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# Bio-organic studies of insect olfaction

Glenn D. Prestwich

Department of Chemistry, State University of New York
Stony Brook, New York 11794-3400, USA

Abstract - Pheromones interact with binding and catabolic proteins in insect olfactory sensilla, and the activated dendritic receptors are important in the transduction of a chemical binding event into an electrical signal. Selected results will be presented in which high specific activity tritium-labeled pheromones and pheromone analogs are synthesized and used to study proteins from insect antennae which bind and degrade pheromone molecules.

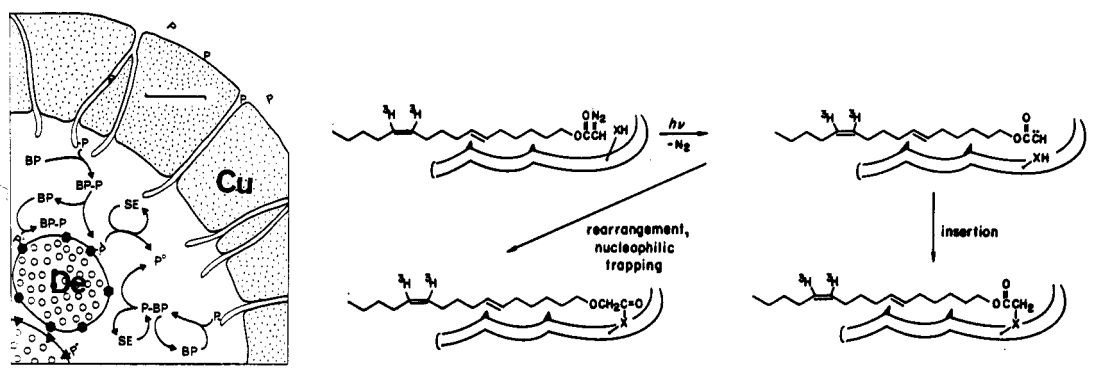
## INTRODUCTION

A basic understanding of the biochemical events involved in pheromone biosynthesis, release, reception, transport, transduction, and degradation in insect systems is still lacking. This new discipline of **pheromone biochemistry** is the subject of a recent monograph (ref. 1). The exploration of olfactory binding proteins and transductory mechanisms in vertebrate systems has also received considerable attention recently (ref. 2). Our focus at Stony Brook is the use of pheromone components stoichiometrically radiolabeled with tritium to investigate the antennal proteins involved in binding and catabolism. In addition, we have employed chemically reactive and photoactivated affinity analogs of pheromones to modify pheromone-processing proteins covalently.

In the sections below, I describe recent results from several species of moths and beetles. This survey highlights how different chemical functionalities present in pheromones, e.g., epoxides, alcohols, aldehydes, acetates, ketones, and cyclic ethers, undergo biotransformations *in vivo*.

## DIAZOACETATE PHOTOAFFINITY LABEL: *Antheraea polyphemus*

Long-chain unsaturated acetates are the most ubiquitous pheromone components emitted by female moths. Male giant silk moths have large antennae with exquisitely sensitive and selective detectors for low concentrations of (*E,Z*)-6,11-hexadecadienyl acetate, the major pheromone component. Pheromone molecules (P) enter through pores in the cuticle (Cu) and interact with a soluble esterase (SE) and a soluble binding protein (BP) enroute to the receptors in the membrane of the olfactory dendrite (De). We therefore prepared a tritium-labeled photoaffinity analog of a this pheromone, [11,12-$^3H_2$]-(*E,Z*)-6,11-hexadecadienyl diazoacetate, to identify pheromone binding proteins in moth antennal tissues (ref. 3).

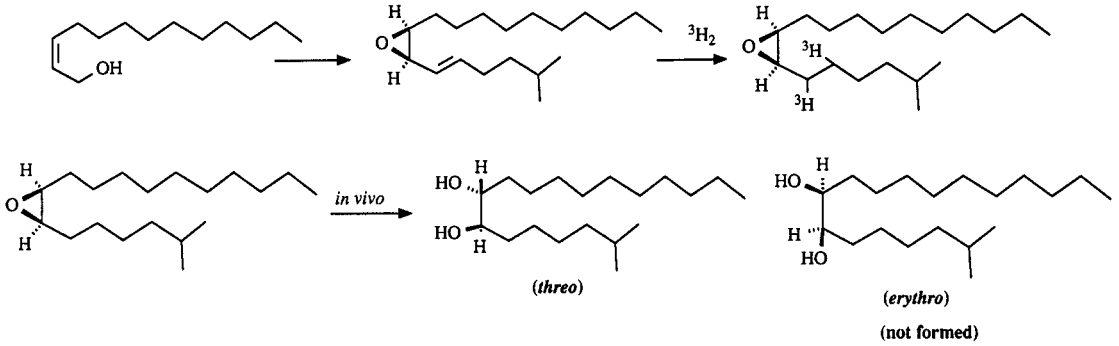



Using purified sensillum lymph and sensory dendritic membranes of *A. polyphemus*, we have been able to photoaffinity label a 15-kDa soluble protein (BP) and a 69-kDa membrane protein (solid hexagons) (ref. 4). Tritium-labeled covalently modified proteins can be conveniently detected in denaturing polyacrylamide gels by fluorescence autoradiography. These two proteins were found only in males, and were uniquely present in antennal tissues. The substrate specificity was demonstrated by a protection experiment, in which photoattachment of the tritiated analog is blocked by co-incubation with a 100-fold excess of the unlabeled pheromone during the irradiation at 254 nm. These experiments constitute the first direct evidence for a membrane protein of a chemosensory neuron interacting in a specific fashion with a biologically relevant odorant, and we propose that this low-abundance membrane protein is in fact the sex pheromone receptor protein for this insect.

## EPOXIDE BINDING AND HYDRATION: *Lymantria dispar*

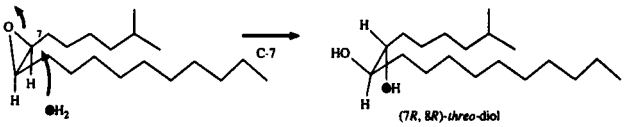
The female gypsy moth *L. dispar* produces (+) disparlure, (7*R*,8*S*) 7,8-epoxy-2-methyloctadecane, as a single pheromone component. However, the male gypsy moths detect both enantiomers via separate sensory hair receptor cells (ref. 5); the (+) enantiomer is attractive and the (-) enantiomer inhibits attraction (ref. 6). We were interested to know what antenna-specific proteins were involved in recognition, binding, and metabolism of the two disparlure enantiomers. Thus, we prepared each enantiomer in >95% e.e. with stoichiometric tritium labeling to have specific activities of 58 Ci/mmol (ref. 7) for optimal sensitivity in detecting binding and degradation products at nanomolar concentrations.

The radiolabeled epoxides are both cleanly converted to a single product by antenna-specific enzymes of adult male gypsy moths. Using protein homogenates from 100 antenna to convert ca. 50% of 2 mg of disparlure (ca. 10 μM epoxide substrate), we obtained a 7,8-diol product, as determined by GC-MS of the bis(TMS ether), the bis(acetate), and the *n*-butylboronate. This diol product was chromatographically identical to the authentic racemic *threo*-diol prepared by alumina-acetic acid induced opening of the *cis* epoxide followed by hydrolysis of the mixture of mono- and diacetates. The *erythro* diastereomer was produced analogously from the *trans* epoxide; this compound and its derivatives were cleanly separated from the *threo* diastereomers on capillary GC and TLC. Hydration of disparlure under substrate saturation conditions (10 μM) produced no detectable *erythro* diol (<0.3%); furthermore, incubation of the pure radiolabeled enantiomers at physiological concentrations (< 200 nM) afforded only the $^3$H-labeled *threo* diol as the product.

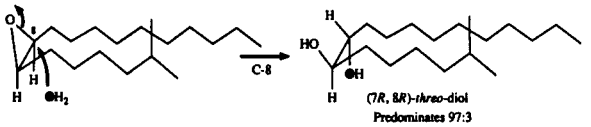



Analytical separation of the *threo*-diol enantiomers can be accomplished with a chiral capillary column (ref. 8), providing access to direct stereochemical analysis of the absolute stereochemistry and regiochemistry of the epoxide hydrolase reaction. Thus, in collaboration with Dr. W.A.. König, the *threo*-bistrifluoroacetates of diols produced by hydration of the racemic, (+)- and (-)- disparlure substrates by male *L. dispar* antennal enzymes were separated on a 30-m column coated with XE-60-L-valine-(*R*)-α-phenylethylamide. As shown below, both the (+) and (-) enantiomers are converted to the (7*R*,8*R*)-diol (authentic (7*S*,8*S*)-diol was a gift of Dr. Y. Masaki).

Hydration of (-)-Disparlure Occurs By Attack at C-7



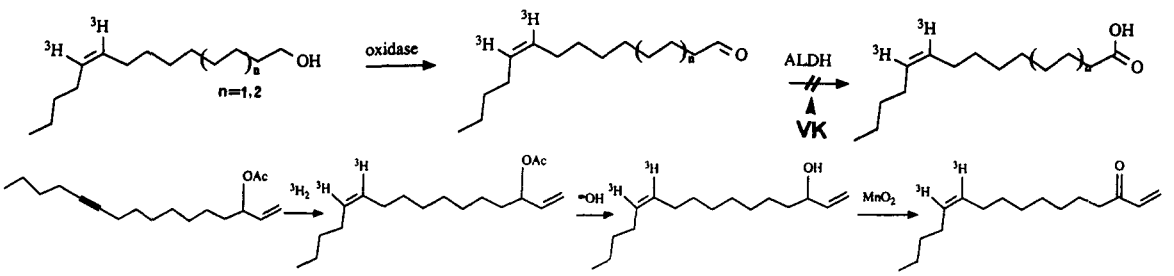

Hydration of (+)-Disparlure Occurs By Attack at C-8




Using proteins and mRNAs extracted from the antennae of male antennae from five days before emergence to two days post-emergence, Dr. R.G. Vogt characterized two male-specific binding proteins in *L. dispar* antennae in equal abundance (ref. 10). From the purified proteins, we have obtained partial amino acid sequences and antisera which recognize pheromone binding proteins from a variety of moth species. Surprisingly, only one of these two 14-kDa proteins binds tritium-labeled disparlure when visualized by fluorography of native-gel separated protein-ligand complexes, and both the (+) and (-) enantiomers bind equally to this one protein.

## VINYL KETONES AND ALDEHYDE OXIDATION: *Heliothis virescens*

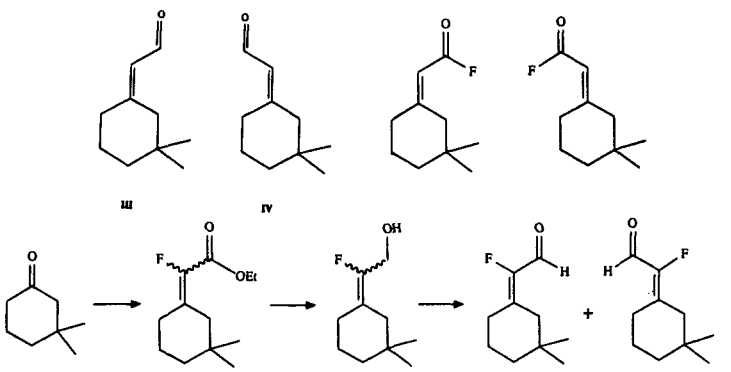
*Heliothis virescens* (the tobacco budworm) use a multicomponent blend of aldehydes as the sex pheromone. These are produced in the adult female pheromone gland by an alcohol oxidase (ref. 11) and de-activated to the carboxylic acids in the male and female antennae by aldehyde oxidizing enzymes, initially defined as aldehyde dehydrogenases (ref. 12). Recently, we described chemical approaches to inhibition of the aldehyde-oxidizing enzymes using cyclopropanols, α-fluoroaldehydes, and vinyl ketones (VK) as active-site directed substrate analogs (ref. 12). Only the vinyl ketones proved to be potent (submicromolar) and irreversible inhibitors of the conversion of $^3$H-Z9-14:Al and $^3$H-Z11-16:Al to the labeled acids.




Further detailed examination of the antennal enzymes has been carried out by M.L. Tasayco J. (unpublished results). She has found the apparent $K_m$ values for oxidation of the two aldehyde pheromones to the corresponding acids to be in the 1-6 micromolar range. However, two enzyme activities could be distinguished: an $O_2$-requiring oxidase and an NAD+-requiring dehydrogenase. The oxidase and dehydrogenase activities can be separated by gel electrophoresis using a benzaldehyde and NAD+ substrates coupled to a tetrazolium dye to visualize the oxidase. This activity is not inhibited by either the vinyl ketones or the addition of NADH; however, the pheromone-processing enzymes are inhibited by both the vinyl ketones and by NADH. Furthermore, covalent modification of the pheromone-processing enzymes with a labeled vinyl ketone (synthesized as shown above) followed by native PAGE clearly shows the dehydrogenase enzymes to be different from the benzaldehyde-visualized oxidases. Finally, we have examined a homologous series of twelve saturated and unsaturated vinyl ketones with each aldehyde substrate to determine the structural parameters important in specific inhibition of the antennal enzymes (ref. 14). Briefly, the $C_{14}$ and $C_{16}$ unsaturated inhibitors are clearly superior, with activity diminishing by either saturation or regiochemical movement of the unconjugated alkene. The $IC_{50}$ values are 30 nM and 90 nM, respectively for the oxidation of $^3$H-Z11-16:Al by Z1,11-16:3-oxo and Z1,19-14:3-oxo; both inhibitors inhibit the $^3$H-Z9-14:Al more effectively with 5 nM and 20 nM values, respectively.

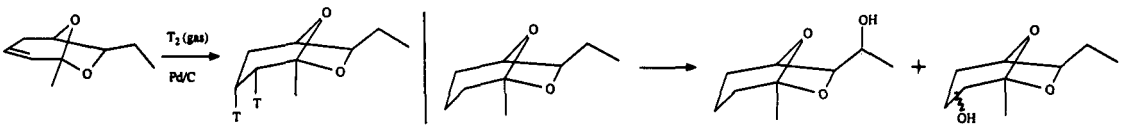
## RESPONSES TO FLUORINATED PHEROMONES: *Anthonomus grandis*

The boll weevil pheromone contains two isomeric enals which attracted our attention as candidates to probe the role of fluorine as isostere of hydrogen in pheromone perception. We thus prepared the electronically perturbed acyl fluorides and α-fluoroaldehydes to determine if they would show altered electrophysiological and behavior properties (ref. 15). The two isomeric acyl fluorides, which proved to be stable to hydrolysis and chromatography, were essentially inactive in eliciting responses from female weevil antennae in an electroantennogram assay. In contrast, the α-fluoroaldehydes were actually more potent at low dosage levels than the natural enals. Finally, using carefully separated III and IV, J.C. Dickens was able to demonstrate that male and female weevil antennal response to the (*Z*)-enal III is 10- to 100-fold weaker than to the (*E*)-enal IV. Similarly, full behavioral responses can be obtained using blends containing only IV instead of III + IV (ref. 16).



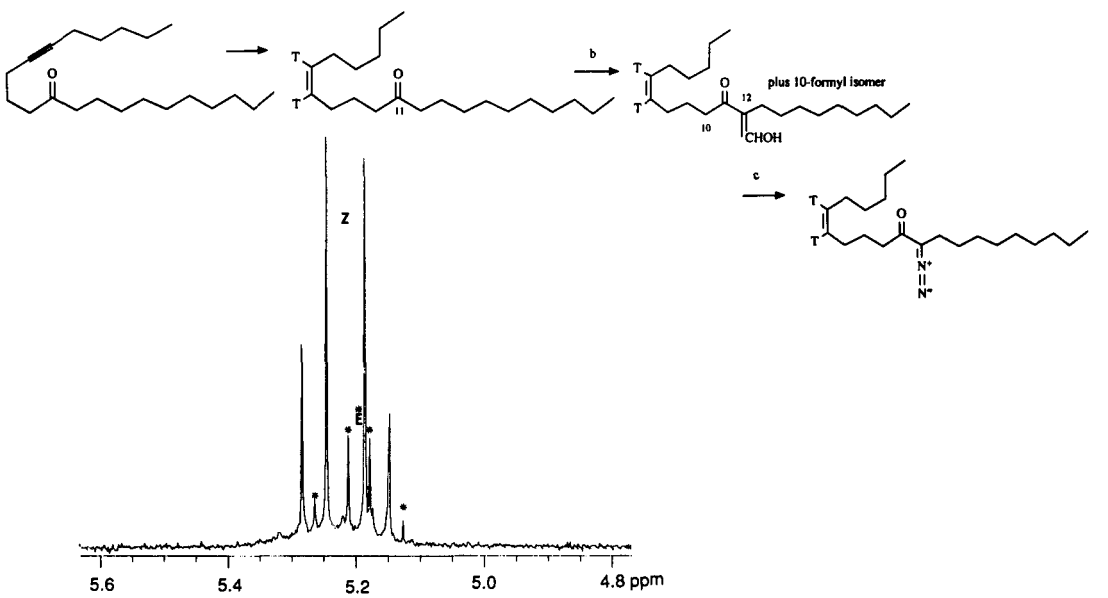
## HYDROXYLATION OF CYCLIC KETALS: *Dendroctonus ponderosae*

Bark beetles are well-known for their cyclic ketal constituents, and we were curious what chemical conversions might be involved in the de-activation of these volatile, nonpolar olfactory stimuli. To this end, we obtained a sample of dehydrobrevicomin and prepared high specific activity tritium-labeled *exo*-brevicomin. In work carried initiated at Stony Brook and continued at the College of Forestry and Environmental Sciences at Syracuse (Dr. F.X. Webster) and at the University of Calgary (D. Crump, E.A.Dixon, and H. Wieser), we discovered that brevicomins are oxidized by intact live pine bark beetles to monohydroxylated products (ref. 16). Neither beetle homogenates nor intact heat-killed beetles catalyzed formation of these products. To obtain material for spectometric measurements, unlabeled brevicomin enantiomers were employed. At high concentrations (1-10 mg/10 beetles), three hydroxyketals are generated; at lower, more physiological concentrations, a single product is obtained. This has been identified by an independent synthesis and NMR studies to be the axial alcohol as illustrated on the next page.




## KETONE PHEROMONE AND TRITIUM NMR: *Orgyia pseudotsugata*

Functional groups important in insect pheromones include acetates, aldehydes, epoxides, hydrocarbons, cyclic ketals, ethers, and ketones. We chose the Douglas fir tussock moth, a lymantriid closely related to the gypsy moth, as a suitable model insect to investigate binding and catabolism of ketonic pheromones. Thus, we prepared tritium-labeled Z6-heneicosen-11-one by catalytic semitritiation of the 6-alkyne, and observed an unexpected AB quartet pattern for the anisochronous vinyl tritons in the 320 MHz tritium NMR spectrum (ref. 18). Moreover, the presence of ca. 15% of the *E* isomer was clearly signalled by a second AB pattern. The ketone (labeled or unlabeled) can be converted by formylation and diazo transfer to a mixture of two diazoketones, which show EAG and behavioral activity in *O. pseudotsugata* males. In addition, irradiation (254 nm, 1 min) of the antennal proteins in the presence of the labeled diazoketones results in covalent photoattachment to a 21-kD protein, analogous to the result seen in photolabeling of the *A. polyphemus* soluble proteins.

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