Biosynthesis of scarab beetle pheromones
Enantioselective 8-hydroxylation of fatty acids

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Chemical communication in scarab beetles (Coleoptera: Scarabaeidae) is achieved with a wide variety of pheromones, but one typical structure is the γ-lactone having a long unsaturated hydrocarbon chain. Several species utilize (*R,Z*)-5-(−)-(oct-1-enyl)-oxacyclopentan-2-one (buibuilactone), (*R,Z*)-5-(−)-(dec-1-enyl)-oxacyclopentan-2-one and (*S,Z*)-5-(+)-(dec-1-enyl)-oxacyclopentan-2-one [(*R*)-japonilure and (*S*)-japonilure]. Using deuterated precursors, we have demonstrated that these compounds are biosynthesized from fatty acids. $(9,10\text{-}d_4)$-Palmitic acid, $(9,10\text{-}d_4)$-stearic acid, $(9,10\text{-}d_2)$-palmitoleic acid, $(9,10\text{-}d_2)$-oleic acid, $(9,10\text{-}d_2)$-8-hydroxypalmitoleic acid and $(9,10\text{-}d_2)$-8-hydroxyoleic acid were readily incorporated by female *Anomala cuprea* into the pheromone molecules, while (*Z*)-$(5,6\text{-}d_2)$-5-dodecenoic acid and (*Z*)-$(5,6\text{-}d_2)$-5-tetradecenoic acid were not. Therefore, the reaction pathway starts from saturated fatty acids, involves their desaturation, followed by 8-hydroxylation, chain shortening and cyclization. The products obtained from racemic $(9,10\text{-}d_2)$-8-hydroxypalmitoleic acid and $(9,10\text{-}d_2)$-8-hydroxyoleic acid were also racemic, implying that the steps following hydroxylation were not stereospecific. Perdeuterated palmitic acid was applied to disclose the mechanism of the unique hydroxylation reaction. Retention of all deuterium atoms implied that this reaction was a direct process mediated by a specific fatty acid hydroxylase, and preceding desaturation or epoxidation was not involved.

Keywords: *Anomala cuprea*; *Anomala osakana*; buibuilactone; japonilure.

The remarkable progress in our understanding of pheromone biosynthesis [1] and its regulation [2] in insects is primarily due to the extensive efforts that have centered on Lepidoptera. Reports from other orders predominantly describe pathways from economically important species, such as the house fly [3], cockroaches [4], bark beetles [5], nitidulid beetles [6] and grain beetles [7]. Numerous species of scarab beetles (Coleoptera: Scarabaeidae) are also devastating pests in many parts of the world. When compared with lepidopteran pheromones, which are primarily straight chain alcohols, aldehydes and acetates, scarab sex pheromones are remarkably diverse. They range from the ubiquitous phenol [8] and anisole [9] to an amino acid derivative [10] and an aromatic alkaloid [11]. One typical pheromone structure is the γ-lactone having a long unsaturated hydrocarbon chain [reviewed in 12], such as (*R,Z*)-5-(−)-(oct-1-enyl)-oxacyclopentan-2-one (buibuilactone) [13] and (*R,Z*)-5-(−)-(dec-1-enyl)-oxacyclopentan-2-one (japonilure) [14]. These γ-lactones are unusual for the occurrence of a double bond adjacent to the lactone ring (Fig. 1). In addition, their stereochemistry plays a pivotal role in chemical communication of some species, because enantiomers have opposite behavioral functions [14,15]. While one enantiomer is released by females to attract males, its antipode acts as a strong behavioral

antagonist, i.e. small amounts of the antipode completely shut off male attraction.

In many insect species biosynthesis of sex pheromones involves addition of only one or two unique enzymes into the general metabolic pathways, such as the Δ^{11}-desaturase [1], which is found only in moth pheromone glands [16]. Using deuterium-labeled precursors, we demonstrate here that the monounsaturated γ-lactone pheromones of *A. cuprea*, *A. osakana* and other scarab species are formed from fatty acids via a unique stereospecific 8-hydroxylation.

EXPERIMENTAL PROCEDURES

Synthesis

(9,10-d_2)-Palmitoleic acid and (9,10-d_2)-oleic acid. Alkynyllithiums of 1-octyne and 1-decyne were coupled with 8-bromooctyl tetrahydropyranyl ether; deprotection, semideuteration (Lindlar, D_2), and oxidation (Jones reagent) gave the desired products in 65% overall yield. Catalytic reduction (PtO_2, deuterium atmosphere) of these compounds yielded $(9,10\text{-}d_4)$-palmitic acid and $(9,10\text{-}d_4)$-stearic acid. Saturated fatty acids were converted to the corresponding methyl esters by reaction with BF_3 in methanol for 20 min at 80 °C.

(Z)-(5,6-d_2)-5-Dodecenoic acid and (Z)-(5,6-d_2)-5-tetradecenoic acid. These were obtained by the coupling of the alkynyllithium of hex-5-ynyl tetrahydropyranyl ether with *n*-hexyl or *n*-octyl bromide, followed by deprotection, semideuteration and oxidation.

(E)-(5,6-d_2)-5-dodecenoic acid. This was obtained by the reduction of 5-dodecyn-1-ol with $LiAlD_4$ in ether (work-up with D_2O), and oxidation (Jones reagent).
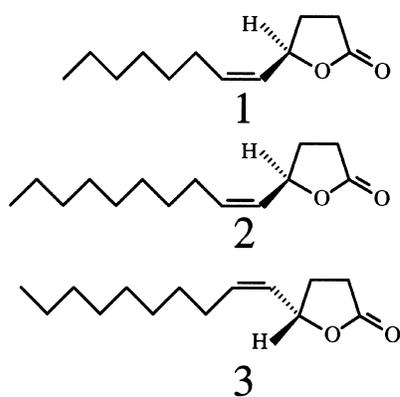
Fig. 1. Structures of the two constituents of the sex pheromone for *A. cuprea*, (*R*)-buibuilactone (1) and (*R*)-japonilure (2), and the sex pheromone for *A. osakana*, (*S*)-japonilure (3).

Methyl (9,10-d₂)-8-hydroxypalmitoleate and methyl (9,10-d₂)-8-hydroxyoleate. 1-Octyne and 1-decyne were treated with ethylmagnesium bromide at −70 °C for 1 h and then coupled with methyl suberyl chloride at −70 °C. Reduction (sodium borohydride) of methyl 8-oxo-hexadec-9-ynoate and 8-oxo-octadec-9-ynoate, followed by semideuteration gave the desired methyl 8-hydroxypalmitoleate and methyl 8-hydroxyoleate (47%).

Application and extraction procedures

Beetles were reared in the laboratory as previously described [17] and kept under a photoperiod of 16L/8D (photophase 06:00–22:00 h) at 25 °C. At the time of pheromone production (14:00–20:00 h), acetone solutions of labeled compounds (20 µg·µL^{-1}) were topically applied (1 µL) on the anal plate (pygidium) of at least three female beetles per replicate (five replicates/experiment). Deuterium-labeled palmitic, stearic, 8-hydroxypalmitoleic and 8-hydroxyoleic acids were applied as methyl esters to facilitate penetration into beetle cuticle, whereas deuterium-labeled palmitoleic, oleic, 5-dodecenoic and 5-tetradecenoic acids were applied as free acids. Two hours later, the beetles were anesthetized with ether, and the anal plate plus the last two sternites were cut off and extracted with hexane (5 min), after internal tissues had been removed. The crude extract was purified on a Pasteur pipette filled with silica gel (Wakogel C-200; Wako, Tokyo, Japan), eluted successively with solutions of ether in hexane (0, 10, 25, 50, 75, 100%), collected in small fractions (300 µL), concentrated and analyzed by gas chromatography–mass spectrometry (GC-MS).

Analytical procedures

GC-MS was carried out either on an HP 6890 Series gas chromatograph linked to a mass selective detector MSD 5973, a Mass Engine 5898B, or on a GC electron ionization detector GCD Series (Hewlett-Packard, Palo Alto, CA, USA). Chromatographic resolution was achieved on an HP-5MS capillary column (30 m × 0.25 mm × 0.25 µm; Hewlett-Packard) operated at 70 °C for 1 min, temperature increased to 270 °C at a rate of 10 °C·min^{-1}, and held at this temperature for 10 min. Vapor phase Fourier transform infrared (FTIR) spectra were recorded with a GC HP6890 coupled to a light pipe interface, FTS-40A, GC/C32 (Bio-Rad, Cambridge, MA, USA). NMR spectra were recorded on a 500 MHz JNM-A500 spectrometer (Jeol, Tokyo, Japan). GC analyses to determine the enantiomeric
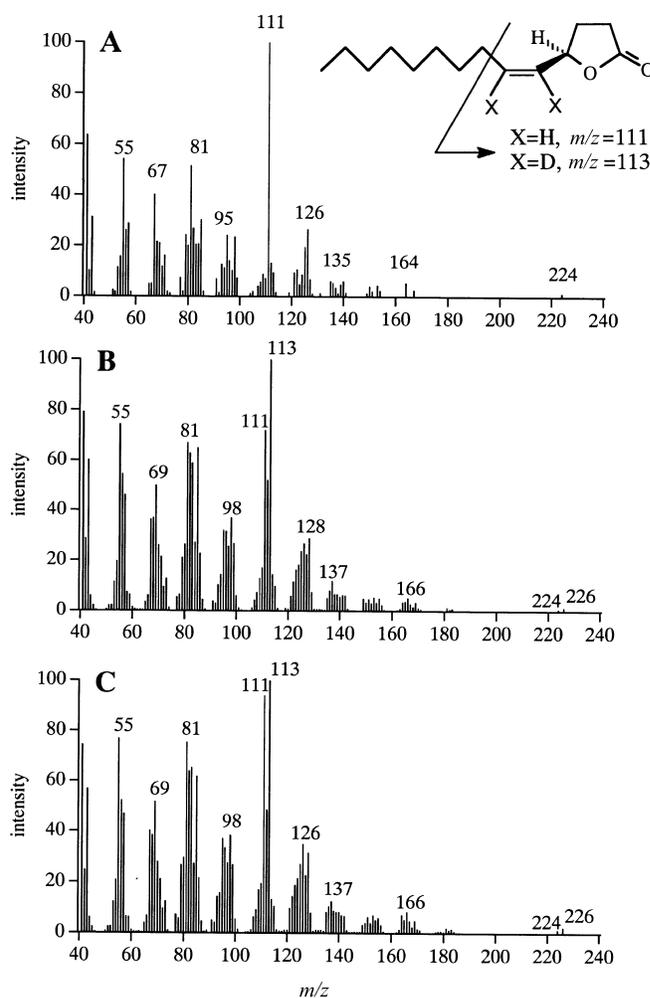

Fig. 2. Mass spectral data for japonilure from *A. cuprea* obtained after application of (A) (*Z*)-(5,6-d₂)-5-tetradecenoic acid (no incorporation), (B) (9,10-d₂)-oleic acid (58% incorporation), and (C) (9,10-d₂)-8-hydroxyoleic acid methyl ester (52% incorporation). When there is little or no incorporation of the intermediate (A) the mass spectrum is indistinguishable from that of the natural japonilure.

compositions were performed on a Hewlett-Packard 5890 II Plus instrument or a Mass Engine operated on selected ion monitoring (SIM) mode. These instruments were equipped with a trifluoroacetylated γ-cyclodextrin-based column, Chiraldex GTA (10 m × 0.2 mm; 0.25 µm; Astec, Whippany, NJ, USA), operated at 135 °C (isothermal) with helium (2 mL·min^{-1}) as a carrier gas. GC with an electroantennographic detector was performed as previously described [18].

RESULTS

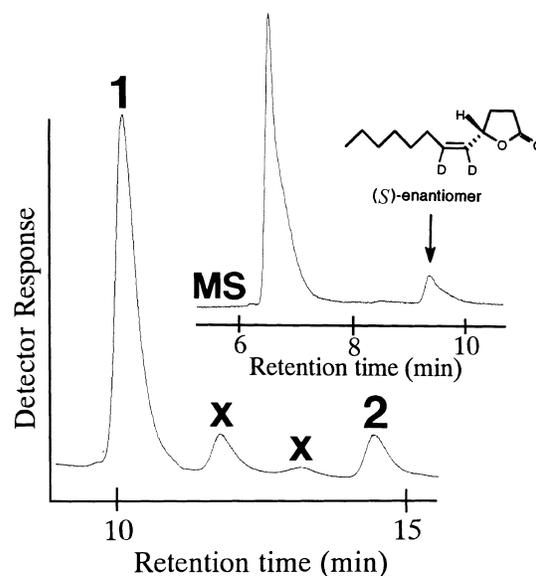The major fragmentation pattern of γ-lactones with a double bond adjacent to the ring [13,19] leads to the formation of a base peak at *m/z* = 111 (Fig. 2A). This provided a very useful diagnostic feature for isotopic labeling experiments. Incorporation of deuterium into the vinyl positions shifted the base peak to *m/z* = 113, which has very low intensity (6.7%) in the natural, unlabeled lactones. In our initial experiments, acetone solutions of (9,10-d₄)-palmitic acid and (9,10-d₄)-stearic acid were applied on female *A. cuprea*, but we were unable to demonstrate incorporation of these saturated precursors into final products. We attributed this failure to insufficient penetration of the
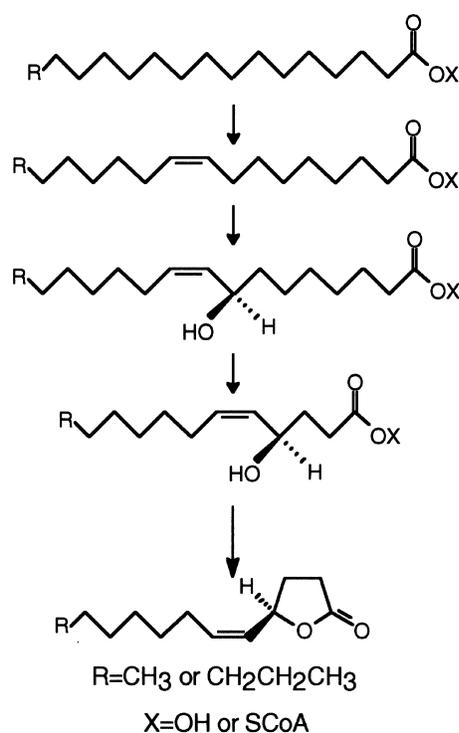
Fig. 3. Proposed pathway for the biosynthesis of buibuilactone and japonilure in scarab beetles.

Fig. 4. Enantiomeric resolution of (5,6-d$_2$)-buibuilactone on a capillary column with a chiral stationary phase. (*R*)-(5,6-d$_2$)-buibuilactone (peak 1) coeluted with the naturally occurring pheromone at 10.09 min, whereas its enantiomer (peak 2) appeared at 14.54 min. By GC-MS operated at SIM mode (insert), these peaks appeared at 6.59 and 9.57 min, respectively. Two contaminants (denoted by 'x') at 11.79 and 13.24 min were not detected by GC-MS.

cuticle by these compounds, which are solid at room temperature. The pheromone-producing cells of scarab beetles do not form a distinct gland but are spread as a single layer on the inner surface of the abdominal integument [20]. Direct application of precursors to the cells *in vivo* is therefore technically very difficult, if not impossible. To circumvent this obstacle we prepared methyl esters of both (9,10-d$_4$)-palmitic acid and (9,10-d$_4$)-stearic acid, which are liquid at room temperature. It has been reported previously that fatty acid methyl esters are readily incorporated into metabolic pathways, either via direct conversion or hydrolysis and then conversion to coenzyme A (CoA) thioesters [21]. This change in strategy led to clear increases in the *m/z* = 113 peak, indicating incorporation of labeled saturated fatty acid into the pheromone molecules (buibuilactone, 13%; japonilure, 24%). Application of the next intermediates in the postulated pathway, (9,10-d$_2$)-palmitoleic acid and (9,10-d$_2$)-oleic acid, dramatically increased the level of incorporation (27 and 58%, respectively). The incorporation of labeled precursors into the pheromone molecules was always higher for the longer homolog (japonilure) (Fig. 2B).

To test the reaction sequence for the remaining steps (hydroxylation and chain shortening), two sets of intermediates were prepared. (*Z*)-(5,6-d$_2$)-5-Dodecenoic acid and (*Z*)-(5,6-d$_2$)-5-tetradecenoic acid would be incorporated into the respective pheromone compounds if chain shortening preceded the hydroxylation, whereas the reverse order would lead to the incorporation of (9,10-d$_2$)-8-hydroxypalmitoleic acid and (9,10-d$_2$)-8-hydroxyoleic acid. Application of labeled (*Z*)-dodecenoic (data not shown) and (*Z*)-tetradecenoic acid (Fig. 2A) to females did not lead to incorporation into the natural pheromones. Unexpectedly, we observed a significant increase in the amount of (*E*)-5-(oct-1-enyl)-oxacyclopentan-2-one and (*E*)-5-(dec-1-enyl)-oxacyclopentan-2-one, and a large proportion of these isomers (if not all) contained deuterium (data not shown). Under natural conditions, the *trans* isomers of the pheromone compounds are also isolated in some species, but in very small

amounts [22]. To investigate further this unexpected phenomenon we prepared (*E*)-(5,6-d$_2$)-5-dodecenoic acid. (*E*)-5-(Oct-1-enyl)-oxacyclopentan-2-one, the unnatural geometric isomer, obtained after application of this precursor contained essentially only labeled molecules. The mechanism of such strong discrimination between the *cis* and *trans* isomers is not clear. It is not even certain whether the enzyme or enzymes that hydroxylate the *trans* isomers also participates in the biosynthesis of the natural *cis* pheromones. However, it appears that the unnatural precursors are metabolized via an alternative pathway, which does not lead to the formation of the natural products.

(9,10-d$_2$)-8-Hydroxypalmitoleic acid and (9,10-d$_2$)-8-hydroxyoleic acid were incorporated into buibuilactone (43%) (data not shown) and japonilure (52%) (Fig. 2C), respectively. This strongly suggests that in the natural pathway the hydroxylation of fatty acid precedes the chain shortening reaction (Fig. 3).

Pheromones obtained from the labeled, hydroxylated fatty acids were analyzed by GC with a chiral stationary phase column (Fig. 4). The synthetic intermediates were racemic; therefore, their fate in the biosynthetic pathway could give insights into the stereospecificity of reactions following hydroxylation. In all cases, labeled racemic precursors were incorporated into both (*R*) and (*S*) enantiomers of both pheromone compounds and, as expected, the unnatural stereoisomer was essentially completely derived from the synthetic deuterium-labeled substrate (data not shown). Therefore, the hydroxylation of fatty acids seems to be the only stereospecific reaction in the pathway and further steps do not discriminate between enantiomers.

In order to test whether pheromones of related species are biosynthesized via analogous pathways, we applied (9,10-d$_2$)-8-hydroxyoleic acid methyl ester to female *A. osakana,* which utilizes (*S*)-japonilure as a sex pheromone. This intermediate was readily incorporated into the pheromone molecule (Fig. 5), indicating that biosynthesis in this species also involves 8-hydroxylation. The pheromone was isolated and further
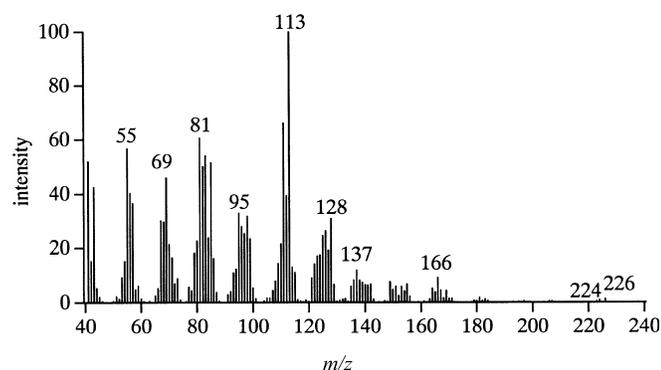
Fig. 5. Mass spectral data for japonilure from *A. osakana* obtained after application of (9,10-d$_2$)-8-hydroxyoleic acid methyl ester.

analyzed by GC and GC-MS with a chiral stationary phase column (Fig. 6). The labeled precursor was incorporated not only in the natural pheromone, (*S,Z*)-japonilure (peak 4), but also gave rise to three more isomers, i.e., (*R,E*)-japonilure, (*R,Z*)-japonilure and (*S,E*)-japonilure (peaks 1, 2 and 3, respectively). Because of the normal bleeding of trifluoroacetylated cyclodextrin columns that generates a high background (peaks at *m/z* 111 and 113) it was not possible to determine accurately the composition of peak 4 in terms of the natural and (5,6-d$_2$)-labeled (*S,Z*)-japonilure. Interestingly, the (*R,E*)-isomer (peak 1) was incorporated to the same extent as the (*R,Z*)-isomer (peak 2, Fig. 6), although the labeled precursor contains only ≈ 5% of the *trans* isomer. These findings suggest that the hydroxylation of fatty acids is the only stereospecific step in the biosynthetic pathway and that the geometry of the double bond of the natural pheromone is controlled by the Δ9 desaturase; the following steps probably do not discriminate *cis* and *trans* isomers.
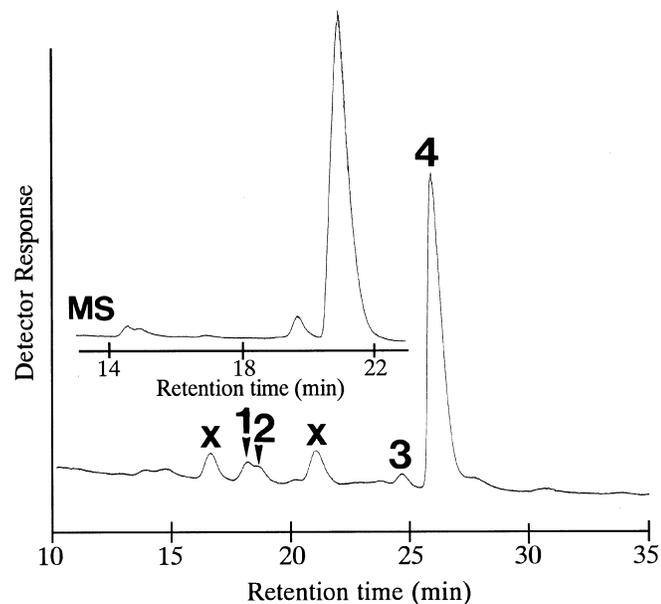

Fig. 6. Enantiomeric resolution of (5,6-d$_2$)-japonilure on a capillary column with a chiral stationary phase. (*S,Z*)-(5,6-d$_2$)-japonilure (peak 4) coeluted with the naturally occurring pheromone at 25.84 min, whereas (*R,E*)-(5,6-d$_2$)-japonilure, (*R,Z*)-(5,6-d$_2$)-japonilure and (*S,E*)-(5,6-d$_2$)-japonilure appeared at 18.1 (peak 1), 18.14 (peak 2), 24.5 min (peak 3), respectively. Phthalate ester contaminants (denoted by 'x') were not detected by GC-MS in the SIM mode (insert).
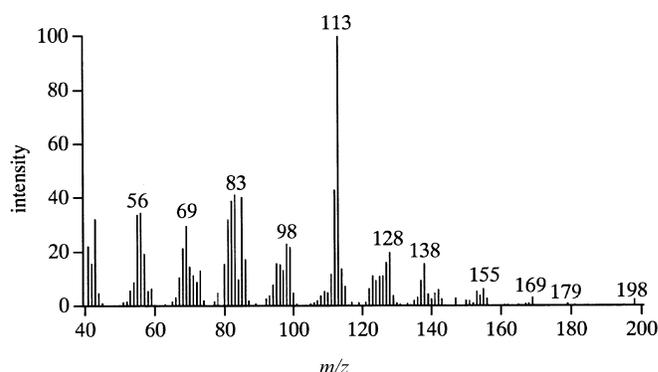

Fig. 7. Mass spectral data for buibuilactone from *A. osakana* obtained after application of (9,10-d$_2$)-8-hydroxypalmitoleic acid methyl ester.

Naturally *A. osakana* produces only (*S,Z*)-japonilure, the 14-carbon lactone; no traces of buibuilactone (the shorter homolog) are detected in extracts from the pheromone glands [15]. However, application of (9,10-d$_2$)-8-hydroxypalmitoleic acid methyl ester to female glands led to the formation of (5,6-d$_2$)-buibuilactone (Fig. 7). On the other hand, no pheromone was detected upon application of (9,10-d$_2$)-palmitoleic acid. In conclusion, it appears that the 8-hydroxylase of *A. osakana* can only use oleic acid as a substrate and does not act on palmitoleic acid. Buibuilactone is not produced because of this high chain length specificity.

In our attempt to disclose the mechanism of the hydroxylation reaction we applied the methyl ester of the commercially available perdeuterated palmitic acid to female *A. cuprea*. This provided benefits in baseline separation of the female-produced perdeuterated lactone and the natural lactone on a GC column (Fig. 8). The suspected perdeuterated product was eluted 0.25 min earlier than the unlabeled buibuilactone. The mass spectrum of the small chromatographic peak associated with buibuilactone closely resembled the spectrum of the natural product in the overall pattern. The spectrum also showed the expected diagnostic features: a base peak at *m/z* = 118 and the molecular ion peak at *m/z* = 216. Its infrared spectrum also contained features similar to those of the natural buibuilactone (*ν*C=O, 1808·cm^{-1}; *ν*C–C(=O)–O, 1206·cm^{-1}; *ν*O–C–C, 1031·cm^{-1}), but it showed no absorption in the C–H stretching region (3100–3000·cm^{-1}). Instead, pronounced peaks appeared at 2216·cm^{-1} (C–D st.) indicating that this molecule was indeed the perdeuterated buibuilactone. When the compound was tested for physiological activity on a gas chromatograph coupled to an electroantennographic detector [11,15,18], it elicited an electrophysiological response in male antennae, similar to that evoked by the natural pheromone (Fig. 8).

The mass spectrum of the perdeuterated buibuilactone clearly demonstrated that no deuterium was replaced by protium during the biosynthetic pathway. The incorporation of the hydroxyl group is therefore a direct process and does not involve preceding desaturation or epoxidation steps. Either of these alternative pathways would inevitably lead to the replacement of two deuterium atoms by protium, producing a base peak at *m/z* = 116 and a molecular ion peak at *m/z* = 214.

DISCUSSION

The postulated biosynthetic route for the lactone pheromones implied three major steps: desaturation, hydroxylation and chain shortening, but their order remained to be determined [12,23]. Position of the double bond fixed with respect to the carboxylate
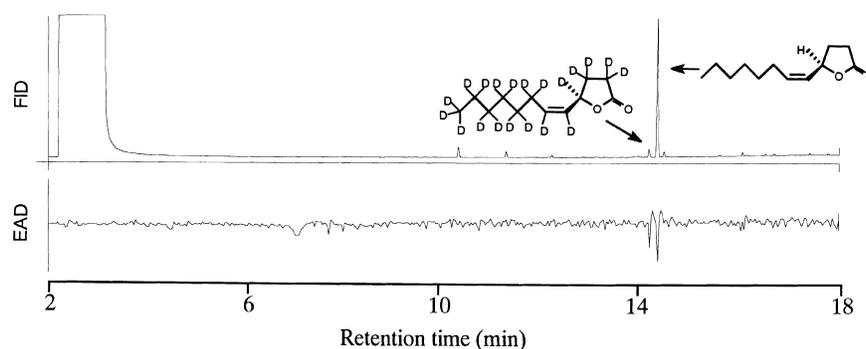
Fig. 8. Electroantennographic detector (EAD) response of male *A. cuprea* antenna and flame ionization detector (FID) response to natural and perdeuterated buibuilactone (6.5% incorporation) biosynthesized by female *A. cuprea*.

rather than the terminal methyl group implied that the desaturation occurred first and was most likely mediated by a common Δ9 desaturase, converting stearic and palmitic acid to oleic and palmitoleic acid, respectively. However, the discovery of γ-lactones with a hydroxy group instead of a double bond in a danaine butterfly [24] indicates that a unique enzyme may be involved.

Two reaction sequences had to be considered for the remaining steps: the chain shortening could either precede or follow the hydroxylation. Incorporation of labeled precursors in our experiments show that the hydroxylation of unsaturated fatty acids occurs first.

Internal hydroxylations of fatty acids in animals are not common. In mammals, hydroxylation most frequently occurs at terminal positions or at the allylic or bis-allylic carbons of polyunsaturated fatty acids [25]. 8-Hydroxy-9,12-octadecadienoic acid has been detected among liver metabolites. Such reactions are usually attributed to cytochrome P450 [25]. In insects, terminal hydroxylation has also been demonstrated, e.g. in the biosynthesis of caste-selective pheromones in honeybees [26]. The formation of macrocyclic lactones from terminally hydroxylated fatty acid was first described in grain beetles [7].

The best studied example of a hydroxylated fatty acid, ricinoleic acid from castor bean, *Ricinus communis*, is biosynthesized from oleic acid by oleate 12-hydroxylase, which shows homology to fatty acid desaturases [27]. It has been known for more than three decades that ricinoleic acid can be converted to γ-lactones by a number of microorganisms, particularly yeast [28]. Recently, it has been demonstrated that (*R*)-γ-decanolactone in *Sporobolomyces odorus* is biosynthesized from oleic acid, which is first hydroxylated to ricinoleic acid [29]. Feron and coworkers [30] have also identified 4-hydroxydecanoic acid as an intermediate during the conversion of ricinoleic acid to γ-decalactone in two *Sporobolomyces* species. Thus, biosynthesis of lactones in yeast and scarab beetles apparently follows the same route, although the position of hydroxylation differs. However, 8-hydroxylinoleic acid have been previously identified in microorganisms [31,32].

In plants, saturated γ-lactones are formed via a different, more complex pathway. Fatty acids are first epoxidized, hydrolysis of the epoxide to the diol is followed by cyclization, dehydration and reduction of the double bonds [33,34].

Although in *A. cuprea* and *A. osakana* the hydroxylation of fatty acids occurs at an allylic position, it cannot be attributed to lipoxygenases, as has been established in the biosynthesis of unsaturated δ-lactones in *Sporobolomyces odorus* [35]. No double bond shift or hydrogen exchange occurs during the biosynthesis of buibuilactone and japonilure. If we assume that the pheromones of closely related species are biosynthesized via analogous pathways, the allylic position does not seem to be required for hydroxylation. The example of (*R,Z*)-7,15-hexadecadien-4-olide, the sex pheromone of the yellowish elongate

chafer, *Heptophylla picea* [18] illustrates that the position can be even shifted from C-8 to C-6. We therefore postulate that this reaction is performed by a specific fatty acid hydroxylase.

It is worth noting that various scarab species have developed pathways to produce unique pheromone molecules by changing either stereospecificity or regiospecificity of the hydroxylation step. *A. cuprea*, *Popillia japonica* and several other species synthesize (*R*)-japonilure and (*R*)-buibuilactone, whereas *A. osakana* produces (*S*)-japonilure and *Heptophylla picea* makes (*R,Z*)-7,15-hexadecadien-4-olide. Closely related species can even be devoid of this enzymatic activity. The sex pheromone of *Anomala rufocuprea* is not a γ-lactone, but a fatty acid ester (methyl 5-tetradecenoate) [36], whereas *A. osakana* does not produce buibuilactone probably because it is devoid of an 8-hydroxylase that acts specifically on palmitoleic acid. All these pathways fall into the general pattern described previously for moths: introduction of a single specific reaction into general metabolic routes can generate impressive diversity of semiochemicals.

ACKNOWLEDGEMENTS

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