Enzymatic processing of pheromones and pheromone analogs

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Summary. Pheromone perception requires rapid enzymatic degradation of the active chemical signal in the sensory hairs. Three insects are used to illustrate chemical approaches to studying the degradation of pheromones by antennal enzymes. First, hydrolysis of acetate and haloacetate esters is examined in the diamondback moth, *Plutella xylostella*. Second, aldehyde oxidation and the selective inhibition of the oxidase and dehydrogenase activities are described for the tobacco budworm moth, *Heliothis virescens*. Finally, a stereochemical analysis of the epoxide-hydrase catalyzed addition of a water molecule to the oxirane ring of disparlure is described for the gypsy moth, *Lymantria dispar*.
Key words. Pheromone biochemistry; enzyme inhibitors; sensory hair enzymes; epoxide hydrase; esterase; aldehyde dehydrogenase; *Plutella xylostella; Lymantria dispar; Heliothis virescens.*

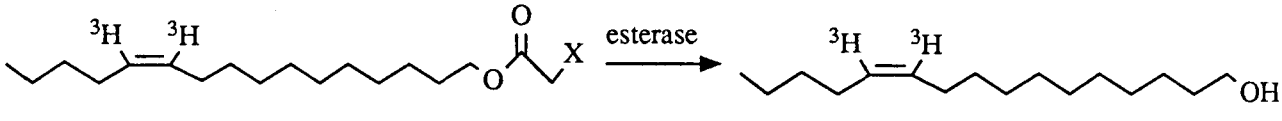
Introduction

Pheromone perception is mediated by interactions of the pheromone molecules with sensory hair binding proteins and enzymes. However, a basic understanding of the biochemical events taking place in insect antennae during pheromone perception is still lacking. The entry of infinitesimal quantities of a precise blend of small lipophilic compounds into the sensillum is just the beginning of a chain of events including transport, receptor binding, degradation, transduction, and ion channel opening which ultimately results in the conversion of a chemical binding energy into electrical signals. Indeed, molecular approaches to insect olfaction are part of a new discipline of pheromone biochemistry, the subject of a recent monograph [19]. The importance of soluble odorant-binding proteins [24] and membrane-associated receptors in vertebrates [11] and in insects [8, 31] can be demonstrated by binding of labeled odorants, activation of transduction, and tissue localization. In addition, the enzymatic degradation of chemical signals [5] seems to be required to minimize the stimulus noise level [26, 30].

At Stony Brook, we have examined the products and enzymes of pheromone catabolism [18], using pheromone components stoichiometrically-labeled with tritium [17] in in vitro enzyme assays. Stoichiometric labeling provides the maximum specific activity obtainable, i.e. 29 Ci/mmol per tritium atom incorporated. In addition, we have used chemically-reactive analogs of pheromones to modify pheromone-processing proteins [4, 31]. In this article, we summarize previously unpublished results in three insects to demonstrate a variety of chemical, biochemical, and analytical techniques. The insects chosen are from three different families in the Lepidoptera and use different chemical functionalities as their primary pheromone components.

Acetate and haloacetate hydrolysis by Plutella xylostella (Yponomeutidae)

The diamondback moth, a pest of cruciferous crops worldwide, employs *Z*11-16:Ac (shorthand for (*Z*)-11-hexadecen-1-yl acetate) as the most abundant component of its pheromone blend [2]. Recently, the ability of male antennae to distinguish halogen-substituted acetate analogs of this pheromone was demonstrated using an electroantennogram assay [20]. We found that while the mono-, di- and trifluoroacetates were well recognized, the corresponding chloro-, bromo-, iodo- and diazoacetates elicited poor responses. The ability of these halogenated acetates to act as competitive inhibitors of the antennal esterase activity in vitro closely paralleled the electroantennogram (EAG) activity results, with poor inhibition by compounds which gave low EAG responses. In the wild silkmoth *Antheraea polyphemus*, the pheromone esterase shows tissue- and sex-specificity [16] as well as partial substrate-specificity [27]. These specificities have not been completely examined for other species. Thus, the esterase activities in various tissues of male and female *P. xylostella* adults were characterized as detailed below. In this tiny moth, filiform antennae are difficult to collect and obtaining a sensory hair preparation analogous to that from *A. polyphemus* was impractical. Thus, we measured the esterase activity of 10,000 × g soluble proteins from crude antennal and leg ho-



X = H, Z11-16:Ac
X = F, Z11-16:FAc
X = Cl, Z11-16:ClAc
X = Br, Z11-16:BrAc

mogenates by monitoring the hydrolysis of [^{3}H]-*Z*11-16:Ac (ca 45Ci/mmol) to labeled *Z*11-16:OH using a thin layer chromatography-liquid scintillation counter (TLC-LSC) assay[20].

Tissue specificity

One-day-old male and female adult insects were narcotized with CO_2, chilled on ice, and their antennae and 3 legs/adult were transferred to four separate 1-ml ground-glass homogenizers. The tissues were homogenized with 10 mM Tris buffer (pH 7.0), sonicated in a cleaning bath for 5 min at 0 °C, centrifuged at 10,000 × g for 5 min, and the supernatants were frozen (−90 °C) in 150–300-µl aliquots for later use in enzyme assays. The male antennae (MA), female antennae (FA), male legs (ML) and female legs (FL) were prepared at 16.6 antennal or 8.3 leg equivalents per 100 µl. Protein concentrations of 1.2 µg/MA, 0.7 µg/FA, 2.4 µg/ML, and 2.7 µg/FL were calculated using a Coomassie Blue dye-binding assay. Assays using 0.6 µM [^{3}H]-*Z*11-16:Ac as substrate were performed on undiluted, 1:3, 1:10, and 1:30 dilutions of the enzyme preparations, and the ratio of the hydrolysis product [^{3}H]-*Z*11-16:OH to the recovered product plus substrate were obtained from the TLC-LSC assay[20]. The results, shown in figure 1 below, show high esterase activity in all tissues, as expected. How then, could we determine the specificity of the esterases for the pheromone?

Kinetic analyses of antennal and leg esterases

A dilution series of twelve concentrations (0.02–5 mM) *Z*11-16:Ac was prepared in ethanol, and 1 µl of each was added to duplicate tubes containing 90 µl of a pH 7.4 sodium phosphate buffer (75 mM) at 4 °C. Next, 10 µl of tissue homogenate was added followed by 1 µl of 20-µM [^{3}H]-*Z*11-16:Ac in ethanol. The tubes were vortexed and incubated at 26 °C for 30 min; the reactions were stopped by chilling to 4 °C and vortexing with 100 µl of ethyl acetate. The percentage hydrolysis was determined as previously described[20], and both the male and female antennal enzymes showed saturation kinetics (fig. 2a); the female hydrolytic activity was 3–5 times lower per antennal equivalent. When the results were plotted using an Eadie-Hofstee plot of velocity/substrate concentration (v/s) against velocity, clear evidence was obtained for two hydrolytic activities: one with high K_M (MA, 35 µM; FA, 23 µM) and high velocity, and one with low K_M (MA, 0.7 µM; FA, 0.7 µm) and lower velocity (fig. 2b, 2c). Surprisingly, the leg enzymes showed very similar Eadie-Hofstee plots (not shown), indicating that both activities are present in non-sensory tissues as well.

Hydrolysis of labeled haloacetates by antennal homogenates

To correlate the inhibitory effects observed earlier[20] for the monohaloacetate analogs of *Z*11-16:Ac with their ability to act as substrates for the esterases, we prepared each one in radiolabeled form. Thus, [11,12-^{3}H]-*Z*11-16:OH was obtained by hydrolysis of the labeled pheromone, and was esterified with monofluoroacetyl chloride, monochloroacetyl chloride, or monobromoacetyl chloride and chromatographed to give the homogeneous [^{3}H]-*Z*11-16:FAc, [^{3}H]-*Z*11-16:ClAc, and [^{3}H]-*Z*11-

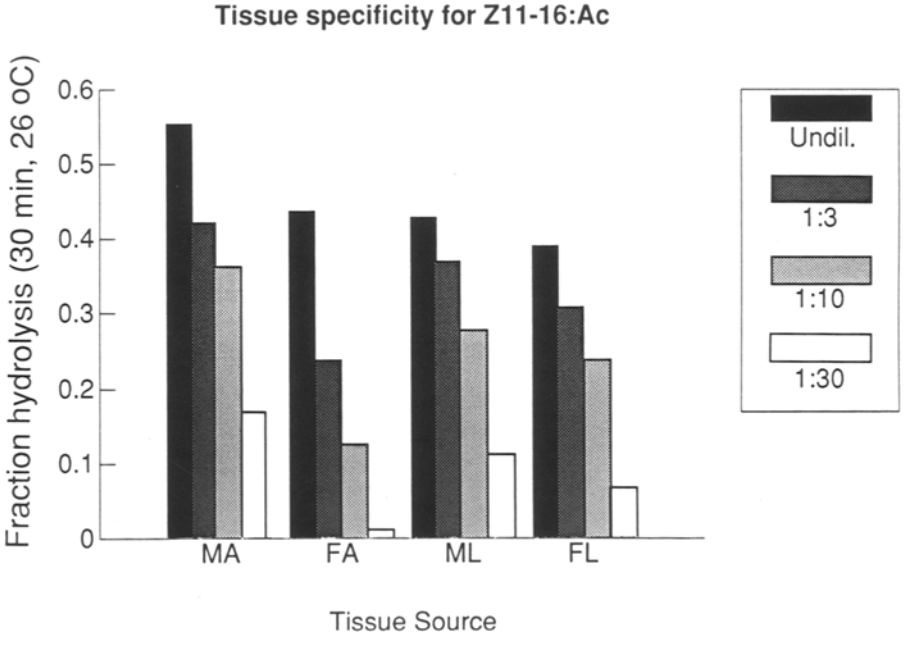


Figure 1. Distribution of esterase activity in legs and antennae of *Plutella xylostella* moths. Hydrolysis of [^{3}H]-*Z*11-16:Ac was measured in tissue homogenates using a radio-TLC assay described in the text.

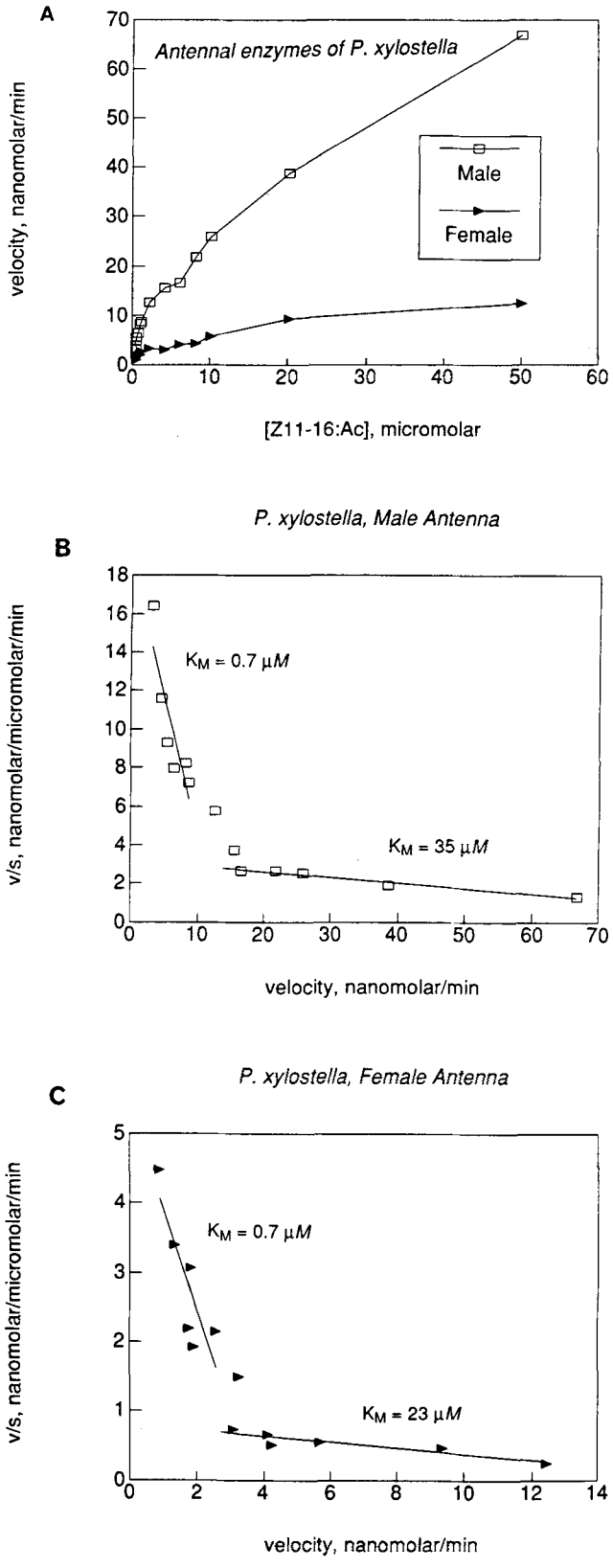


Figure 2. Kinetic analysis of *P. xylostella* antennal esterase activity. Panel (**A**) shows the saturation curves for *Z*11-16:Ac as substrate for male and female antennal enzymes at an equivalent number of antennal equivalents per assay. Two kinetically distinct esterases are apparent in the Eadie-Hostee plots of velocity vs velocity/substrate concentration for the male (**B**) and female (**C**) antennal activities, respectively.

16:BrAc with specific activities 45 Ci/mmol. Next, a dilution series from 0.02 to 5 mM in ethanol was prepared for each unlabeled haloacetate. Following the kinetics experiment outlined above for the acetate pheromone, hydrolysis data were collected, analyzed, and plotted; the results for the male antennal enzymes are illustrated in figures 3A–3C.

Only the monofluoroacetate, which had shown an IC_{50} value of 1 μM as a competitive inhibitor and showed stimulation of antennal receptor cells, showed a biphasic plot suggestive of hydrolysis by both esterase types. The electrophysiologically-inactive chloroacetate and bromoacetate analogs appear to be hydrolyzed only by the non-specific general esterase. These results are consistent with a role of the low K_M enzyme in the clearance of pheromone from sensory and non-sensory tissues.

Comparison with esterases in other moths

Hydrolysis of acetate pheromone components by legs, wings, and antennae was first described for the cabbage looper *Trichoplusia ni* (Noctuidae) by the Ferkovich group[5]. More recently, the importance of fatty acyl esterases in pheromone catabolism in the wild silkmoth *Antheraea polyphemus* (Saturniidae) and several *Heliothis* species (Noctuidae) has been documented using selective enzyme inhibitors, substrate analogs, high specific activity substrates, and electrophoresis combined with biochemical staining methods[21,22]. Similarly, the role of acetate hydrolysis in pheromone biosynthesis has been examined in the spruce budworm *Choristoneura fumiferana* (Tortricidae) and in other species[13]. The general and pheromone esterases seem to be widely distributed on the body surfaces, including wing scales[6,28] and legs[4–6], consistent with the need to degrade conspecific and heterospecific pheromone components which adsorb onto the cuticle surface. The localization of a set of male- and sensory-hair specific esterase isozymes has been demonstrated only for the large saturnid moth *Antheraea polyphemus*[9,26,29].

Aldehyde oxidation by Heliothis virescens (Noctuidae)

The tobacco budworm moth, a pest of cotton, is a primary target for our efforts to achieve selective disruption of pheromone communication using reactive analogs of the two aldehyde components *Z*11-16:Al and *Z*9-14:Al to inactivate receptor or catabolic proteins in sensory tissues[14,22]. For example, we found that acyl fluoride analogs hyperstimulated pre-copulatory behavior in male *H. virescens*[15]. Our examination of the primary enzymatic activities in *H. virescens* tissues using [^{3}H]-*Z*9-14:OH, [^{3}H]-*Z*9-14:Al, and [^{3}H]-*Z*9-14:Ac revealed alcohol oxidase, aldehyde dehydrogenase, and acetate esterase activities in legs, abdominal glands, and antennae of both sexes[3]. Attempts to inhibit the aldehyde dehydrogenase of antennae and legs with electrophilic reagents such as α-fluoro aldehydes, α,β-unsaturated ke-

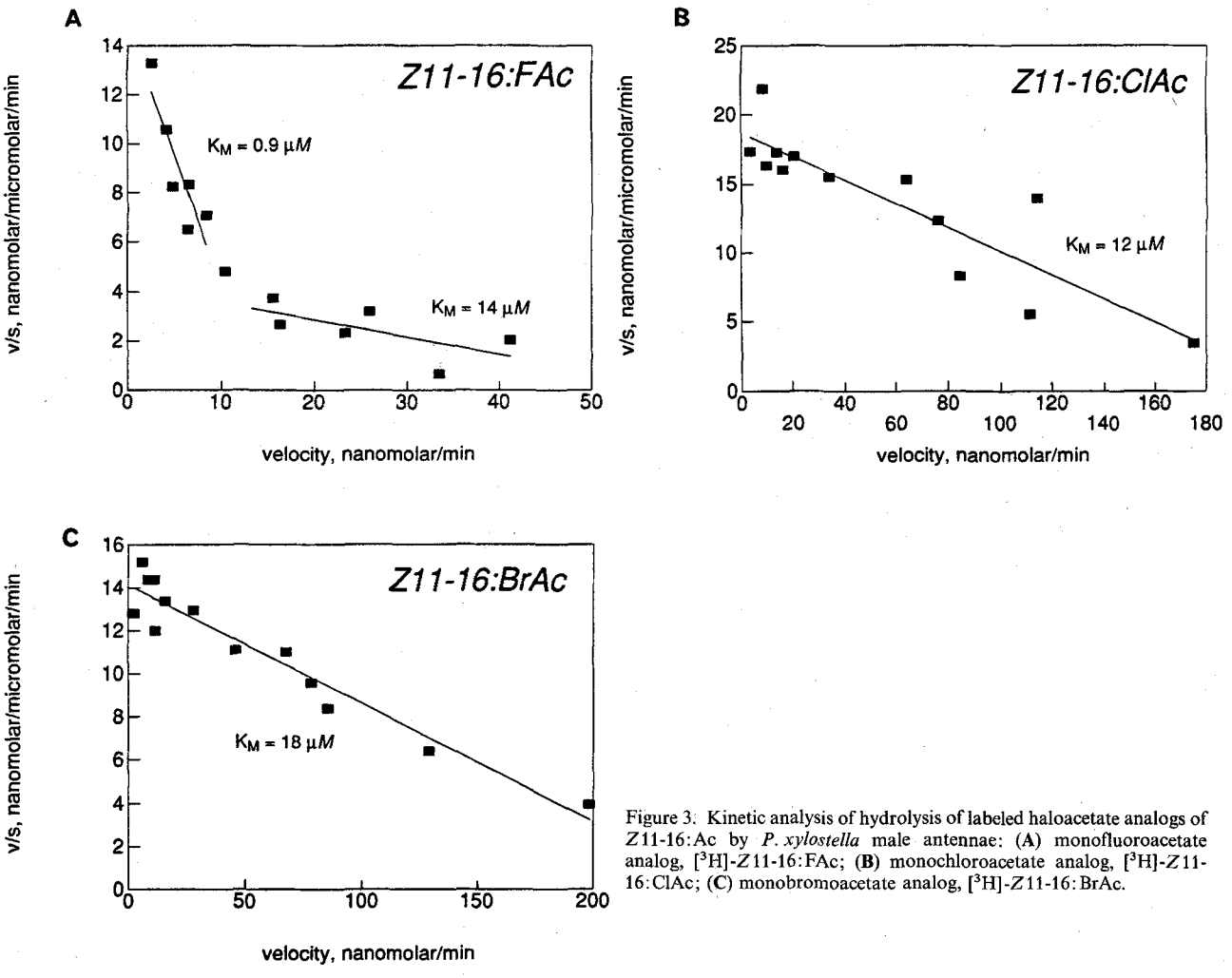
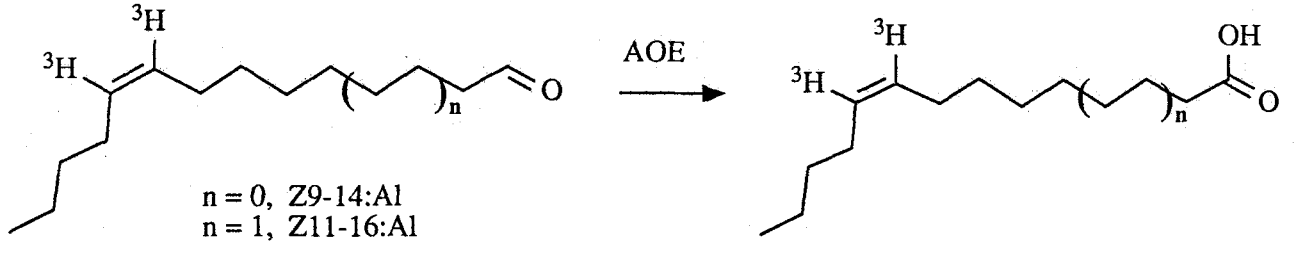


Figure 3. Kinetic analysis of hydrolysis of labeled haloacetate analogs of Z11-16:Ac by *P. xylostella* male antennae: (**A**) monofluoroacetate analog, [^{3}H]-Z11-16:FAc; (**B**) monochloroacetate analog, [^{3}H]-Z11-16:ClAc; (**C**) monobromoacetate analog, [^{3}H]-Z11-16:BrAc.

tones and aldehydes, and cyclopropanols led to the discovery of the vinyl ketone Z1,11-16:3-oxo as a potent, apparently irreversible inhibitor of [^{3}H]-Z11-16:Al oxidation to the carboxylic acid[4]. In addition, we began to see partial inhibition and oxygen requirements suggestive of the existence of more than one type of aldehyde oxidizing enzyme (see below).

To establish the specificity of the vinyl ketone inhibition of the aldehyde oxidizing enzymes, a series of homologous saturated and unsaturated vinyl ketones was prepared (fig. 4)[12]. Each vinyl ketone was assayed at thirteen concentrations from 0.20 µM to 100 µM final concentration using either the [^{3}H]-Z11-16:Al or the [^{3}H]-Z9-14:Al as substrate. The synthetic procedures will be summarized first, and then the assay protocol and results will be presented.

Each vinyl ketone (fig. 4) was prepared from the corresponding aldehyde by addition of vinyl magnesium bromide at low temperature followed by oxidation of the allylic alcohol with either pyridinium dichromate in CH_2Cl_2 or using a slurry of MnO_2 in CH_2Cl_2[4,12]. The electrophilic vinyl ketones were stored below 0 °C and purified by silica gel chromatography prior to use. Stock solutions were prepared in heptane at 10^{-2} M, and serial



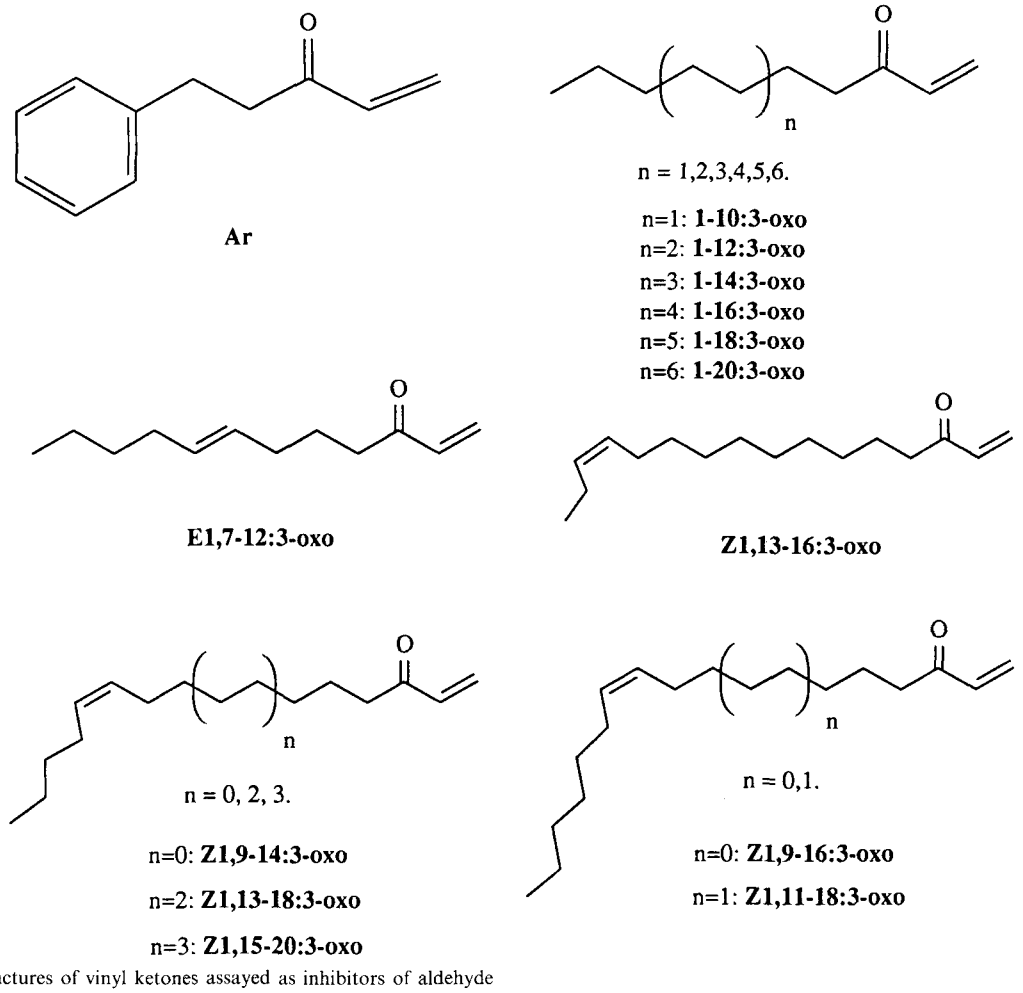


Figure 4. Structures of vinyl ketones assayed as inhibitors of aldehyde oxidation in *Heliothis virescens* tissues.

dilutions in ethanol were prepared immediately prior to use.

Enzyme solutions were obtained by homogenization (pH 7.4, 76 mM sodium phosphate buffer, 0.1 mM NAD^+, 4 °C) of freshly-collected antennae of 1-day-old virgin male *H. virescens* moths which had been reared from pupae at 27 °C in a 16:8 L:D photoperiod and narcotized with CO_2. Enzyme homogenates or antennae could be stored frozen at −90 °C. Assays were performed using 2–10 antennal equivalents per 100-µl assay, such that 20–30% oxidation was observed in the uninhibited controls. The enzyme solution was preincubated with 1 µl of the required ethanolic solution of vinyl ketone for 30 min, incubated for 30 min with 1 µl of ethanolic substrate ([^{3}H]-*Z*11-16:Al or [^{3}H]-*Z*9-14:Al), quenched with 100 µl of ethyl acetate, and duplicate 3-µl radio-thin layer chromatography (RTLC) samples separated (10% ethyl acetate-hexane) and counted (liquid scintillation). Data were normalized to be in the range from 0 to 100% (nonenzymic blank and inhibitor-free control, respectively).

The results of these assays are summarized in figures 5 and 6, which show the IC_{50} values (and percentage inhibition at 100 µM) for vinyl ketone inhibition of aldehyde oxidation for *Z*11-16:Al as substrate (fig. 5) and for *Z*9-14:Al (fig. 6)[12]. The percentage values at the top of each bar show that many vinyl ketones do not completely inhibit aldehyde oxidation. This residual activity is due primarily to the activity of a second set of enzymes, the aldehyde oxidases, as described below. Not shown in this scheme are the original two vinyl ketones, *Z*1,11-16:3-oxo and *Z*1,9-14:3-oxo, which were measured in a modified assay system. Their IC_{50} values[21] were 30 nM and 90 nM for oxidation of [^{3}H]-*Z*11-16:Al, and 5 nM and 20 nM for oxidation of [^{3}H]-*Z*9-14:Al (M. L. Tasayco J., unpublished results). In comparing chain length requirements for the saturated vinyl ketones, the greatest inhibition is achieved for the C_{12}, C_{14}, and C_{16} analogs for *Z*11-16:Al (fig. 5a) and the C_{14} and C_{16} analogs for *Z*9-14:Al (fig. 6a). For the most alkenyl-substituted vinyl ketones, figure 5b demonstrates high inhibitory potency for virtually all alkenyl-substituted vinyl ketones for inhibition of [^{3}H]-*Z*11-16:Al oxidation, with IC_{50} values below 5 µM for the majority of compounds. The aryl analog showed the lowest activity. The potency of the unsaturated analogs in blocking the oxidation of

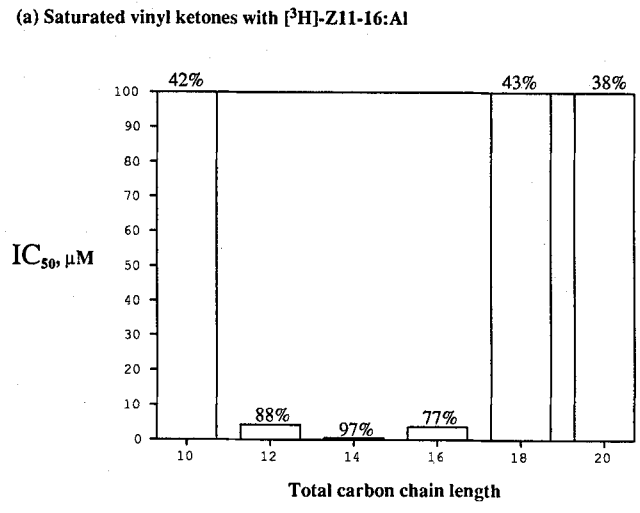


Figure 5. Inhibition of oxidation of [^{3}H]-*Z*11-16:Al by *H. virescens* male antennal enzymes: (a) saturated chain analogs, (b) unsaturated chain analogs. Percentage values on bars indicate % inhibition at IC_{50} or [I] = 100 µM.

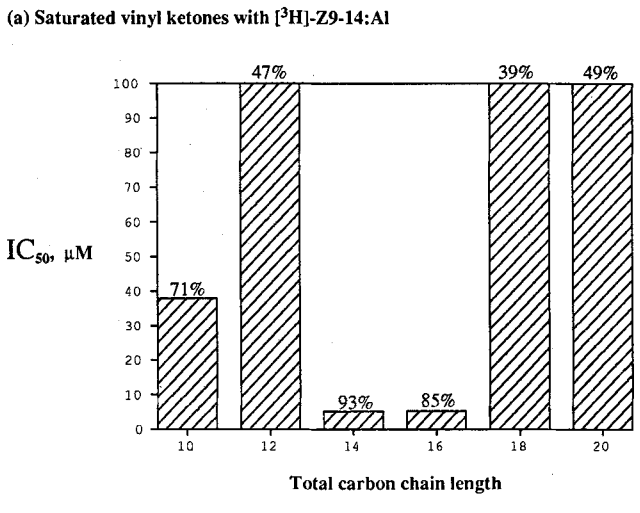


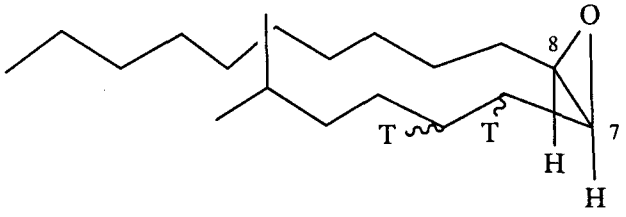
Figure 6. Inhibition of oxidation of [^{3}H]-*Z*9-14:Al by *H. virescens* male antennal enzymes: (a) saturated chain analogs, (b) unsaturated chain analogs. Percent values indicate % inhibition at IC_{50} or at [I] = 100 µM.

[^{3}H]-*Z*9-14:Al is more difficult to interpret in a systematic fashion.

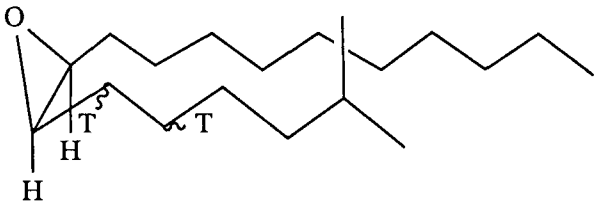
Recently, we have been re-investigating the nature of the aldehyde-oxidizing enzymes[21,25]. Antennal tissues contain both O_2-requiring aldehyde oxidase (AO) activity and an NAD^+-dependent aldehyde dehydrogenase (ALDH) activity. The AO activity can be detected in enzymatic assay using [^{3}H]-*Z*11-16:Al or [^{3}H]-*Z*9-14:Al in the absence of exogenous NAD^+. The ALDH can be detected directly by a spectrophotometric assay for appearance of NADH, although tissue requirements are high for this assay, and AO activity can be measured by an enzyme-coupled spectrophotometric assay of H_2O_2 using horseradish peroxidase. In electrophoretic gels (native PAGE or IEF), multiple AO isozymes can be visualized with formazan dye using benzaldehyde as the gel-staining substrate or by staining for H_2O_2 production using horseradish peroxidase. Additionally, the putative ALDH enzymes can be visualized in different locations on the gel by using the [^{3}H]-labeled vinyl ketones to covalently modify the protein bands (M. L. Tasayco J., unpublished results). In short, this painstaking re-examination shows AO activity localized in the antennal tissue of both sexes, and shows more efficient metabolism of the C_{14} substrate than the C_{16} substrate. In contrast, the ALDH activity is found in legs, head, and antennae (but not hemolymph). These data are consistent with the hypothesis that one or more AOs are the primary enzymes involved in pheromone degradation in sensory tissues, while the more widely distributed ALDHs are important in degrading adventitious absorbed aldehydes.

Epoxide hydration by Lymantria dispar (Lymantriidae)

The gypsy moth, a major forest defoliator, is unusual in having essentially a single-component pheromone. This



(-)-(7*S*,8*R*)-disparlure



(+)-(7*R*,8*S*)-disparlure

epoxide, disparlure, exists in the two enantiomeric forms. Female *L. dispar* moths produce only the (7*R*,8*S*) enantiomer, but males can detect both enantiomers via separate sensory cells [7]; the (+) enantiomer is attractive and the (−) enantiomer inhibits attraction [1]. We prepared each enantiomer in tritium-labeled form (58 Ci/mmol) by reduction of alkenyl oxiranes known to be >98% enantiomerically pure [23].

The radiolabeled epoxides are both cleanly converted to a single product by leg and antennal tissues of both male and female moths; the male antennae show the highest activity (fig. 7). Using homogenates from 100 antennae to convert 2 mg of disparlure (10 μM), we obtained sufficient 7,8-diol to unambiguously assign the *threo* structure by comparison with authentic material [23]. Thus, only the expected S_N2 product was produced, with not detectable amounts of the *erythro* diol.

We have now documented that the same single diol enantiomer is produced from hydration of either enantiomer by the male antennal enzymes [21]. Figure 8 shows the enantioselective hydration at C-8 to give a >97:3 ratio of (7*R*,8*R*) to (7*S*,8*S*)-diol (S. McG. Graham and W. A. König, unpublished results). By using a chiral-phase fused-silica capillary gas chromatography column [10], the *threo*-bis(trifluoroacetate) derivatives of the diol enantiomers can be separated and quantified. The

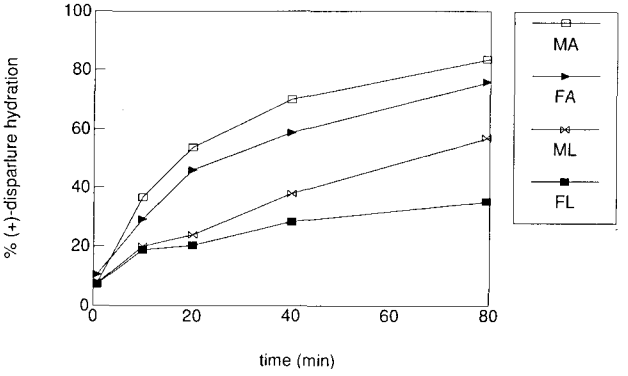


Figure 7. Tissue course of epoxide hydrase activity in *Lymantria dispar* male and female moths detected by radio-TLC assay of homogenates from four tissues with 200 nM [^{3}H]-(+)-disparlure, using six antennae or legs per 300 μl assay. Key: MA, male antenna; FA, female antenna; ML, male leg, FL, female leg.

absolute stereochemistry of disparlure opening by selected tissues is now being investigated using chiral disparlure and *meso*-substrate mimics.

Practical applications

Manipulation of insect behavior with pheromones is emerging as a key strategy in integrated pest manage-

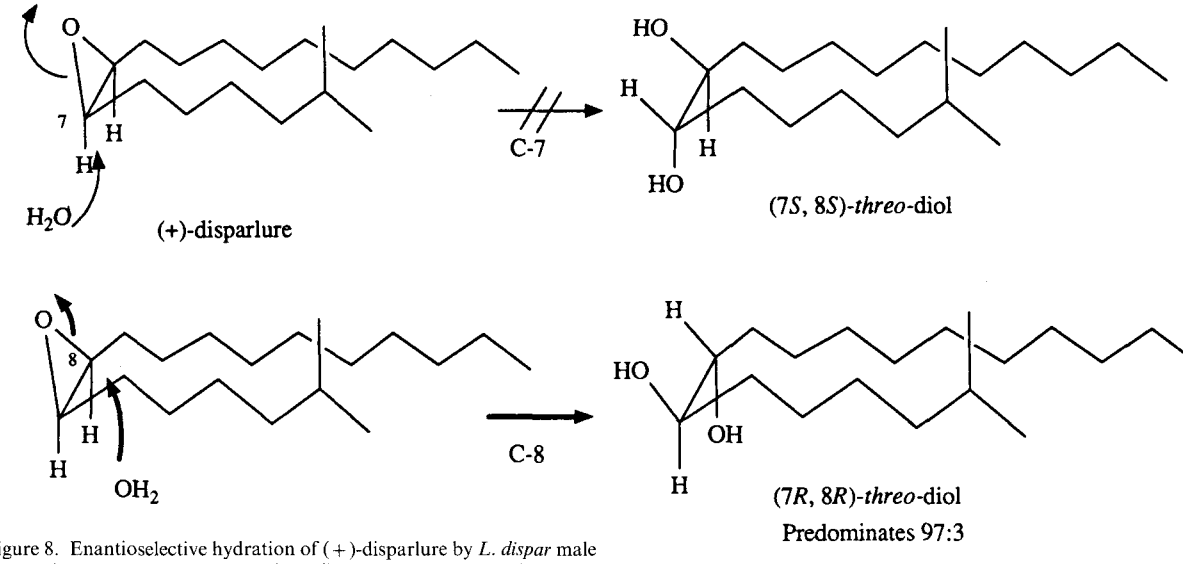


Figure 8. Enantioselective hydration of (+)-disparlure by *L. dispar* male antennal enzymes occurs by attack at C-8 to give the (7*R*,8*R*)-diol, as determined by chiral GC analysis.

ment. Aside from the uses of pheromones for monitoring population levels of crop pests and occasional efforts at mass trapping, the major application appears to be disruption of communication. Pheromonal-based 'confusion' requires relatively large quantities of pheromone to saturate the atmosphere above a plot, thereby making mate location impossible. Two clear drawbacks of this approach are the quantity of pheromone required and the problem of immigration from outside the plot by additional pests; mating at plot edges could then increase. We envision the use of specific enzyme-targeted pheromone mimics to inactivate the sensory system either by irreversibly modifying pheromone binding or receptor proteins, or by disabling the catabolic enzymes which degrade pheromone. To implement such a biochemical strategy, we have described herein several efforts to design reactive pheromone mimics. In the future, we hope our approach will 1) provide fundamental new information on pheromone sensory biochemistry, and also 2) provide lead structures for development of biochemically-based mating disruptants.

Acknowledgments. We thank the U.S. National Science Foundation, the Herman Frasch Foundation, the U.S. Department of Agriculture, and the Rohm and Haas Company for grants in support of this research. We are grateful to Dr R. G. Vogt and Dr Y.-S. Ding for their guidance and advice for the enzyme assays, Dr W. A. König for chiral GC analyses, Ms H. Mei for help in conducting *Plutella* assays, and Drs G. E. Drake (*Heliothis*), C. P. Schwalbe (*Lymantria*), and K. D. Biever (*Plutella*) for providing insect pupae.

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