Chemistry of Pheromone and Hormone Metabolism in Insects

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Chemical evidence is needed in both insect endocrinology and sensory physiology to understand hormone and pheromone action at the molecular level. Radiolabeled pheromones and hormones have been synthesized and used to identify binding and catabolic proteins from insect tissues. Chemically modified analogs, including photoaffinity labels and enzyme inhibitors, are among the tools used to covalently modify the specific acceptor or catalytic sites. Such targeted agents can also provide leads for the design of growth and mating disruptants by allowing manipulation of the physiologically important interactions of the chemical signals with macromolecules.

THE CHEMICAL STRUCTURES OF INSECT PHEROMONES AND hormones have been known in some cases for as long as two decades. More recently, the technologies of microscale protein and nucleic acid sequencing and of recombinant DNA have enabled substantial probing of the molecular actions of hormones and pheromones. However, no single discipline can answer the question of how, for example, a simple sesquiterpene hormone triggers a change in a complex developmental sequence, or how a simple fatty acid–derived pheromone triggers a complex behavioral sequence. The research I describe here focuses on the use of high specific activity radioligands and chemically modified pheromone and hormone analogs to dissect the biochemical events that occur in the specific insect tissues that are the targets of these chemical signals.

Juvenile Hormone Bio-Organic Chemistry

Juvenile hormones (JHs) are biosynthesized from acetate and propionate in insects through the mevalonate and homomevalonate pathways by paired neurosecretory organs, the corpora allata, and are then released into the hemolymph for transport to target tissues (*1*). The two primary effects of juvenile hormone are (i) the morphogenetic effect, that is, the modulation of larval growth and development and the inhibition of premature metamorphosis (*2*), and (ii) the gonadotrophic effect, that is, the regulation of several aspects of reproductive physiology including vitellogenesis (*3*). In (i), JH acts as a repressor, whereas in (ii), it activates DNA transcription and the translation of new messenger RNAs into proteins. A third effect of JH has been found in social insects, namely, the regulation of caste determination; for example, JH induces worker to soldier molts in termites (*4*).

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The discovery of JH, the chemical identification and synthesis of JH, and the study of the role of JH in insect physiology and molecular biology now span more than a half-century. Five naturally occurring JH homologs have been identified (*5*), and literally thousands of JH analogs (JHAs) have been prepared and evaluated as insect growth regulators (*6*). Some of these are greater than 1 million times more active than JH and exhibit high species specificities. However, there is still an inadequate understanding of how the JH homologs and JH analogs work at the molecular level. The detection of macromolecules that bind JH with high affinity requires ligands that are radioactively labeled with high specific activity, that is, at levels near 2000 Ci/mmol for carrier-free ^{125}I or greater than 58 Ci/mmol for carrier-free $^{3}H_2$. Synthesis of radioactively labeled JH homologs and analogs, including radioimmunoassay (RIA) antigens, has been reviewed recently (*7*). The efforts of the previous decade focused on the preparation and use of relatively low specific activity radioligands for the study of JH and JHA distribution and metabolism (*8*). Recently, my co-workers and I have prepared four new types of radioactively labeled ligands for studying JH biochemistry. These ligands are used in locating JH-specific carriers and receptors in over 20 insect and tick species from five orders. The four types include:

1) Enantiomerically enriched JH I, JH II, and JH III, in both the naturally occurring and the unnatural enantiomeric forms (*9*), have been prepared with tritium labels with specific activities of 15 to 60 Ci/mmol and have been assayed with JH binding proteins (JHBP).

2) Enantiomerically enriched and racemic photoaffinity-labeled analogs of JH I, JH II, and JH III have been prepared with specific activities of 10 to 60 Ci/mmol (*10*). These diazoacetates allow covalent and highly specific attachment to JHBP and also allow modified proteins to be analyzed by denaturing and proteolytic techniques (*11, 12*).

3) Tritium-labeled hydroprene and methoprene have been prepared by two separate synthetic routes to yield JHAs with specific activities in excess of 55 Ci/mmol for the study of the receptor

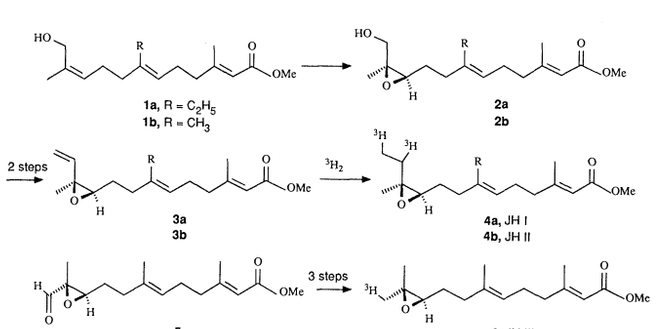

Fig. 1. Synthesis of the high specific activity, enantiomerically enriched juvenile hormones JH I (**4a**), JH II (**4b**), and JH III (**6**).

binding properties of these commercial dienoate insect growth regulators (IGRs) (*13*).

4) Iodine-125 labeled analogs of JH I (12-iodo-JH I) and of methoprene (iodovinylmethoprenol, IVMA) have been synthesized and are valuable tools for JH receptor characterization (*14*).

JH homologs. Since the first chemical synthesis of JH homologs in 1970, many stereoselective preparations have been reported and the enantiomers have been chemically and enzymatically separated. JH homologs labeled with tritium at 10 to 15 Ci/mmol have been commercially available in racemic form and are obtained by sodium borotritide reduction of the 11-chloro-10-ketones (*15*). However, none of the early chemical syntheses allowed access to high specific activity tritium-labeled JH homologs that were enantiomerically pure. Enantiomerically enriched JH I (**4a**) and JH II (**4b**) with specific activities of 58 Ci/mmol for both the natural and unnatural optical isomers have been synthesized (*7*) (Fig. 1). The key intermediates in this synthesis are the (*Z*)-allylic alcohols **1**, which can be converted in high chemical yield to the epoxy alcohols **2** in a ratio greater than 98:2 of natural (10*R*,11*S*) to unnatural (10*S*,11*R*) enantiomers. Both the JH I (R = C_2H_5) or JH II (R = CH_3) series can then be obtained by the catalyzed homogeneous tritiation of the vinyl oxiranes **3** to the ditritioethyl oxiranes **4**. However, this

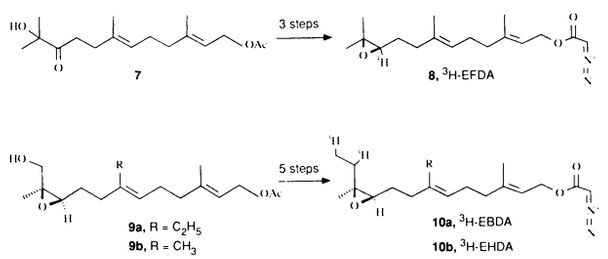

Fig. 2. Synthesis of tritium-labeled photoaffinity labels for insect JH binding proteins: EFDA (**8**); EBDA (**10a**); and EHDA (**10b**); Ac denotes acetyl group.

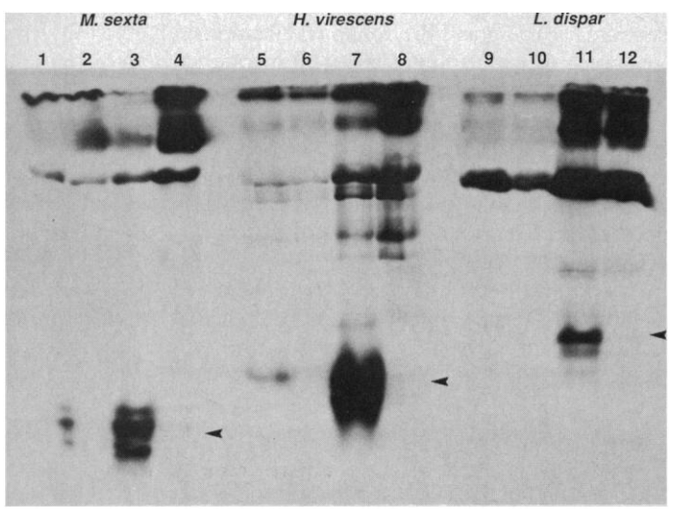

Fig. 3. Photoaffinity labeling of JH binding proteins from the hemolymph of three lepidopterous larvae: lanes 1 to 4, *M. sexta*; lanes 5 to 8, *H. virescens*; lanes 9 to 12, *L. dispar*. For each species the first pair of samples is unphotolyzed and the second pair is photolyzed for 30 seconds at 254 nm. Lanes 3, 7, and 11 were irradiated with [^{3}H]EFDA (**8**) alone, whereas lanes 4, 8, and 12 were irradiated with [^{3}H]EFDA and a 30-fold excess of unlabeled JH I. Specific labeling is displaced by this protection experiment. Proteins were separated by native PAGE in a 5 to 20% gradient gel (5 to 20%); the labeled bands were visualized by fluorography on x-ray film. All labeled proteins showed molecular weights in the 28- to 32-kD range on SDS-PAGE (*18*) but clearly differ in charge and native conformation, or both. [Figure courtesy of P. Kulcsar, Stony Brook, New York]

method cannot be used to make the most abundant JH homolog, JH III (**6**), which lacks the terminal ethyl group. Natural (10*R*)-JH III has now been prepared at 12 Ci/mmol by the reduction of a chiral epoxyaldehyde (**5**) with sodium borotritide followed by a two-step conversion to the tritiomethyl compound (*16*). Optically pure, high specific activity JH III is also being synthesized by selective tritiation of an 8,9-dehydro JH III (*17*).

Competitive binding studies with the radioactively labeled JH I and JH II enantiomers were performed with the hemolymph JH-binding proteins of *Manduca sexta, Trichoplusia ni, Heliothis virescens*, and *Lymantria dispar* (*18*) and for characterization of an epidermal JHBP in *Galleria mellonella* (*19*). The natural (10*R*,11*S*) enantiomers are bound with two- to tenfold higher affinity than the unnatural (10*S*,11*R*) enantiomers in each case. In addition, labeled natural JH II is hydrolyzed by purified JH esterase (*20*) from *T. ni, M. sexta, H. virescens*, and *Heliothis zea* up to three times faster than the unnatural enantiomer (*21*). Labeled and unlabeled JHs with the natural epoxide configuration are being used by other research groups in the development of JH radioimmunoassays (*22*).

Photoaffinity labels. In 1982, our laboratories introduced the first photoactivatable analogs of JH III and their relative binding affinities for cockroach JHBP were described (*23*). Subsequently, the highest affinity analog, epoxyfarnesyl diazoacetate (EFDA) (**8**), was prepared with a [10-^{3}H] label with specific activities of 5 to 11 Ci/mmol (*7, 10*) (Fig. 2). The tritium label is introduced by sodium borotritide reduction of the hydroxy ketone **7**. Tritium-labeled EFDA (**8**) has been successfully used to characterize JHBP in a variety of insects (*11*), including cockroaches (*24*), grasshoppers (*25, 26*), cultured *Drosophila* cells (*27*), *M. sexta* and other lepidopterous larvae (*18*), and termites (*28*).

The [^{3}H]EFDA-labeled hemolymph JHBP from *M. sexta* larvae

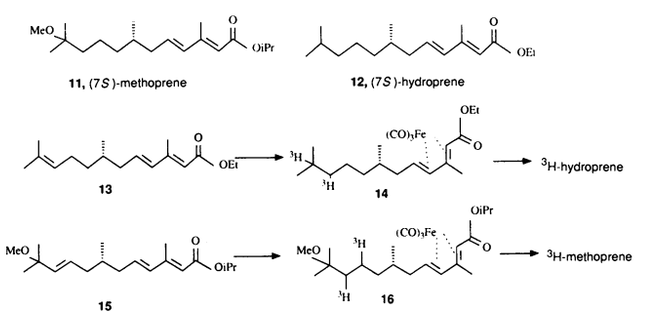

Fig. 4. Synthesis of tritium-labeled dodecadienoate JH analogs [^{3}H]hydroprene (**12**) and [^{3}H]methoprene (**11**) with an iron tricarbonyl protecting group. Abbreviations: Me, methyl; Et, ethyl; iPr, isopropyl.

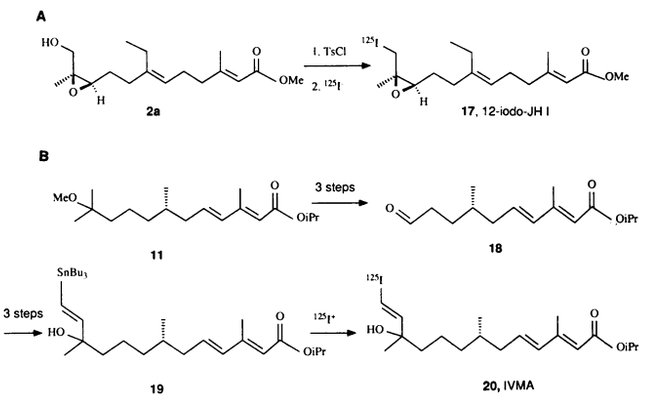

Fig. 5. Synthesis of two radioactive iodine–labeled JH analogs; (**A**) 12-iodo-JH I (**17**) and (**B**) IVMA (**20**). Abbreviations are as in the legend to Fig. 4 plus TsCl, tosyl chloride; $SnBu_3$, tributyltin.

has been purified by reversed-phase high-performance liquid chromatography (HPLC); 35 of the NH_2-terminal amino acids have been sequenced (*29*). The results confirm the 18 amino acid residues determined by the Law group (*30*) and indicate that EFDA does not covalently modify an amino acid residue in this region of the macromolecule. Current efforts focus on (i) the isolation and sequencing of proteolytic fragments of the labeled *M. sexta* JHBP and mapping of the amino acids at the active site, (ii) NH_2-terminal sequencing of EFDA-labeled JHBP of *H. virescens* and other caterpillar JHBPs, and (iii) immunochemical comparisons of the JHBPs from different sources. Figure 3 shows a comparison of the [^{3}H]EFDA-labeled JHBPs from the hemolymph of three lepidopterous larvae after separation on a native polyacrylamide gel and autoradiography (*31*). The multiple bands for EFDA-labeled JHBP can also be observed in two-dimensional gel electrophoresis [isoelectric focusing (IEF) then sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)]. NH_2-terminal amino acid sequences of the different electromorphs are obtained after electroblotting the proteins onto activated glass fiber paper (*32*).

We have used [^{3}H]EFDA in competition with methoprene or JH III for labeling hemolymph proteins of ticks and termites. We used two-dimensional electrophoresis to observe two new proteins in the hemolymph of mated female dog ticks (*Dermacentor variablis*) that could be photolabeled with [^{3}H]EFDA; the photoaffinity label could be displaced by a tenfold excess of JH III (*33*). Moreover, these two proteins were absent from the hemolymph of virgin female ticks. With the Eastern subterranean termite *Reticulitermes flavipes*, Robles and Mohamed (*28*) showed that worker termites that were kept isolated from soldiers developed a new hemolymph protein that can be labeled with [^{3}H]EFDA. The labeling can be prevented by competitive displacement with JH III but not with methoprene. This new binding protein is absent in workers in normal colonies with soldiers and is absent in soldiers and presoldiers. The protein can also be induced in workers by application of methoprene, which is known to cause workers to differentiate into presoldiers. These two examples illustrate the extension of the photoaffinity labeling technique to species in which JHBPs were previously uncharacterized and for which JH titers are unknown.

If a racemic, moderate specific activity analog of JH III is useful, then chiral, high specific activity analogs of each JH homolog would be even more useful (Fig. 2). Tritium-labeled EHDA (**10b**), which is the diazoacetate analog of JH II, was recently synthesized with a specific activity of 58 Ci/mmol from an enantiomerically pure epoxy alcohol precursor **9b** (*7*). The JH I analog [^{3}H]EBDA (**10a**) was synthesized from the related precursor **9a** (*34*). The more lipophilic JH analogs have increased affinity for the JHBP of lepidopterous larvae such as *T. ni, H. virescens*, and *M. sexta*, and give both improved selectivity and sensitivity for JHBP detection (*31*).

The dialkylacrylate chromophore of the natural JHs (λ_{max} = 217 nm) can be photochemically excited with 254-nm light. This excited species has a small probability of radical coupling to a binding site

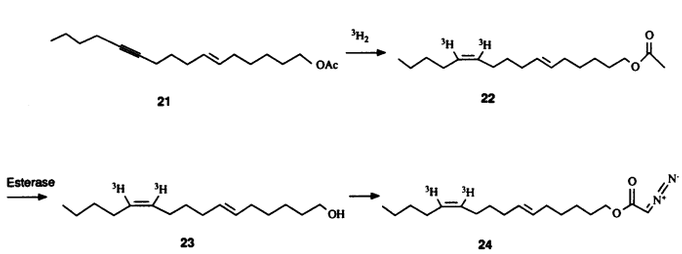

Fig. 6. Chemical synthesis of [^{3}H]*E*6, *Z*11-16:Ac (**22**) and a diazoacetate photoaffinity (**24**) label. The hydrolysis of the acetate **22** to alcohol **23** also occurs enzymatically in antennae. Ac denotes the acetyl group.

residue instead of relaxation to the ground state. Racemic JH III with a specific activity of 10 Ci/mmol was photoattached to JHBPs of *M. sexta* and *Locusta migratoria* hemolymph (*35*). Labeling by photoexcitation requires long irradiation times (more than 8 minutes) to produce a 1 to 3% attachment with less than 40% specificity. In contrast, less than 30 seconds irradiation with 100 n*M* of racemic [^{3}H]EFDA gives 15% attachment with only 1.4% bound in the presence of 13 μ*M* JH I. The labeled racemic EFDA can be directly compared with the tritiated natural enantiomers of JH I and II by using the hemolymph JHBP of *M. sexta*. Tritiated natural JH I at 100 n*M* produced only 1.8% bound, whereas 0.7% was still bound in the presence of 13 μ*M* unlabeled JH I (*36*). Thus [^{3}H]EFDA is quantitatively and qualitatively superior as a specific photoaffinity label in the cases examined.

Tritiated dodecadienoates. The first commercial dodecadienoate JHA, methoprene (**11**, shown in the biologically active *7S* configuration), has been prepared in both [5-^{14}C] and in [10-^{3}H] forms (*37*) for the study of its in vivo metabolism and distribution. Despite its extensive use as a JHA in physiological and biochemical experiments, the molecular basis of the action of the dodecadienoate JHAs is still unclear. We prepared tritiated (*7S*)-hydroprene ([^{3}H]-**12**) with high specific activity as shown in Fig. 4 (*13*). The dienoate moiety of 10,11-dehydrohydroprene (**13**), which was obtained by elimination of methanol from (*7S*)-methoprene **11** followed by ester exchange, was first protected as the iron tricarbonyl diene complex. This prevented hydrogenation of the more accessible 4,5-double

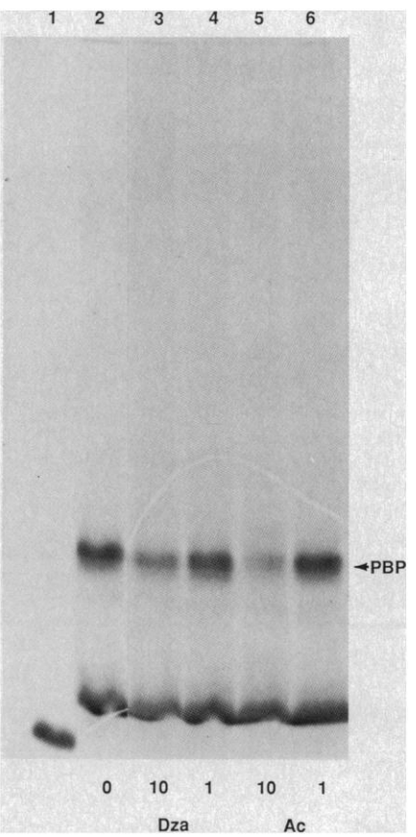

Fig. 7. Photoaffinity labeling of the pheromone-binding protein of *A. polyphemus* with [^{3}H]*E*6, *Z*11-16:Dza (**24**), as seen in the fluorogram of an SDS-PAGE separation of isolated sensory hair proteins after irradiation at 254 nm with 150 n*M* of photolabel. Lane 1 is unirradiated. Lane 2 is irradiated with no competitor. Competition is shown in pairs of lanes at 10 μ*M* (lanes 3 and 5) and 1 μ*M* (lanes 4 and 6) for the unlabeled diazoacetate (Dza) (lanes 3 and 4) and the acetate (Ac) pheromone (lanes 5 and 6); PBP, pheromone binding protein.

bond, and allowed selective reduction of the 10,11-olefin under homogeneous hydrogenation (tritiation) conditions to yield the tritiated complex **14**. The iron tricarbonyl group was removed by a mild oxidation. Recently, [^{3}H](*7S*)-methoprene was synthesized with a specific activity greater than 70 Ci/mmol by selective reduction of the 9,10-double bond of the trienoate **15**. After complexation and selective hydrogenation (tritiation) to give complex **16**, [^{3}H]methoprene was obtained by deprotection with ceric ammonium nitrate (*38*).

Iodine-125–labeled JH and JHA. In anticipation of finding receptor proteins present in low copy numbers and that have subnanomolar dissociation constants, two iodinated JH analogs were synthesized. In one a methyl group was mimicked by an iodine atom, and in a second an iodovinyl group was appended to a dodecadienoate. Each was initially prepared in unlabeled form for determination of relative binding affinities in vitro and JHA effects in vivo; subsequently, the no-carrier-added radioactive iodine-labeled ligands were synthesized (Fig. 5).

The 12-iodo-JH I (**17**) was obtained from a chiral epoxy alcohol (**2a**) by conversion to a *p*-toluenesulfonate ester and subsequent nucleophilic displacement with iodide (Fig. 5A). Since the rate of this reaction is dependent on the concentration of both reactants, a large excess of sodium iodide is required for the unlabeled material (24 hours, 40°C). This is impractical for the labeled material, so excess substrate was used with Na^{125}I as the limiting reagent; this reaction required 14 to 21 days and gave only a 2% radiochemical yield in addition to a volatile radioactive byproduct, 1-iodopropan-2-one (*39*). The yield of labeled 12-iodo-JH I (**17**) was increased to 48% by addition of unlabeled sodium iodide to yield a radiochemically pure compound with a specific activity of 50 Ci/mmol. The unlabeled 12-iodo-JH I had 10% of the relative binding affinity of JH II in a competitive binding assay for *M. sexta* JHBP and a tenfold lower activity in the black *Manduca* assay (*40*). Consistent with this tenfold difference, the ^{125}I-labeled 12-iodo-JH I showed saturable binding to the hemolymph JHBP of *M. sexta* with a dissociation constant (K_D) of 800 n*M* (*39*).

The iodovinyl analog of methoprene was synthesized in ten steps from (*7S*)-methoprene (**11**) through the aldehyde **18** (Fig. 5B) (*14*). The key step was the electrophilic iododestannylation of the vinylstannane (**19**), a reaction which was more rapid, more efficient, and more readily adapted to the incorporation of radioactive iodine than the nucleophilic displacement method that was necessary in the preparation of 12-iodo-JH I. Iodovinylmethoprenol (IVMA, **20**) showed high biological activity in the black *Manduca* assay [median effective dose (ED_{50}) of 3.3 pmol] and the epidermal commitment in vitro assay (ED_{50} = 35 n*M*) (*40*). IVMA was ten times more potent than methoprene in these assays and within a factor of 2 as potent as natural JH II. [^{125}I]IVMA is taken up into the nuclei of epidermal cells obtained from first day, fifth instar *M. sexta* larvae; two independent, saturable binding sites that have dissociation constants (K_D) of 4 and 59 n*M* have been identified. High-affinity IVMA binding is absent in wandering larvae (fifth instar, fifth day) but is present in pupal epidermis 24 to 36 hours after ecdysis. Finally, natural JH I does not compete for the dienoate binding sites in these nuclear preparations; a separate high-affinity binding component is present for tritiated (10*R*,11*S*)-JH I, which shows no affinity for the dodecadienoates (*40*). IVMA is the first radioactively iodinated JH analog that has both high specific activity and high biological activity, and it has provided evidence for different acceptor sites for JH homologs and JH analogs in a target tissue of an insect.

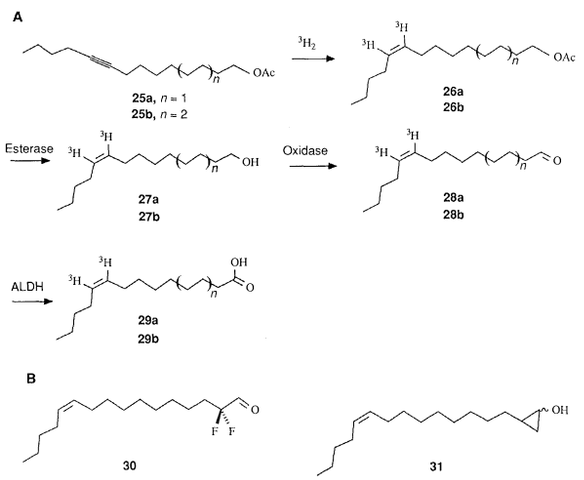

Fig. 8. (**A**) Synthesis of [^{3}H]*Z*9-14:Al (**28a**) and [^{3}H]*Z*11-16:Al (**28b**) and catabolism to the corresponding acids (**29a** and **29b**) by the aldehyde dehydrogenases of *Heliothis*. (**B**) Compounds **30** and **31** are aldehyde dehydrogenase inhibitors in vitro. Ac denotes the acetyl group.

Pheromone Bio-Organic Chemistry

There is still only a rudimentary understanding of how pheromones act at a molecular level to produce changes in behavior. If effective strategies for disruption of insect mating are to be imple-

Table 1. Dilution series for two soluble antennal enzymes of *H. virescens*. All values, which are percent conversion to product, were measured in duplicate or triplicate TLC assays and were corrected for nonenzymatic hydrolysis or oxidation. The concentration of isotopically labeled substrate was 100 n*M*. [Adapted from (*49*) with permission of Academic Press]

Antennal equivalent (per ml solution)	Esterase (%)				Aldehyde dehydrogenase (%)			
	Male		Female		Male		Female	
	*Z*9-14:Ac	*Z*11-16:Ac	*Z*9-14:Ac	*Z*11-16:Ac	*Z*9-14:Al	*Z*11-16:Al	*Z*9-14:Al	*Z*11-16:Al
360	27	14	30	19				
120	10	10	5	19				
80	0		0		62	51	53	54
36		3		12				
26.67					50	45	33	49
12		0		5				
8.0				0	50	40	33	45
2.67					31	29	15	27
0.8					10	17	15	22
0.267					0	0	0	8
0.08								0

mented, it is important that research priorities include the basic biochemistry of pheromone production, transport, binding, and catabolism (*41*). The isolation of odorant binding proteins in insects (*42, 43*) and in vertebrates (*44, 45*) is the beginning of a new effort to elucidate the mechanisms of olfactory reception. An understanding of the conversion of peripheral sensory stimuli to electrical impulses and subsequently into "odor images" in the brain involves yet a higher level of biochemical complexity (*46*). The involvement of adenylate cyclases and guanosine triphosphate (GTP)–binding proteins in vertebrate olfactory cilia (*47, 48*) has encouraged the search for the existence of analogous biochemical components in olfactory transduction in insects.

The synthesis of chemically reactive pheromone analogs was integrated with the preparation of tritium-labeled insect pheromones. With these materials, the activity and localization of proteins that bind and catabolize pheromones is being probed (*49*). I now focus on three insects: (i) the wild silkmoth, *Antheraea polyphemus*; (ii) the tobacco budworm, *H. virescens*; and (iii) the gypsy moth, *L. dispar*. Three of the major functional groups involved in pheromone chemistry, esters, aldehydes, and epoxides, are included by selection of these insects. Selected recent results with labeled pheromones and pheromone analogs for bees, bark beetles, Japanese beetles, boll weevils, and termites will be mentioned in the next section.

Antheraea polyphemus. The sensillum lymph from antennae of males of *A. polyphemus* contains several soluble proteins involved in pheromone binding and metabolism (*42, 43, 50*). These include a highly active sensillar esterase that has several electrophoretically different forms (55,000 to 85,000 daltons) and an abundant pheromone-binding protein (15,000 daltons). The interaction of these two soluble proteins in the deactivation of (*E,Z*)-6,11-hexadecadien-1-yl acetate (denoted *E*6,*Z*11-16:Ac, in a nomenclature commonly used for fatty acid–derived insect pheromones) and the possible roles of these proteins in pheromone perception have been described (*50, 51*). Under physiological conditions, the esterase, which has a Michaelis constant K_M of 2 μM, hydrolyzes the acetate pheromone **22** (Fig. 6) to the alcohol **23** with an estimated half-life in vivo of 2 to 20 msec. This suggests a molecular model for reception in which the highly concentrated binding protein (greater than 10 mM in sensillum lymph) acts as a carrier or sequestering agent and in which the enzyme acts as a rapid inactivator to maintain a low stimulus noise level in the vicinity of the dendrite. The binding protein and the esterase have been studied at physiologically relevant pheromone concentrations (0.1 nM to 1000 nM) with a tritium-labeled pheromone that has a high specific activity. The synthesis of [11,12-$^3H_2$]*E*6,*Z*11-16:Ac (**22**) (by partial reduction of the triple bond in enynyl acetate **21** with carrier-free tritium gas in the presence of a poisoned catalyst) provided the labeled pheromone with specific activities in the range of 25 to 58 Ci/mmol (Fig. 6) (*52*).

The hydrolysis of a 100 nM solution of the tritiated *E*6,*Z*11-16:Ac to the alcohol **23** by the purified sensillar esterase was subject to competitive inhibition by the corresponding diazoacetate *E*6,*Z*11-16:Dza (**24**) but not by the (6*Z*)-stereoisomer or by the 11-alkynyl analog (*51*). The serine esterase inhibitor 1,1,1-trifluoro-2-tetradecanone, which is a transition-state analog inhibitor that presumably forms a stable hemiketal at the active site (*53*), showed a median inhibitory dose (I_{50}) for *E*6,*Z*11-16:Ac hydrolysis of 5 nM, whereas most serine esterase inhibitors were ineffective even at 100 μM. Clearly, the enzyme preferentially binds fatty alcohol esters with the correct chain length and geometry. A study of the hydrolysis of tritiated pheromone analogs also confirmed the importance of a single *Z*-olefinic linkage and the acetate (but not diazoacetate) for hydrolysis to occur at an appreciable rate.

The diazoacetate analog of the *A. polyphemus* pheromone was first prepared in 1978 in unlabeled form and had more than 10% of the electrophysiological activity of the parent acetate (*54*). The tritium-labeled photoactivatable diazoacetate **24** was synthesized with a specific activity greater than 40 Ci/mmol (*52*) so that the binding protein in the receptor lymph could be photoaffinity labeled. In addition, R. G. Vogt and I hoped to covalently modify the putative receptor protein that has been postulated to be located in the dendritic membrane.

Irradiation of sensory hair homogenates that contained 100 nM of tritium-labeled *E*6,*Z*11-16:Dza (**24**) resulted in selective photolabeling of male antennal proteins in both soluble and membrane-associated fractions. Photoaffinity labeling of these proteins can be suppressed by competitive displacement with *E*6,*Z*11-16:Ac and does not occur in the absence of irradiation (*43, 49, 55*). Figure 7 shows a fluorogram of an SDS-PAGE gel that shows the pheromone-displaceable photoaffinity labeling that occurs in male antennae but not in female antennae or other male tissues.

Both the binding protein and the esterase show what appears to be considerable allelic variation among individual male moths from sympatric and allopatric populations. The structural and kinetic properties of these proteins may well be a direct consequence of sexual selection, since their interaction and efficacy determines the

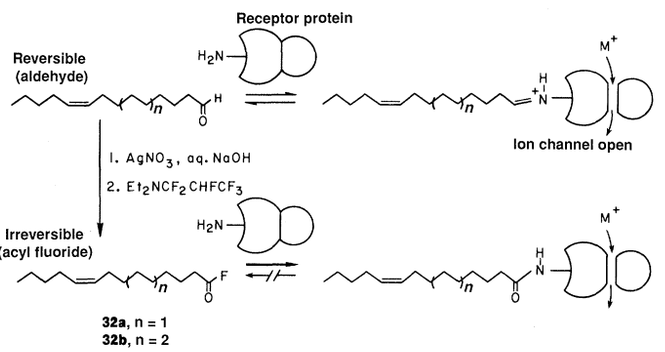

Fig. 9. Hypothetical model for the interaction of acyl fluoride pheromone analogs **32a** and **32b** with receptor molecules. [Adapted from (*66*) and reprinted with permission of Birkhäuser Verlag]

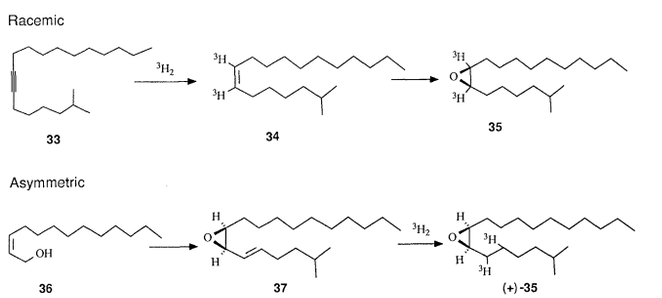

Fig. 10. Synthesis of racemic and enantiomerically enriched [^{3}H]disparlure (**35**).

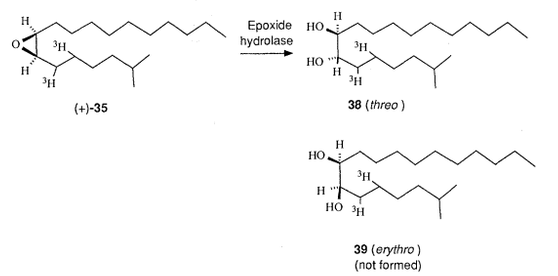

Fig. 11. Reaction of natural (+)-[^{3}H]disparlure (**35**) with antennal epoxide hydrolase of *L. dispar*.

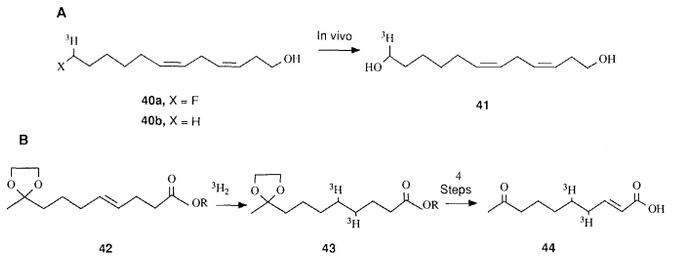

Fig. 12. Labeled compounds used for studying social insects. (**A**) Termites [*R. flavipes* (Isoptera)] metabolize **40a** and **40b** to diol **41**. (**B**) Honeybee queen substance **44** of *A. mellifera* (Hymenoptera); R denotes an alkyl group.

mating success of the male moth (*56*). The 30 NH_2-terminal amino acids of the two electrophoretically distinguishable forms of the binding protein from isolated populations of *A. polyphemus* (Long Island, New York, and Wisconsin) have been sequenced (*43*). A single difference at residue 24 (Asn for the New York moths versus Ser for the Wisconsin moths) was observed in one pair of sequences.

Heliothis virescens. The two major aldehyde components of the female-produced pheromone, *Z*9-14:Al (**28a**) and *Z*11-16:Al (**28b**), were synthesized with two tritiums per molecule (Fig. 8A) (*57*) for investigating pheromone binding and catabolism in this economically important noctuid, commonly known as the tobacco budworm. Partial tritiation of the alkynyl acetate (**25**) was performed at 720 torr of carrier-free tritium gas with a poisoned catalyst (*58*). Higher specific activities (>52 Ci/mmol) were obtained in this reduction when tetrahydrofuran was used as the solvent instead of methanol (*49*) because of the reduced exchange of the allylic and vinylic hydrogens. The high specific activity pheromones and analogs are best stored below −20°C in heptane-toluene mixtures to retard radiolytic decomposition. The tritiated acetates (**26**) appear to be the best form for storage, with less than 10% decomposition per year; the alcohols (**27**) degrade at about 10% per month and the aldehydes (**28**) are over 50% decomposed within several weeks. Of the reagents available for partial oxidation of primary alcohols, only pyridinium dichromate (PDC) afforded the desired aldehyde on the scale of 200 nmol (about 10 mCi) of labeled alcohol.

The tissue specificity of the acetate esterase, alcohol oxidase, and aldehyde dehydrogenase was investigated in both male and female moths (*57*). These three types of enzyme activity are also involved in pheromone biosynthesis and degradation in the spruce budworm moth *Choristoneura fumiferana*, as determined by radiochemical and luminescence assays (*59*). Soluble enzyme activities in *Heliothis* were determined by radio–thin-layer chromatography (TLC) assays; the relative esterase and aldehyde dehydrogenase activities for male and female antennae are shown in Table 1.

Aldehyde dehydrogenase (ALDH) was the primary enzyme activity that was found in leg and antennal tissues of males and females; NAD^+ is required as a cofactor. This is important in its implications for pheromone clearance from cuticular surfaces as well as from sensillum lymph. Conversion by ALDH of *Z*9-14:Al **28a** to the acid **29a** was detectable at tissue concentrations of 0.04 antennal equivalent (AE) per assay in 1 hour, whereas the esterase activity on the corresponding acetate required more than 5 AE per assay. Females had a threefold lower ALDH activity for the conversion of *Z*11-16:Al **28b** to acid **29b** than did males, but the activities for *Z*9-14:Al metabolism were the same.

Alcohol oxidase activity is low in antennae, higher in legs, and highest in glandular tissues for both sexes. Indeed, the oxidase shows relatively low substrate specificity and appears to be localized in the cuticular surface of the pheromone gland epithelium of the female (*60*). As in the spruce budworm, this enzyme is essential for the production of the aldehyde pheromone components. In *Heliothis* species, aldehyde production and release by females is under photoperiod-regulated neuroendocrine control (*61*). Esterase activity is low in the antennae as well as other tissues; this is in contrast with the high esterase activity in the sensillum lymph of *A. polyphemus*, which uses an acetate pheromone.

An important goal is to alter the aldehyde functionality to produce a chemically reactive analog that is capable of causing sensory disruption. Two modes of action can be imagined: (i) tight (preferably irreversible) binding to a receptor protein on the dendritic membrane to produce either a permanent hyperagonistic or an antagonistic effect on precopulatory behavior; or (ii) inhibition (preferably irreversible) of a pheromone-degrading enzyme to effectively prevent the signal-causing pheromone from being removed from its receptor site. Only a few examples of this strategy have been examined.

For example, the 2,2-difluoro analog (**30**) of *Z*11-16:Al was prepared and its activity as an inhibitor of the oxidase and ALDH was examined (Fig. 8B) (*62*). The strongly hydrated difluoroaldehyde showed only modest inhibition of the oxidase and the ALDH activities. An alternative plan was to prepare alkenyl-substituted cyclopropanones as transition-state analog inhibitors (*63*) of the pheromone ALDH. These materials were extremely unstable and could not be characterized at room temperature. However, the cyclopropanol **31** (Fig. 8B) is a stable precursor of the desired cyclopropanone inhibitor. It does not inhibit the oxidase but does show inhibition of ALDH activity in a mixed enzyme preparation; the inhibition appears to require oxidase activity as predicted (*64*).

It was postulated that the acyl fluorides **32** (Fig. 9) would act as reactive mimics of the corresponding aldehydes and, in a mechanism reminiscent of the acylation of an ε-amino group of a lysine residue by retinoyl fluoride (*65*), would covalently modify the dendritic receptor protein. In principle, either anosmia (odor blindness) or hyperactivation could result if the ion channel postulated in chemoelectrical transduction mechanisms (*46*) was forced closed or open. Indeed, the *Z*9-14:Acf (**32a**) and *Z*11-16:Acf (**32b**) are unusual

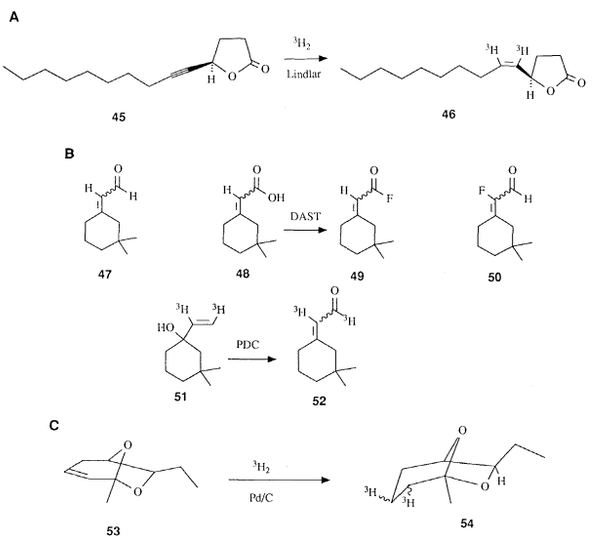

Fig. 13. Labeled pheromones and analogs used for studying beetles (Coleoptera): (**A**) Synthesis of the tritium-labeled pheromone **46** of the Japanese beetle (*P. japonica*); (**B**) acyl fluoride (**49**) and α-fluoroaldehyde (**50**) analogs of the cyclohexylidene aldehyde compound (**47**) of the pheromone of the boll weevil (*A. grandis*), and synthesis of the tritium-labeled aldehyde, pheromone **52** (DAST, diethylaminosulfur trifluoride; PDC, pyridinium dichromate); (**C**) synthesis of tritium-labeled racemic *exo*-brevicomin (**54**), an aggregation pheromone for the bark beetle *D. ponderosae*.

hyperagonists and caused aphrodisia and disorientation in male moths (*66*). The effect was similar to the *Z*9-14:Al–induced interspecific couplings between *Heliothis* species observed in the field. The maximal effect for the acyl fluorides included irreversible extrusion of the male genitalia and hair pencils as well as locking of the male and female during copulation. These effects, however, appear to be reversible at lower doses, perhaps because the acyl fluorides are hydrolyzed in air with a half-life of 2 to 4 hours; they are thus unlikely to be commercially useful mating disruptants.

Lymantria dispar. More than 14 years ago, radioactively labeled racemic disparlure (**35**, 2-methyl-*cis*-7,8-epoxyoctadecane) was prepared by partial tritiation of an alkyne (**33**) to a *Z*-alkene (**34**) followed by epoxidation (*67*). Subsequent experiments with whole antennae of male gypsy moths showed conversion of the racemic epoxide to uncharacterized polar metabolites with a half-life of 2.5 minutes (*68*). The binding and catabolism of the two enantiomeric epoxides were of interest to my research group, since the proteins that are involved in perception and clearance are expected to reside in separate sensory cells (*69*).

During the past year tritium-labeled racemic disparlure and both the attractive (+)-disparlure and inhibitory (−)-disparlure enantiomers (with enantiomeric excess of greater than 95% and specific activities of 58 Ci/mmol) have been synthesized (*49*). The optically active pheromones were prepared from the allylic alcohol **36** by the Sharpless asymmetric epoxidation method, chain extension, and homogeneous tritiation of the alkenyl oxirane [for example, (+)-**37**] to give the [5,6-$^3H_2$]-labeled disparlure enantiomers **35** (Fig. 10) (*70*).

Pheromone degradation occurs exclusively in the microsomal fraction of the antennal tissues of male and female gypsy moths and can be further localized to the sensory hair homogenate (for males). After incubation of 100 n*M* solutions of labeled disparlure with buffered homogenates of leg tissue, hemolymph, whole antennae, or male sensory hairs, metabolites are extracted and analyzed by TLC. A single polar product is produced by the male antennal homogenate, but only the starting epoxide is detected after several hours with nontarget tissues.

The product of disparlure catabolism is expected to be the *threo*-7,8-diol (**38**, Fig. 11; only one possible enantiomer is shown) from an anti S_N2 type opening of the *cis*-epoxide by water at the enzyme catalytic site. This *threo* diol has been unambiguously identified as the product through a series of chemical derivatizations and analysis by mass spectrometry and co-chromatography experiments (TLC, capillary gas chromatography). Both the authentic *threo* (**38**) and *erythro* (**39**) diols were prepared synthetically to make these comparisons (*70*). The (+)-enantiomer of disparlure (**35**) was hydrated to the *threo* diol **38** twice as fast as the racemic or the (−)-disparlure isomers. In performing the epoxide opening chemically, it became apparent that this hydrolytic opening must indeed be specifically enzyme catalyzed. Boiling ethanolic 12*N* KOH would not open the epoxide ring, and even acidic opening of the oxirane was slow compared with the neutral enzymic reaction.

The sensory hairs of the male gypsy moth can be readily isolated in a form free from hemolymph and epidermal contamination by using the freeze-fracture method that was used before for *A. polyphemus* (*71*). Native polyacrylamide gels show two abundant proteins specific to the male sensory hairs with mobilities similar to the *A. polyphemus* binding protein. These proteins are synthesized continuously in the late pupal stages (beginning 3 days before adult eclosion) and throughout the short life of the adult moth (*72*). The NH_2-terminal amino acid sequences (30 residues) of the two 15,000-dalton bands both show approximately 50% homology to the *A. polyphemus* sensillar binding protein (*43*). Current efforts are directed at chemical modification and the developmental biology of the binding proteins and the epoxide hydrolase that are unique to the male sensory hairs.

New Results with Other Insect Pheromones

Several new examples are discussed here in the hope of stimulating further interest in the biochemistry of insect chemoreception.

Termite trail pheromone. An unexpected ω-oxidation of a dienol trail pheromone analog (**40b**) to the diene diol (**41**) was observed in antennal tissues and intact termites of the Eastern subterranean termite *R. flavipes* (Fig. 12A) (*49, 73*). This oxidation at the terminal methyl also occurred for the 12-fluorododecadienol **40a**, thus detoxifying a potential delayed-action termiticide.

Queen bee substance. The tritium-labeled queen substance of the honeybee *Apis mellifera*, [4,5-$^3H_2$](*E*)-9-oxo-2-decenoic acid (**44**), was synthesized for studying ovarian binding by this primer pheromone (Fig. 12B) (*74*). The binding and catabolism in the antennae of honeybee drones, for which the same compound acts as a sex pheromone, will also be examined. The synthesis requires introduction of the tritiums in non-enolizable positions of ketal ester **43** by tritiation of unsaturated precursor **42**. Desaturation by sequential selenation and oxidation, followed by ester and ketal hydrolysis, produced the labeled pheromone **44** with a specific activity greater than 80 Ci/mmol.

Japanese beetle pheromone. By using the enantiomerically enriched alkynyl lactone **45** (*75*), the doubly labeled alkenyl lactone **46**, which is the natural enantiomer of the pheromone produced by female *Popilla japonica*, was prepared (Fig. 13A). The ^3H nuclear magnetic resonance (NMR) spectrum of this sample clearly shows that the major product is the *Z*-alkene with tritons in both vinylic positions. In addition, the proton-decoupled ^3H NMR spectrum and the ^1H NMR spectrum both show evidence of molecules with a triton in only one of each of the vinylic positions (about 10% per site) (*76*).

Boll weevil pheromone. The unexpectedly stable acyl fluorides **49** (both *E* and *Z* isomers) were prepared from the isomeric acids **48** as mimics of the two isomeric cyclohexylidene aldehyde components **47** of the pheromone blend of *Anthonomus grandis* (Fig. 13B). In addition, the isomeric α-fluoro aldehydes **50**, which bear a vinylic fluorine, were also synthesized (*77*). The acyl fluorides elicit only very weak responses in electroantennogram (EAG) assays, but the α-fluoro compounds cause EAG responses equal to or greater than those from the parent aldehyde isomers **47** (*77*). The tritium-labeled enal **52** was prepared through the oxidative rearrangement of tritiated allylic alcohol **51** to provide a substrate for catabolism and binding assays in this economically important beetle (*78*). Unambiguous evidence of the presence of the vinylic and aldehydic tritons in both (*Z*) and (*E*) isomers is seen in the ^3H NMR spectrum (*76*).

Pine bark beetle aggregation pheromone. The racemic dehydrobrevicomin (**53**) has been tritiated to produce [^3H]*exo*-brevicomin (**54**), the mass attack pheromone produced by pioneer bark beetles of the species *Dendroctonus ponderosae* (Fig. 13C) (*79*). This is the first tritiated cyclic ketal pheromone and the most volatile high specific activity pheromone that we have prepared thus far. Surprisingly, no metabolites could be observed with homogenates of beetle antennae, hemolymph, or legs. In fact, degradation of the two other labeled beetle pheromones described above could not be observed with tissue homogenates. However, tritium-labeled brevicomin was cleanly converted to three new hydroxylated metabolites during an overnight incubation with live beetles; these products were not produced in the presence of heat-killed beetles or in empty vials (*79*).

Conclusion

The synthesis of radioactively labeled insect hormones and pheromones with high specific activity makes it possible to approach the characterization of the receptor and catabolic proteins that convert chemical signals to biochemical events. The design of reactive (or latently reactive) analogs of these intra- and interorganism chemical messengers will allow covalent modification of the proteins of interest. The information derived from this approach will aid in the dissection of the molecular processes involved in olfactory transduction and in JH-mediated gene regulation. Moreover, a better understanding of the chemical parameters for these processes can lead to more selective and environmentally acceptable modes of pest control.

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