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exo-Brevicomin Biosynthesis in the Fat Body of the Mountain Pine Beetle, *Dendroctonus ponderosae*

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Abstract *exo*-Brevicomin (*exo*-7-ethyl-5-methyl-6,8-dioxabicyclo[3.2.1]octane) is an important semiochemical for a number of beetle species, including the highly destructive mountain pine beetle, *Dendroctonus ponderosae*. It also has been found in other insects and even in the African elephant. Despite its significance, little is known about its biosynthesis. In order to fill this gap and to identify new molecular targets for potential pest management methods, we performed gas chromatography–mass spectrometry analyses of cell cultures and *in vitro* assays of various *D. ponderosae* tissues with *exo*-brevicomin intermediates, analogs, and inhibitors. We confirmed that *exo*-brevicomin was synthesized by "unfed" males after emerging from the brood tree. Furthermore, in contrast to the paradigm established for biosynthesis of monoterpenoid pheromone components in bark beetles, *exo*-brevicomin was produced in the fat body, and not in the anterior midgut. The first committed step involves decarboxylation or decarbonylation of ω-3-decenoic acid, which is derived from a longer-chain precursor via β-oxidation, to (*Z*)-6-nonen-2-ol. This secondary alcohol is converted to the known precursor, (*Z*)-6-nonen-2-one, and further epoxidized by a cytochrome P450 to 6,7-epoxynonan-2-one. The keto-epoxide is stable at physiological pH, suggesting that its final cyclization to form *exo*-brevicomin is enzyme-catalyzed. *exo*-Brevicomin production is unusual in that tissue not derived from ectoderm apparently is involved.

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Introduction

exo-Brevicomin (*exo*-7-ethyl-5-methyl-6,8-dioxabicyclo[3.2.1]octane) was first identified as a sex pheromone in the western pine beetle, *Dendroctonus brevicomis* (Silverstein et al. 1968) and has since has been shown to be a semiochemical for several other *Dendroctonus* beetles, (El-Sayed 2013; Wood 1982). It has been reported to be present in other insects (Borden et al. 1987; Perez et al. 1996) and even in the African elephant (Goodwin et al. 2006). Male *D. ponderosae* produce *exo*-brevicomin as they emerge from a brood tree, with production declining when they arrive at a new host tree and mate with pioneer females (Libbey et al. 1985; Pureswaran et al. 2000).

The biosynthetic steps to *exo*-brevicomin are not well understood. For reference, the proposed pathway is herein divided into five steps (Fig. 1, Roman numerals). Vanderwel et al. (1992) determined that (*Z*)-6-nonen-2-one (Step III) is an intermediate. However, the source of (*Z*)-6-nonen-2-one is unclear, with contributions from tree, fungal, or endogenous metabolism being proposed (Borden 1985; Vanderwel and Oehlschlager 1987). A more likely possibility is *de novo* synthesis or modification of a ω-3 mono-unsaturated fatty acyl-CoA precursor to a (*Z*)-7-decenoyl intermediate (Step I) (Vanderwel et al. 1992). This 10-carbon precursor may be oxidized to a (*Z*)-3-keto-7-decenoyl intermediate by β-oxidation (Step II, left side) (Skiba and Jackson 1994). Alternatively, (*Z*)-7-decenoyl-CoA could be converted to (*Z*)-3-nonene via the oxidative decarbonylase pathway (Step II, right side) (Qiu et al. 2012), then hydroxylated to the secondary alcohol and oxidized to (*Z*)-6-nonen-2-one (Step III). Conversion of (*Z*)-6-nonen-2-one to *exo*-brevicomin probably

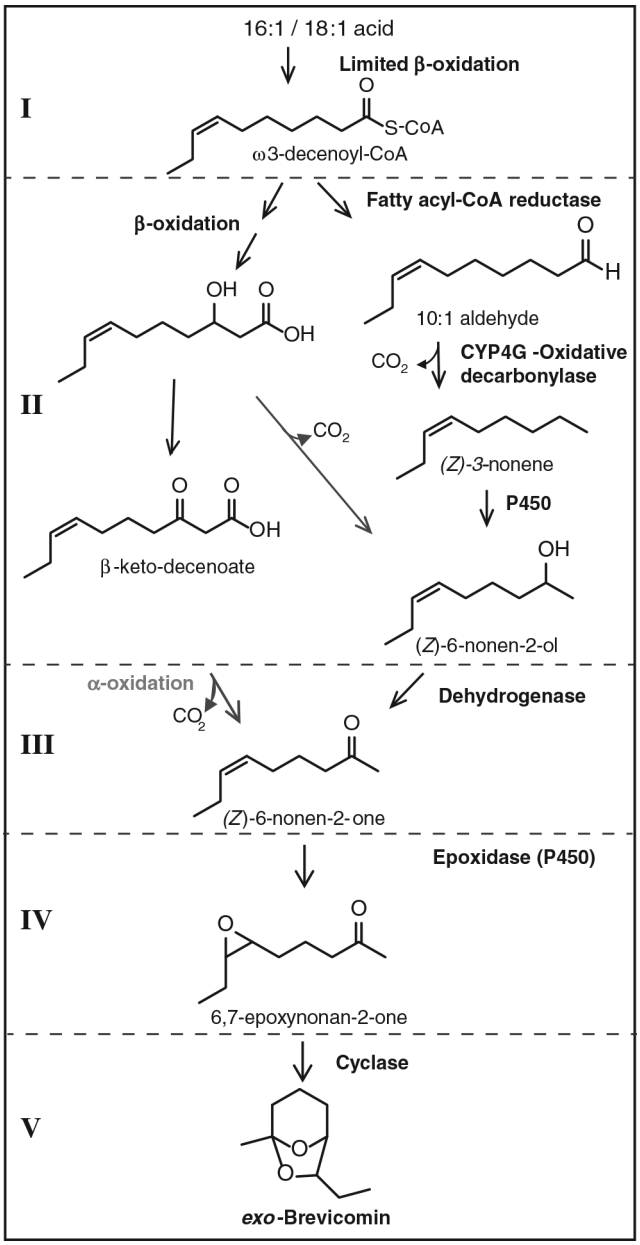

Fig. 1 Proposed biosynthetic pathway to *exo*-Brevicomin. For Step II, the 3-nonene intermediate may arise from decarboxylation of β-oxidation intermediates by α-oxidation (left, *grey arrows*), or via hydrocarbon biosynthesis followed by hydroxylation to 3-nonen-2-ol and oxidation by a dehydrogenase (right, *solid arrows*)

involves cytochrome P450-mediated formation of a keto-epoxide intermediate (Step IV) that is subsequently cyclized (Step V). However, the biochemical source of (*Z*)-6-nonen-2-one and the location of its synthesis and conversion to *exo*-brevicomin remain unknown. Understanding bark beetle pheromone production may guide future management strategies by identifying new molecular targets and helping our understanding of how pheromone systems evolve.

In order to address these issues, we performed *in vivo* experiments with isolated tissues. Based on precedents (Barkawi et al. 2003; Hall et al. 2002a, b), we hypothesized that *exo*-brevicomin is synthesized in the midgut of unfed males that have just emerged from the brood tree. Here, we confirm that *exo*-brevicomin is present in unfed males, and not in females, and show that *exo*-brevicomin is produced by male fat bodies, but not by carcasses or digestive tissue.

Methods and Materials

Reagents and Chemicals Hink's TNM-FH Medium (1x Supplemented Grace's Medium) was from Mediatech, Inc. (Herndon, VA, USA). Unlabeled (*Z*)-6-nonen-2-one and *exo*-brevicomin were purchased from PheroTech Inc. (Delta, BC, Canada). Agarose was from Bio-Express (Keysville, UT, USA). NAD^+, $NADP^+$, NADPH, piperonyl butoxide (PBO), [10,10,10-D_3]-Decanoic acid, BF_3/MeOH, phenylmethylsulfonyl fluoride (PMSF), and protease inhibitor cocktail were from Sigma-Aldrich (St. Louis, MO, USA). 2X NADPH regeneration system was from AAT Bioquest (Sunnyvale, CA, USA).

(*Z*)-[1,1,1,3,3-6-D_5]-6-Nonen-2-one and the corresponding pentadeuterated epoxide and (*Z*)-6-nonen-2-ol were prepared through a base catalyzed deuterium-hydrogen exchange of (*Z*)-6-nonen-2-one, and used without further purification. (*Z*)-6-Nonen-2-one (0.100 g, 0.713 mmol) was added dropwise to a solution of CD_3ONa in CD_3OD (~ 1.0 M) at 0 °C. After stirring for 1 hr, the reaction was diluted with D_2O (3 ml), extracted with diethyl ether (2×3 ml). The ether layer was dried with anhydrous Na_2SO_4 and then concentrated under reduced pressure to provide (*Z*)-[1,1,1,3,3-D_5]-6-nonen-2-one [0.0574 g, 55.7 %, ≥ 95 % purity (~ 3 % D_3) via GC/MS) as a yellow oil that was used without further purification. To prepare deuterium-labeled (Z)-6-nonen-2-ol, (*Z*)-[1,1,1,3,3-D_5]-6-nonen-2-one (0.030 g, 0.207 mmol) was dissolved in CD_3OD (1 ml), and sodium borohydride (0.018 g, 0.414 mmol) was added slowly at 0 °C. The mixture was left to stir at 0 °C for 25 min, quenched with saturated sodium bicarbonate (0.85 ml) and dichloromethane (1.25 ml), and stirred for 5 min at 0 °C. The biphasic mixture was warmed to room temperature and extracted with dichloromethane (4×2 ml). The combined organic phase was dried over anhydrous Na_2SO_4, filtered through cotton wool, and concentrated under reduced pressure to provide (*Z*)-[1,1,1,3,3-D_5]-6-nonen-2-ol as a light yellow oil (0.0153 g, 50.2 %, ≥ 95 % purity via GC/MS) that was used without further purification.

The deuterated keto-epoxide was prepared by in 7 % yield by the oxidation of (*Z*)-[1,1,1,3,3-6-D_5]-6-nonen-2-one using *meta*-chloroperoxybenzoic acid. *meta*-Chloroperoxybenzoic acid (0.0840 g, 0.379 mmol, 77 % purity) was added to a solution of (Z)-[1,1,1,3,3-6-D_5]-6-nonen-2-one (0.050 g, 0.34 mmol) in dichloromethane (1.4 ml) at 0 °C. After stirring

for 24 hr at room temperature, the reaction was diluted with dichloromethane and washed with saturated sodium bicarbonate. The organic layer was dried with anhydrous Na_2SO_4 and concentrated under reduced pressure to provide the crude product as a light yellow oil. The crude product was purified by flash column chromatography (pentane:ether) using neutral aluminum oxide to yield the product [1,1,1,3,3-6-D_5]-6,7-epoxynonan-2-one (0.004 g, 7.3 %) as a clear oil.

Beetles Mountain pine beetles were obtained from ponderosa pine bolts collected from the Sierra Nevada in California and Nevada, USA, and maintained as described previously (Aw et al. 2010). Only healthy beetles were selected for experiments, and all groups of beetles were dissected on the same day. For unfed samples, males and females were held separately in plastic cups with the lids allowing air and moisture to enter, and were covered with moist paper in the dark at room temperature overnight as described by Keeling et al. (2004). For tissue distribution studies, males were starved overnight and then dissected under a microscope in 100 mM sodium phosphate buffer pH 7.8. Fat bodies, midguts, and carcasses were transferred into 0.5 ml ice-cold cell lysate buffer (100 mM sodium phosphate pH 7.8, 1.1 mM EDTA, 0.1 mM DTT, 0.5 mM PMSF, 1/1,000 v/v Sigma protease inhibitor cocktail, 20 % glycerol) or culture medium (20 μg/ml gentamycin, 4/1,000 v/v Sigma protease inhibitor cocktail, 15 μg/ml tetracycline, 1 mM PMSF and 12 mM glucose in Hink's TNM-FH medium) and used immediately. Ten beetles were used for carcass and midgut groups, and 20 beetles for fat body groups for each replicate, with four replicates in total. To check *exo*-brevicomin production in males or females, whole bodies from seven insects were pooled for each of three replicates for each group.

exo-Brevicomin Extraction Whole bodies or isolated fat bodies, midguts, and carcasses were homogenized in 0.5 ml ice-cold cell lysate buffer and extracted twice with pentane: ether (1:1) spiked with 250 ng/ml 1-octanol as internal standard. The organic phase was collected and concentrated to approximately 100 μl with N_2 gas, then directly analyzed by GC/MS at the Nevada Proteomics Center (UNR). A Thermo Finnigan Polaris Q ion trap was used with a molecular weight scanning range of 40–180 atomic mass unit (amu) at an ionization potential of 70 eV. A Trace gas chromatograph containing a 60 m×0.25 mm (ID), 0.25 μm film thickness Agilent HP–INNOWAX column (P/N 19091N-136, J&W Scientific, Palo Alto, CA, USA) was programmed for an initial temperature of 50 °C (1 min hold) and increased to 240 °C at 5 °C/min (20 min hold). The injector was split (10:1) at 225 °C with a column flow of 1.2 ml He/min. The detector was set at 200 °C. *exo*-Brevicomin produced by beetles was determined by comparing GC retention times and MS fragmentation patterns with an authentic standard, and was quantified according to the concentration of internal standard (1-octanol) and the ratio of areas of GC traces of *exo*-brevicomin and 1-octanol. The total protein in each reaction was quantified by BCA assay (Thermo Scientific, Rockford, IL, USA) according to the supplier's protocol using BSA as standard. *exo*-Brevicomin levels were calculated as ng *exo*-brevicomin per mg total protein.

Tissue Culture Assay Isolated fat bodies from unfed males were pooled and cultured with or without 23.3 mM (2 mg) [10,10,10-D_3]-decanoic acid in 0.5 ml culture medium in a six-well tissue culture plate at 27 °C in a moist chamber overnight. [10,10,10-D_3]-Decanoic acid (2 mg) in 0.5 ml culture medium without tissue was used as a control. Samples were extracted as per Bligh and Dyer (1959). Thus, the overnight cultures were mixed with 0.5 ml extraction solvent ($CHCl_3$: MeOH: H_2O 1: 2: 0.8), homogenized, and centrifuged at 10,000 × *g* for 2 min at 4 °C in a microcentrifuge to remove debris. The supernatants were transferred into clean glass tubes and extracted with $CHCl_3$. The organic phase was placed in a 60 °C sand bath and dried to near dryness under N_2. Methanolic NaOH (100 μl; 0.5 N NaOH in methanol) was added, and the samples were incubated at room temperature for 5–10 min to dissolve lipids. This mixture was transferred into a clean screw cap glass tube, mixed with an equal volume of 14 % BF_3 in methanol, and then heated in a 60 °C sand bath for approximately 1 hr. Saturated NaCl (0.5 ml) was added, and the mixture was extracted twice with pentane. The organic phase was concentrated under N_2, and analyzed by GC/MS. All steps after the first centrifugation were performed in glass containers. Twenty beetles were used for each replicate, with three replicates in total.

Enzyme Assays Fat bodies were isolated from unfed males and homogenized as described above. Reactions with unlabeled (*Z*)-6-nonen-2-one were conducted in 500 μl volumes containing 468 μl of homogenized fat bodies, 284 μM unlabeled substrate and 300 μM NADPH (final concentrations). Reactions (*N*=3) were incubated for 30 min in a 30 °C water bath. Reactions with (*Z*)-[1,1,1,3,3-D_5]-6-nonen-2-one (284 μM final concentration) were performed in 500 μl volumes containing 250 μl of homogenized fat bodies and 250 μl 2X NADPH regeneration system with 6 hr incubation in a 30 °C water bath (*N*=1). For reactions in the presence of P450 inhibitor, homogenized fat bodies were incubated with 2.5 mM PBO at room temperature for 30 min before performing enzyme assays. Fat bodies without PBO also were incubated at room temperature for 30 min. Fat bodies with or without PBO were mixed with the substrate, and reactions were initiated with the addition of NADPH or 2 X NADPH regeneration system. Experimental conditions included reactions run without the substrate. Assays then were extracted twice with pentane: ether and analyzed by GC/MS as

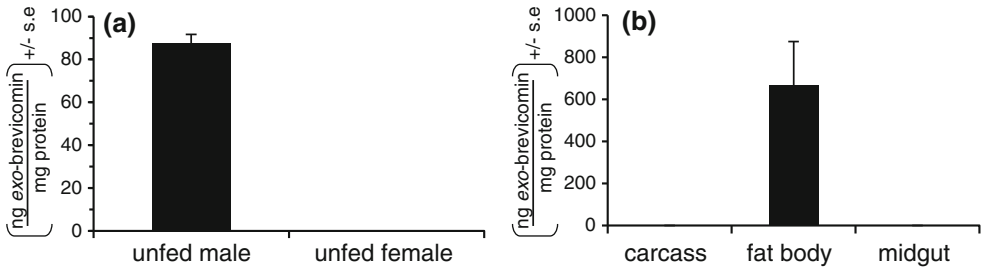

Fig. 2 GC/MS analyses of extracts from whole body homogenates (**a**) or isolated tissues (**b**). *exo*-Brevicomin was detected in unfed males, but not females (**a**), and only in fat bodies, but not midguts or carcass (remainder of tissue) of unfed males (**b**). Each sample contained seven pooled beetles (**a**) or tissues pooled from 10 to 20 unfed males (**b**). Values are means ± standard errors, *N*=3

described above. For assays with unlabeled substrate, 12 beetles/sample/replicate were used.

When the [1,1,1,3,3-D_5]-6,7-epoxynonan-2-one was used as the substrate (*N*=1), enzyme assays contained 468 μl homogenized fat bodies, 600 μM labeled epoxide, with or without 300 μM NAD^+ or $NADP^+$. The mixtures were incubated in a 30 °C water bath for 2 hr. Experimental conditions included fat bodies only and fat bodies with NAD^+ or $NADP^+$ in the presence of epoxide. The reactions were terminated and extracted with pentane:ether and analyzed by GC/MS as described above. Products were identified by comparing retention times and mass spectra with their controls and an authentic standard of [1,1,1,3,3-D_5]-6,7-epoxynonan-2-one or unlabeled *exo*-brevicomin. The percentage conversion of substrate to product was calculated from the areas of GC traces by dividing the amount of product by the total amount of products and substrates (product/(products + substrates)).

Epoxide Cyclization The reaction mixture in pentane:ether from assays in which fat bodies were incubated with (*Z*)-[1,1,1,3,3-D_5]-6-nonen-2-one as described above was treated with acetic acid at different pH values. Briefly, 50 μl of sample in pentane: ether were mixed well with 200 μl water, followed by incubating separately with either 200 μl glacial acetic acid, or acetic acid to pH 3.0, 4.0, 5.0, or 6.0 for 2 hr at room temperature. Each sample was extracted twice with pentane: ether, and analyzed by GC/MS as described above. The untreated reaction mixture was used as a control.

Results and Discussion

Tissue Distribution Extracts of whole insects revealed *exo*-brevicomin in unfed males (average 87.6±4.1 ng/mg protein, *N*=3), but not in females (Fig. 2a). *exo*-Brevicomin was detected in male fat bodies (approximately 668±207 ng/mg protein), but not in male carcasses or midguts (Fig. 2b). This was surprising as numerous studies have located bark beetle pheromone component biosynthesis in the anterior midgut (Blomquist et al. 2010). The difference may be due to the fact that previous studies investigated terpenoid pheromone components, whereas *exo*-brevicomin is fatty acid-derived. Thus, different pheromone components may be biosynthesized in different tissues. While it is possible that all or portions of *exo*-brevicomin biosynthesis are done in other tissues, it is unlikely because the fat body

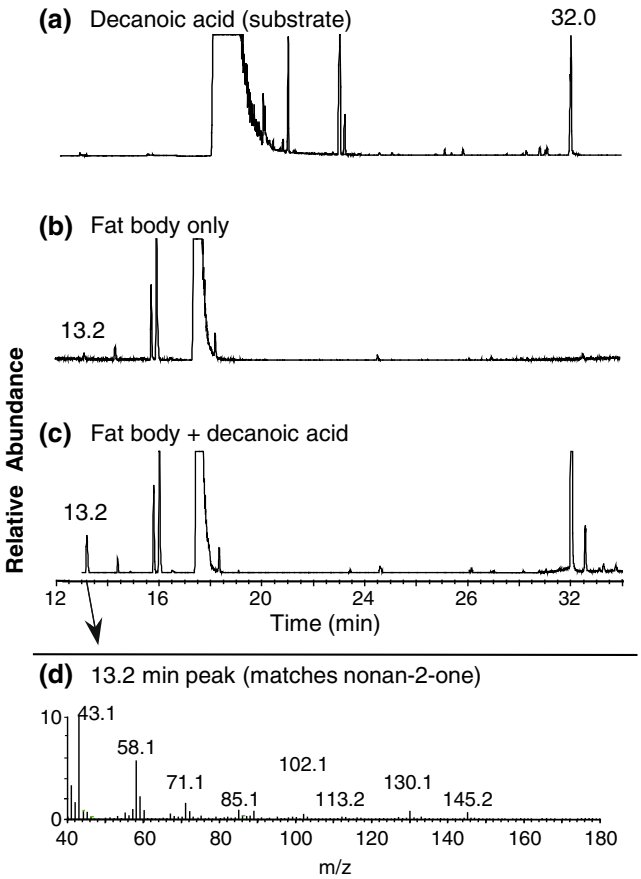

Fig. 3 GC/MS analyses of extracts of unfed male fat body tissue cultures incubated with decanoic acid (ω-3-decenoic acid analog) as substrate. Traces show single ion monitoring at *m/z*=43. The peak at 17.58 min is 1-octanol (internal standard). **a** substrate standard at 32.0 min; **b** negative control; **c** experimental sample. **d** Mass spectrum of the product at 13.2 min matching that of synthetic 2-nonanone

catalyzes several reactions (see below). Pheromone production in the fat body, which is derived from mesoderm, would be unprecedented. However, we note that ectodermally-derived cells, including oenocytes, are sometimes associated with the organ (Lockey 1988), making it a heterogeneous tissue (Arrese and Soulages 2010). We noted fat body heterogeneity during dissections. For example, some portions appeared more tightly connected to the midgut than others (data not shown). Thus, we speculate that *exo*-brevicomin biosynthesis may be sub-compartmentalized in the fat body. Interestingly, (*Z*)-6-nonen-2-one, the precursor of *exo*-brevicomin, was not detected in any beetles or tissues (data not shown). This result likely reflects low (*Z*)-6-nonen-2-one concentrations and/or limits of our assay.

Biosynthetic Pathway We used a series of assays to investigate each of the five steps committed to *exo*-brevicomin biosynthesis, as outlined in Fig. 1.

Step I

There are at least two possible routes to the 10–carbon fatty acyl intermediate, ω-3-decenoyl-CoA. One is from a long chain (16 or 18-carbon) fatty acyl precursor through

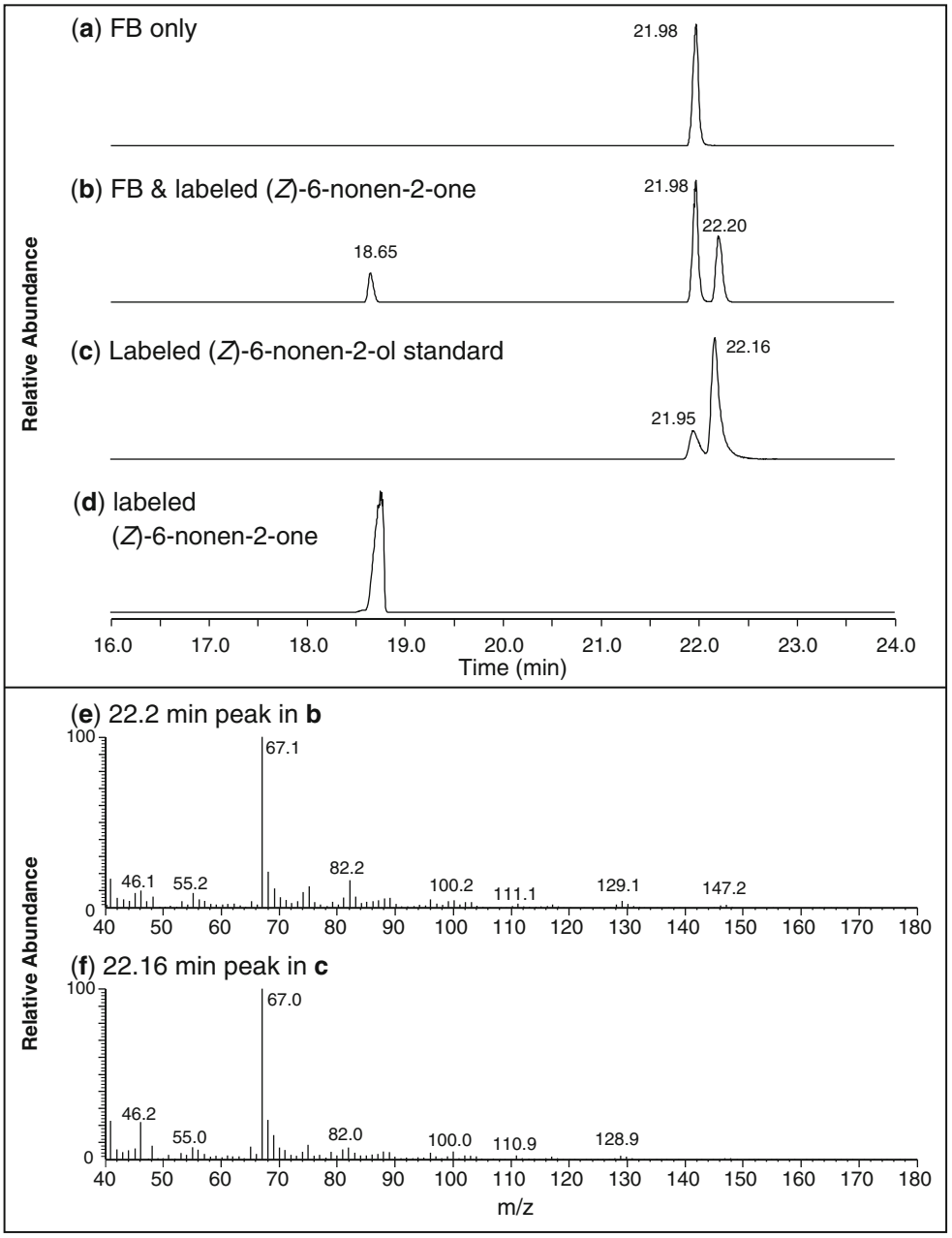

Fig. 4 GC/MS analysis with single ion monitoring at *m/z*=67 of unfed male fat body homogenates (*N*=1) incubated with (*Z*)-[1,1,1,3,3-D_5]-6-nonen-2-one as substrate. Thirty beetles were used in total. Reactions contained 2X NADPH regeneration system and fat bodies only (**a** negative control) or fat bodies with substrate (**b**). Incubations with substrate yielded a product that eluted at 22.20 min, essentially identical to that of [1,1,1,3,3-D_5]-nonen-2-ol (**c** standard) (22.16 min). **d** Pure substrate. The 21.95–22.98 min peak is 1-octanol. **e**, **f** mass spectra from the 22.2 min product peak in (**b**) and 22.16 min standard peak in (**c**), respectively

desaturation and limited chain shortening by β-oxidation, as proposed for pheromone production in Lepidoptera (Tsfadia et al. 2008). Alternatively, the 10-carbon intermediate may be produced directly by curtailing fatty acid elongation during synthesis. Fat bodies of unfed males that were incubated with [10,10,10-D_3]-decanoic acid did not yield a detectable unsaturated metabolite (Fig. 3), suggesting that a short-chain desaturase is not active. Thus, a longer chain unsaturated fatty acyl-CoA is likely required upstream of ω3-decenoyl-CoA, signifying that ω3-decenoyl-CoA arises from chain shortening of a longer chain, unsaturated precursor.

Step II

Fat bodies of unfed males that were incubated with [10,10,10-D_3]-decanoic acid (Fig. 3) yielded a product with a GC retention time (Fig. 3c) and mass spectrum (Fig. 3d) identical to that for 2-nonanone (NIST#: 114362 ID#: 7482). This product was not observed in negative controls (Fig. 3b). While decanoic acid is not a precursor for (*Z*)-6-nonen-2-one, it is an analog yielding the corresponding saturated product, 2-nonanone. These data thus strongly suggest that unfed male fat bodies can produce (*Z*)-6-nonen-2-one from a 10-carbon precursor.

The conversion of ω-3-decenoyl-CoA to the known precursor, (*Z*)-6-nonen-2-one, remains one of the most elusive steps of *exo*-brevicomin biosynthesis. Two routes are plausible, each proceeding from ω-3-decenoyl-CoA. The first relies on α-oxidation of either the 3-hydroxydecenoic- or 3-ketodecenoic β-oxidation intermediates of ω-3-decenoyl-CoA. α-Oxidation would release the carboxyl group, producing either (*Z*)-6-nonen-2-ol or (*Z*)-6-nonen-2-one. A second route involves oxidative decarbonylase (Qiu et al. 2012), which would convert ω-3-decenoyl-CoA to (*Z*)-3-nonene via an aldehyde intermediate. The unsaturated hydrocarbon could then be hydroxylated, likely by a cytochrome P450, to (*Z*)-6-nonen-2-ol, which would then be oxidized to the ketone by an oxidoreductase (Step III).

Experimental evidence for either pathway is not yet available. The first (α-oxidation) parallels a similar reaction leading to (*Z*)-10-heptadecen-2-one in *Drosophila mulleri* (Skiba and Jackson 1994). It requires release of the β-oxidation intermediate(s) as free acid(s), e.g., 3-ketodecenoic acid. We suspect this scenario is unlikely because the product, (*Z*)-6-nonen-2-one, bypasses the requirement for (*Z*)-6-nonen-2-ol oxidation, which we have observed (Song et al. unpublished). In support of the second pathway (oxidative decarbonylase), we note that hydrocarbon biosynthesis in oenocytes via oxidative decarbonylation is conserved across the Insecta (Qiu et al. 2012), and a sibling species, *D. jeffreyi*, uses a cytochrome P450 to hydroxylate heptane to 1- and 2-heptanol (Paine et al. 1999). An orthologous P450 in *D. ponderosae* may hydroxylate short-chain alkenes, including (*Z*)-3-nonene to yield (*Z*)-6-nonen-2-ol. Further experiments are required to distinguish between these two proposed pathways.

Step III

Unfed male fat bodies incubated with (*Z*)-[1,1,1,3,3-D_5]-6-nonen-2-one yielded a product with a similar GC retention time and identical mass spectrum to that of labeled (*Z*)-[1,1,1,3,3-D_5]-6-nonen-2-ol (Fig. 4). This product was not detected in the assay with fat bodies only (Fig. 4a) or in the substrate (Fig. 4d), suggesting that this alcohol product results from oxidoreductase activity in the fat body. This is in agreement with previous observations that male and female MPB exposed to (*Z*)-6-nonen-2-one vapor produced (*Z*)-6-nonen-2-

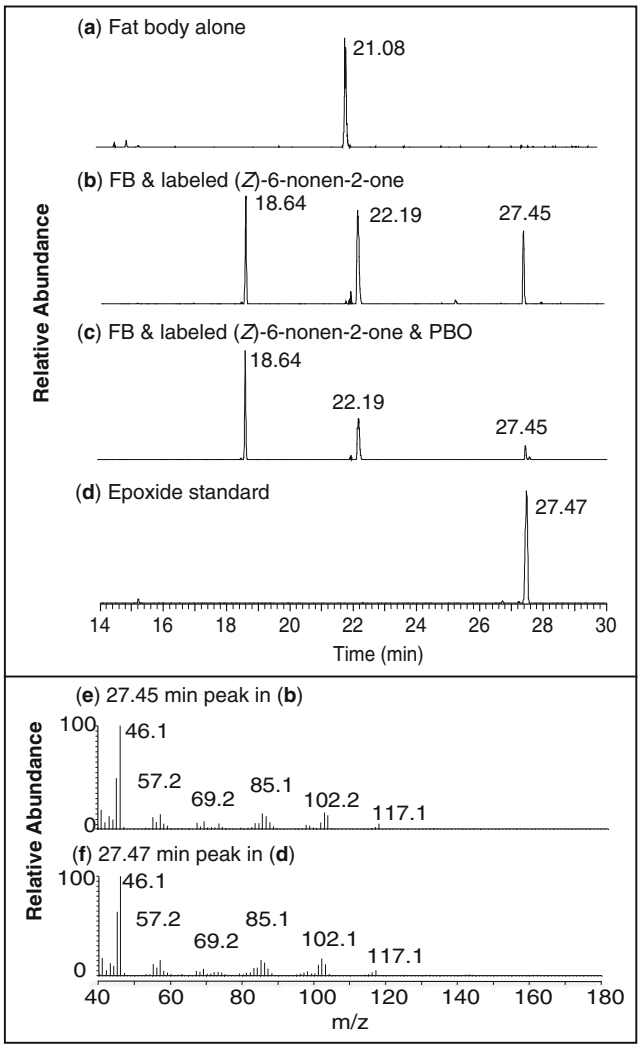

Fig. 5 GC/MS analyses of tissue cultures incubated with (*Z*)-[1,1,1,3,3-D_5]-6-nonen-2-one (18.64 min) yielded a product that eluted at 27.45 min (**b**), essentially identical to that of 6,7-epoxy-nonan-2-one (**d**). The amount of keto-epoxide recovered was lower in incubations with PBO (**c**). The mass spectrum (**e**) of the metabolite at 27.45 min in (**b**) is identical to that of the deuterium-labeled standard 6,7-epoxy-nonan-2-one (**f**)

ol (Vanderwel et al. 1992). These data imply that (*Z*)-6-nonen-2-ol may be the precursor of (*Z*)-6-nonen-2-one and, an oxidoreductase in the fat bodies may be required for the production of (*Z*)-6-nonen-2-one. If this hypothesis is true, 2-nonanol may be present in assays of fat bodies incubated with decanoic acid (Fig. 3). We did not detect 2-nonanol in those incubations, possibly because the 1-octanol peak on the GC overlaps that of 2-nonanol. Additionally, 2-nonanol may be converted rapidly to 2-nonanone. Similarly, (*Z*)-6-nonen-2-ol may be converted rapidly to (*Z*)-6-nonen-2-one under physiological conditions, as the alcohol was observed only in fat body incubations in the presence of (*Z*)-6-nonen-2-one. Nevertheless, our data strongly suggest that (*Z*)-6-nonen-2-ol, regardless of its origin (Step II), is the direct precursor to (6Z)-nonen-2-one.

Step IV

exo-Brevicomin arises through cyclization of 6,7-epoxynonan-2-one (Vanderwel et al. 1992). Unfed male fat bodies incubated with (*Z*)-[1,1,1,3,3-D_5]-6-nonen-2-one yielded a product with a GC retention time (Fig. 5b) and mass spectrum (Fig. 5e) identical to that of [1,1,1,3,3-D_5]-6,7-epoxynonan-2-one (Fig. 5d, f), that was not present in fat

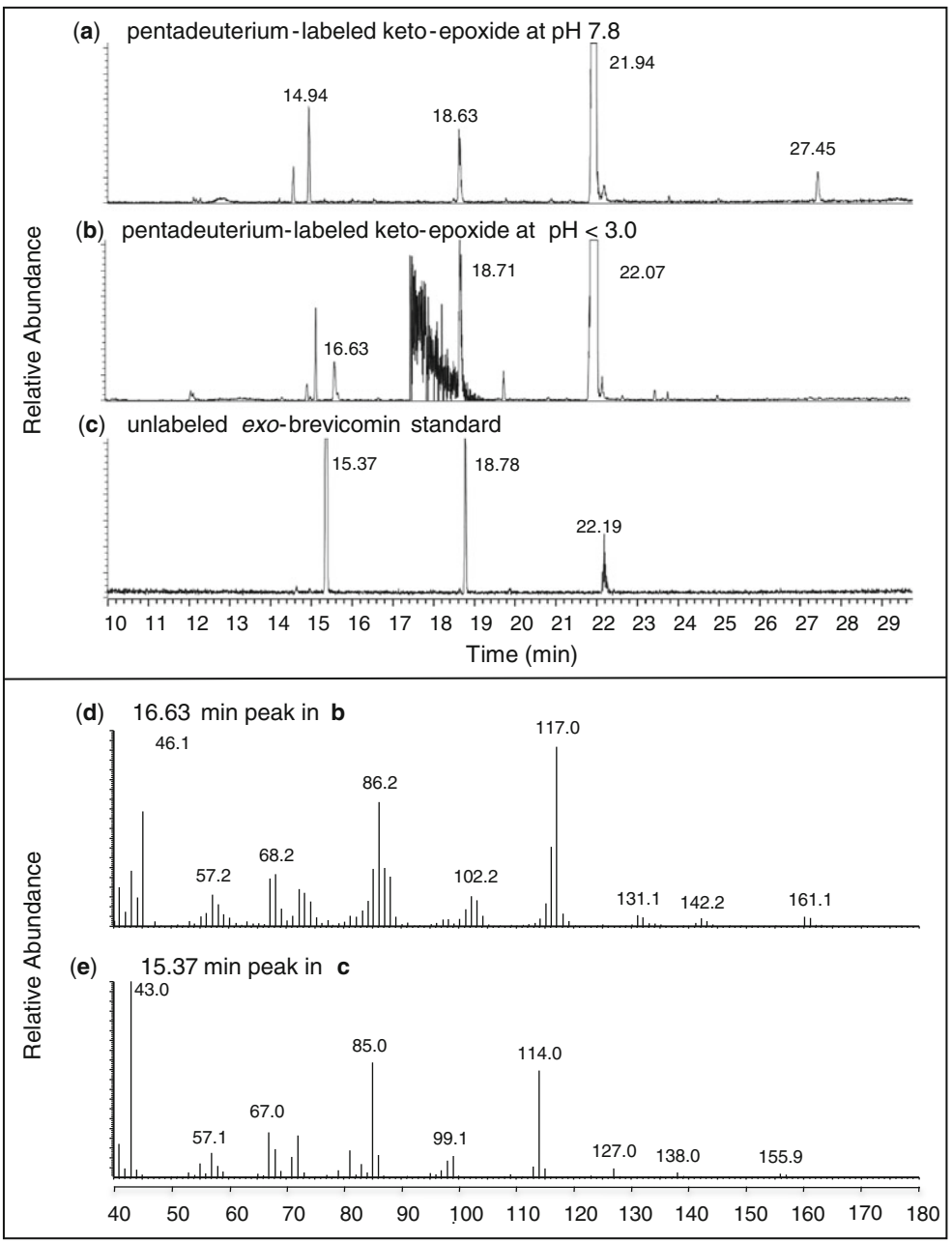

Fig. 6 GC/MS analysis of the conversion of [1,1,1,3,3-D_5]-6,7-epoxynonan-2-one (reactant at 27.45 min) in tissue culture buffer at physiological pH (**a**) or treated with acetic acid to pH<3.0 (**b**). The expected *exo*-brevicomin product (16.63 min) was only observed in acidic incubations. The mass spectrum of deuterium-labeled *exo*-brevicomin (**d**) had peaks shifted up to 3 amu compared to those of the unlabeled standard (**e**). All traces are with single ion monitoring at *m/z*=85

bodies incubated without the substrate (Fig. 5a). This confirms that fat bodies perform this step of *exo*-brevicomin biosynthesis. Furthermore, the epoxidation was inhibited if the incubation included the P450 inhibitor, PBO (Fig. 5c), suggesting that the reaction was catalyzed by a cytochrome P450. Deuterium-labeled *exo*-brevicomin, which would have eluted before (*Z*)-6-nonen-2-one, was not observed in any of these reactions (Fig. 5b, c).

Step V

The final step of *exo*-brevicomin biosynthesis is cyclization of 6,7-epoxynonan-2-one, which could, in theory, happen spontaneously (Bellas et al. 1969; Silverstein et al. 1968; Vanderwel et al. 1992). We incubated [1,1,1,3,3-D_5]-6,7-epoxynonan-2-one at room temperature in aqueous buffer at different pH values and monitored *exo*-brevicomin production with the base peak *m/z*=85, as this is the major fragment for both *exo*-brevicomin and the epoxide. We observed *exo*-brevicomin only in samples incubated at pH<3 (Fig. 6b and not shown). Incubations in cell culture buffer at physiological pH did not show cyclization (Fig. 6a). Unfed male fat body homogenates incubated with [1,1,1,3,3-D_5]-6,7-epoxynonan-2-one suggested *exo*-brevicomin production from this precursor, but were inconclusive (data not shown) due to either inactivation of cyclase activity during lysate preparation, and/or contamination issues in the epoxide preparation. Thus, while we were unable to show reliably cyclase activity in fat bodies, the stability of 6,7-epoxynonan-2-one at physiological pH suggests that an enzyme catalyzes the final step of *exo*-brevicomin production.

In summary, *exo*-Brevicomin is an important semiochemical in various *Dendroctonus* spp. and other insects. With the exception of the identification of (*Z*)-6-nonen-2-one as a precursor, there are no experimental data regarding the location of biosynthetic steps and the corresponding enzymes. Here, we present several lines of evidence regarding the process of *exo*-brevicomin production in *D. ponderosae*. In contrast to monoterpenoid pheromone components, fatty acid-derived *exo*-brevicomin was synthesized in fat bodies. Early, non-specific steps including fatty acid synthesis, desaturation, and possibly some β-oxidation may not be localized to the fat body, but it is reasonable to expect that ω-3-decenoyl-CoA production from a longer chain unsaturated precursor localizes to the fat body (Step I). Decenoyl-CoA then is likely converted to (*Z*)-6-nonen-2-ol by an as-yet undetermined series of reactions (Step II). An oxidoreductase likely converts the secondary alcohol to the methylketone (Step III), which then serves as a substrate for an epoxidase (cytochrome P450; Step IV). The final cyclization is probably enzyme-catalyzed (Step V). The information provided here can reasonably be extended to other taxa.

Interestingly, Aw et al. (2010) noted a cluster of three genes with an expression pattern consistent with *exo*-brevicomin production (high mRNA levels in unfed males relative to all other developmental states). One of these encodes an oxidoreductase, while the other encodes a cytochrome P450. Functional analyses of these recombinant enzymes implicate their involvement in *exo*-brevicomin biosynthesis (Song et al. unpublished).

The discovery that *exo*-brevicomin biosynthesis takes place in the fat body raises new insight into the evolution of bark beetle pheromone production and poses an interesting question as to how the semiochemical is moved into the alimentary canal for release in the frass (Bedard et al. 1969; Bellas et al. 1969). This is not an issue for other bark beetle pheromone components that are synthesized in the midgut and thus have a direct exit. Fat body-derived chemicals may require transport into the alimentary canal, possibly via the malpighian tubules. Alternatively, tight association between fat body and the midgut suggests a possible more direct path.

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