 × 150 μL).

2.5. Analytical procedures

NMR spectra of alkaloid extracts were recorded in $CDCl_3$ on a Bruker Avance DPX-400 spectrometer operating at 400.13 and 100.62 MHz for ^{1}H and ^{13}C, respectively. In order to assign ^{13}C signals within the complex alkaloids extracts, HSQC and DEPT experiments were carried out to correlate ^{1}H and ^{13}C signals. Acquisition was first done by zg30 (^{1}H) and zgpg30 (^{13}C) pulses. In an attempt to improve sensitivity, a second experiment with a zgig30 pulse (relaxation time, 20 s) was carried out (Guillermo Moyna, pers. com.). Following both procedures, ^{13}C signals were clearly detected and assigned only for euphococcinine (**5**). However, comparisons with the corresponding control beetle samples showed no evidence of differential enrichment in any of the ^{13}C signals when [1-^{13}C]-sodium acetate was fed to the beetles.

Extracts of pooled individuals were also analyzed by GC–MS on a HP 5791 [Elite-5; 30 m, 0.32 mm i.d., 0.50 μm film thickness; splitless injection in CH_2Cl_2), 60 °C (4 min) −300 °C (8 min) at 10 °C/min. Injector and interphase temperature: 300 °C]. In the case of hemolymph extracts, GC–MS analyses were performed on a Shimadzu QP 5050 or a Shimadzu QP 2010 [Carbowax 20 M; 25 m, 0.32 mm i.d., 0.50 μm film thickness; splitless injection in CH_2Cl_2, 60 °C (6 min)–240 °C (20 min) at 10 °C/min]. Injector and interphase temperature: 250 °C]. In all GC–MS analyses, detection of the alkaloids in hemolymph extracts was achieved by both total ion current (TIC) and single ion monitoring (SIM) of the characteristic ion clusters of the alkaloids: 98–99–100–101–102–103 (characteristic of alkaloid **1**), 110–111–112–113–114–115–116 (characteristic of alkaloids **2** and **3**), 153–154–155–156–157–158–159–160 [cluster including the M^+ ions of **1** (m/z = 155), **2** and **3** (m/z = 153) (Camarano et al., 2006, 2009). Validation of using the SIM and TIC detection modes for incorporation studies had been previously obtained by comparing results from samples run under both detection modes, with no statistically significant differences in the incorporation percentages obtained. However, ions with lower abundances, such as those corresponding to molecules with many labels, can only be detected by the SIM mode (Camarano et al., 2009). Calculations of the enrichment of alkaloid with ^{13}C were performed using freely available software (http://sx102a.niddk.nih.gov/iso.html, accessed 2007) (Hess et al., 2002). The program calculates the enrichment as MPE% (mol percentage excess of the incorporated isotope, ^{13}C in this particular case), as well as the maximum number of labels incorporated in a sample, comparing MS data from isotope-enriched and control samples.