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PHOTOAFFINITY LABELS FOR INSECT JUVENILE HORMONE BINDING PROTEINS

SYNTHESIS AND EVALUATION *IN VITRO*

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Abstract—The interactions of insect juvenile hormones (JH) with proteins are critically important to titer regulation, transport, and hormone action at a molecular level. We have synthesized several JH analogs bearing photolabile diazocarbonyl groups as potential photoaffinity labels for JH binding proteins (JHBP). The most promising compound, 10, 11-epoxyfarnesyl diazoacetate (2) (EFDA) competes with JH III for The JH binding site of JHBP from *Leucophaea* hemolymph and ovaries and from cultured *Drosophila* cells. Moreover, irradiation of protein solutions containing micromolar amounts of EFDA gave irreversible loss of [^{3}H]-JH III binding capacity with no change in binding affinity of the unlabelled protein. The protein could be protected against photoinactivation by the presence of equimolar JH III during irradiation. High specific activity [10-^{3}H]-EFDA was prepared and used to demonstrate specific, finite binding of EFDA to the JH III receptor site of the binding protein. Further applications of photoaffinity labelling technique to characterization and cellular localization of the JHBP are discussed.

The study of insect juvenile hormones has progressed rapidly from the elucidation of chemical structures (Fig. 1) and gross morphogenetic effects[1] to the examination of the detailed regulation of macromolecular events by these unique sesquiterpenoid messengers.[2,3] While the literature of the first decade of chemical work emphasized stereoselective synthesis and JH analog synthesis, that of the current decade will likely feature elucidation of hormone-protein and receptor-gene interactions on a molecular level.[4] Two main motives determine this direction: (1) the need to understand hormone action to design selective biorational control agents, and (2) the challenge of working out one of Nature's most intricate biochemical control schemes for reading and expressing different genes. The necessity of developing new biochemical and chemical tools to study hormone-protein-gene systems will be of both fundamental interest in macromolecular insect physiology and in turning up new leads for selective insecticides.

Juvenile hormone-protein interactions are involved at all levels of titer regulation,[2,3a,5] biosynthesis and transport,[2,6] excretion and metabolism,[2,7] and cellular mechanisms of action.[2,3b,4] It is the interaction of juvenile hormones with three types of protein which we desired to examine: the JH-specific esterases (JHE), the high-affinity, selective JH carrier proteins (JHCP), and the high affinity cytosolic and nuclear receptor proteins (JHRP). In the three subsections which follow, we will summarize key information on these three protein types and on the application of photoaffinity labelling to hormone-receptor interactions. Following this background material, we will report the successful synthesis of diazocarbonyl-containing JH analogs and their evaluation *in vitro* as photoaffinity labels.[8]

Juvenile hormone specific esterases (JHE). The bioinactivation of JH by deesterification and by epoxide hydrolysis was first established by Slade and Zibbitt[1] and most recently reviewed by Hammock *et al.*[2,7] In *Manduca sexta*, JHE activity occurs in two pulses,[9] pre-wandering and prepupation. These were first proposed[10] to be the primary source of JH titer decline in late 5th instar larvae, but it was later found

*Author to whom correspondence should be addressed. Fellow of the Alfred P. Sloan Foundation (1981–83) and Camille and Henry Dreyfus Teacher-Scholar (1981–86). This paper is dedicated to Dr. Ulrich Weiss on the occasion of his 75th birthday in recognition of his contributions to bioorganic chemistry and his encouragement of young researchers.

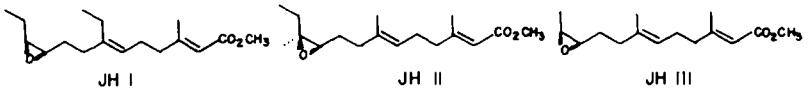


Fig. 1. Insect juvenile hormones.

that JHE activity increased *after* JH to allow cellular reprogramming [3b] to take place with the first ecdysone peak which initates wandering.[12] JHE is inducible by JH in adult moths[13] and in larval cabbage loopers;[14] indeed, in *T.ni* only the second JHE peak is inducible, and is needed to counteract the weak JH pulse which prevents precocious wing and eye development in the pupa. JHE have been surveyed in six insects,[15] and their timing, occurrence, and specificity are very variable.

The JH specific esterase is relatively insensitive to 10^{-4} M DFP, which inhibits the general esterases which co-occur in insects,[10,14] and it is capable of hydrolyzing both JHCP-bound and unbound JH.[10] Homogeneous esterases of MW 68,000 have been purified from *Manduca* hemolymph,[16] from *Tenebrio*, *Trichoplusia* and *Musca* hemolymph,[17] and from various tissues of *T.ni* (all JHE were identical).[18] Inhibition of JHE by organophosphates, carbamates, JHA, epoxygeranyl phenyl ethers, and trifluoromethyl ketone transition state analogs[19] has been extensively investigated.[7,17]

Juvenile hormone binding (carrier, receptor) proteins. The interaction of JH with carrier proteins is another key step in JH titer regulation and delivery of the hormone to target sites. JHCP from *Manduca* has been extensively studied by Law *et al.*[2,4,5,20a] and by Goodman *et al.*[20b] The high-affinity ($K_D \approx 4 \times 10^{-7}$ M), low-capacity JHCP is small (MW 28,000),[21] and its specifity for binding JH isomers, homologs, and analogues was examined.[22] The lipophilicity of JHs (I > II > III) paralleled their relative binding affinities for JHCP.[23] Also, the 10(*R*) enantiomer of JH III was shown to bind better than racemic or 10(S)-JH III.[23] Farnesyl methyl ketone (RCOMe rather than RCO_2Me) and inverted ester (epoxyfarnesyl acetate) show affinities relative to JHI identical to that of JH III ($K_{rd} \approx 0.1$).[20] The high bioactivity of the methyl ketones[2,24] and (in the methoprene and hydroprene series) diazo methyl ketones[25] suggests both good binding and accessibility to the active site for ultimate hormone action. JHBPs have also been used for competitive binding assays for detection of hormones in insects (Goodman *et al.* in ref. 2).

Although low MW, high affinity JHBP have been found in other lepidopterans,[2,6] the coleopterans appear to possess high MW(> 80,000) low-specificity ($K_D \approx 10^{-5}$ M), high capacity lipoproteins to transport JH and other lipids in the hemolymph.[26] In contrast to *Manduca* JHCP, these offer little protection from esterases, and bind JHA, free fatty acids, and JH degradation products. Their role in JH titer regulation is not as clearly defined.

A second group of high affinity JHBP have been postulated as cellular receptors. Following the scheme for steroid hormones in mammals (Schwartz *et al.* in ref. 2), one would expect (1) JH binding to a cytoplasmic receptor, (2) translocation of the hormone-protein complex to the nucleus, (3) binding of the complex to an acceptor site on nuclear chromatin, and (4) activation of transcription to give new mRNA. In the case of ecdysone, many of these events have been observed,[3] including identification of nuclear recptors, mRNA synthesis, and chromosome puffing. For JH, only four reports have appeared documenting the first step, and little molecular data is available on the molecular basis for reprogramming (but see Riddiford[3b]). Thus, Engelmann (in ref. 4) has isolated two JH-saturable proteins ($K_D = 2 \sim 5 \times 10^{-9}$ *M* and $3 \sim 8 \times 10^{-8}$ *M*) from the fat bodies of mated adult female cockroaches (*Leucophaea maderae*). One of these (10^{-8}) appears to be the same as the JHCP from hemolymph. This system is identical to that being studied by Koeppe *et al.*,[27] in which high-affinity proteins are obtained from hemolymph and ovaries of adult females.

Chang *et al.*[28] have purified a JHBP from the cytosol of *Drosophila melanogaster* Kc cells which shows MW 80,000 and $K_D = 1.6 \times 10^{-8}$ *M* for JHI. It does not bind the JH diol or JH acid and exhibits saturability, specificity and affinity appropriate for a true receptor. Only 2500 protein molecules/cell are isolated. The larval integument of *Drosophila hydei* has afforded high affinity binding sites for JHI[29] using very high specific activity chiral JHI.[29b] These workers[29] found two proteins with $K_D = 3.6 \times 10^{-9}$ *M* and $\approx 10^{-7}$ *M* for 10R, 11S-JHI. The former was postulated to be the receptor protein (8 pmol/mg cytosol protein) and shows all the expected properties for a true receptor. Finally, a nuclear receptor protein from *Manduca* epidermis was reported[30] but its role has not been clarified. A more recent report by Chang demonstrates nuclear receptors ($K_D \approx 1.8 \times 10^{-9}$ *M* for JH III) in *Drosophila* Kc cells (*ca.* 40 receptor proteins/nucleus).[31]

Photoaffinity labels. The investigation of enzyme structure and function using photochemically-activated irreversibly-binding substrate mimics was pioneered by Westheimer[8b,32] and by Knowles.[8a] The diazocarbonyl group is easily incorporated[33] into substrates with minimal stereoelectronic perturbation, and provides a readily accessible high-energy acylcarbene when irradiated with UV light. These carbenes can rearrange and/or insert into C–H, O–H, S–H and N–H bonds of protein or solvent.

Recently, Nakanishi *et al.*[34] extended the use of photoaffinity labelling to the preparation of (6E, 11Z)-6,11-hexadecadienyl diazoacetate, a mimic of the sex pheromone of *Antherea polyphemus*. The mimic is behaviourally and neurophysiologically active, and can be photolyzed in < 10 sec at 254 nm. This tantalizing preliminary report has not yet been followed by an *in vitro* labelling of the antennal sensilla receptor protein, although this is the presumed intention. The pheromone receptor protein is membrane-bound and joined by two soluble proteins which transport and hydrolyze the pheromone[35] in the same moth. In analogy to our results with diazo JHA photoaffinity labelling of JHBP as described below, we expect that a high specific activity diazoacetate pheromone mimic could well selectively label the two antennal proteins.

The use of photoaffinity labels in the study of vertebrate steroid hormone-receptor interactions has provided valuable information on estrogenic,[36] corticosteroid,[37] and other receptors.[8c] Diazocarbonyl analogs of retinal have recently been prepared and used to form unbleachable visual[48] pigments. The interaction of ponasterone A with *Drosophila* cells has shown the presence of molting hormone (MH) receptor sites in the cytoplasm.[38] More recently, endogenous ecdysterone has been photoactivated in

salivary glands of *D.melanogaster*[39a] and *Chironomus*,[39b] resulting in covalent linking to the polytene chromosomes. The direct involvement of ecdysterone with the sequentially induced chromosomal puffs was shown by indirect immunofluoroescence microscopy. Thus, ecdysterone binds specifically to the genes which are hormonally controlled.[40] Moreover, the hormone receptor protein for ecdysterone in *D.melanogaster* has also been characterized.[39c]

It is desirable to employ analogous techniques to identify the nuclear JHRP and the site at which JH functions in preventing transcription of the adult genome in larval insects and in activating vitellogenesis in adult female insects. To this end, we have designed several JH mimics bearing diazocarbonyl functionalities (diazo JHA, Fig. 2) which can serve as photoaffinity labels for JHBP.[41] Because of their similarity to authentic JHs and to bioactive JHA which compete with JH for protein binding, the diazo JHA act as JH mimics. After photolysis, the reactive acylcarbenes would be expected to covalently attach to the binding sites and thereby inhibit JH transport and action in a novel antijuvenile hormone sense. The synthetic simplicity of these compounds allows the incorporation of high specific activity radiolabels, so that JH binding proteins can be rapidly, specifically, and irreversibly labelled.

The mode of action of a JHBP photoaffinity label is shown schematically in Fig. 3. Thus, photoactivation of the diazocarbonyl releases N_2 and an acylcarbene. The carbene may (1) diffuse out before reacting with the protein, (2) directly insert into an adjacent C–H, O–H, N–H, or S–H bond, or (3) undergo an alkoxy-Wolff rearrangement to the alkoxyketene. The ketene can in turn diffuse out and be quenched by water, or act as an electrophile in acylating a nucleophilic residue at the binding site. Both modes of covalent attachment to the protein involve retention of the [^{3}H]-labelled juvenoid moiety.

Separation of specific and nonspecific binding, as well as demonstration that the photoactivatable analog binds to the same site as the natural JH III, can be accomplished by a protection experiment (Fig. 4). In **A**, the unprotected JHBP is in equilibrium with the diazo JHA (e.g. EFDA) and could bind at either the JH III site (wedge) or another site (rectangular cut). Photolysis would give unchanged protein, wasted (hydrolysed) photoaffinity label, and EFDA irreversibly bound to whichever site it occupied. In case **B**, the JHBP is protected by excess JH III, which as the better ligand forces EFDA to bind to the "other" sites. Photolysis results in the release of unaffected JHBP, wasted EFDA, and EFDA covalently bound to non-JH III binding sites, causing possible allosteric diminution of the binding by JH III. In this case, partial protection would be observed. Finally, in **C**, the JH III and EFDA compete for the same site, with JH III being a substantially better ligand. Upon photolysis, the JH III-JHBP is unaffected, while the small amount of irreversibly inactivated protein decreases as the JH III concentration increases or as the RBA of the competitor diminishes.

RESULTS AND DISCUSSION

Synthesis. The synthesis[41,42,44] of the four diazo JHAs **1b–4b** is shown in Scheme 1. 10,11-Epoxyfarnesol[22] **6** is readily obtained from (*E*,*E*)-farnesol **5** by acetylation, bromohydration of the terminal alkene, and epoxide closure with concomi-

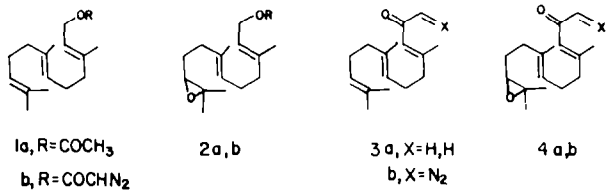


Fig. 2. Diazocarbonyl JH analogs for photoaffinity labelling of JH binding proteins.

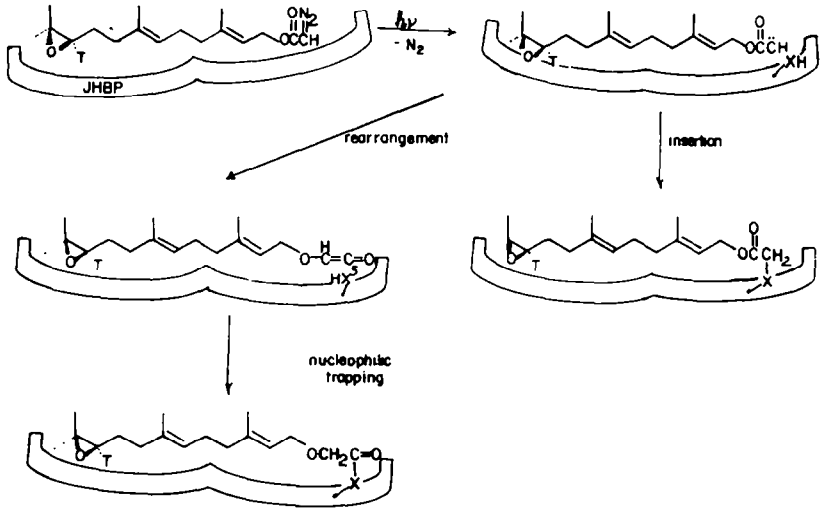


Fig. 3. Photoaffinity labelling of JHBP by EFDA.

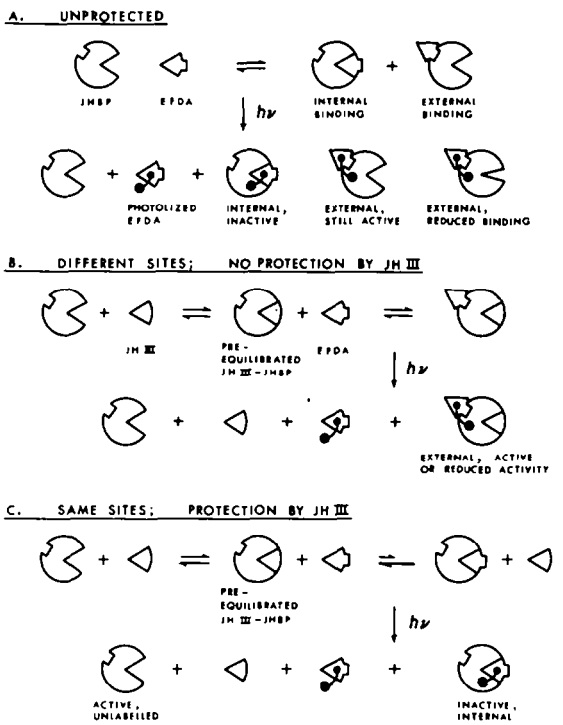
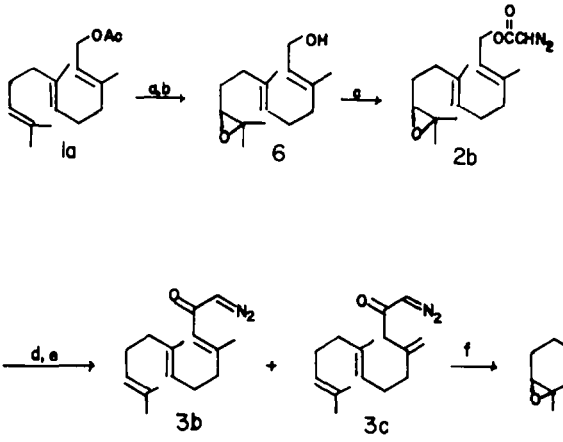


Fig. 4. Protection of JHBP from photoinactivation by diazo JHA (EFDA) using JH III to block binding site: A, Unprotected, no JH III; B, Unprotected, JH III present (implies different binding sites); C, Protected, JH III present (implies same binding site). Refer to text for detailed explanation.



Scheme 1. Synthesis of diazo JHA photoaffinity labels.[41,42] Reagents: (a) NBS, THF-H_2O; (b) K_2CO_3, CH_3OH; (c) ClCOCHNNHTs, 2 equiv. $(C_2H_5)_3N$, CH_2Cl_2 for **2b**, Ac_2O, C_5H_5N for **2a**; (d) $(COCl)_2$, hexane, 80°; (e) CH_2N_2, $(C_2H_5)_2O$; (f) MCPBA, CH_2Cl_2.

tant deacetylation with methanolic K_2CO_3. Acylation of **6** with the acid chloride of glyoxylic acid tosylhydrazone[43] in triethylamine-dichloromethane gives an acylated product, which on treatment with additional triethylamine affords the diazoacetate **2b** (EFDA). This route to diazoacetate **2b** has been described by us[41] and Krafft *et al.*,[44] who introduced a ^{14}C label *via* [1-^{14}C]-glyoxylic acid.

The diazoacetate **1b** was prepared in an analogous manner from farnesol **5**. Diazoacetates were easily purified by flash chromatography (ethyl acetate-hexane) under ambient light conditions. The diazoacetate **1b** showed $\lambda_{max}^{hexane} = 244$ nm (ϵ 10,500), 211 nm (ϵ9100) and is less than 10% degraded (TLC, UV) by irradiation with a 275 watt sun lamp for 1 hr in hexane solution. However, photolysis of diazoacetate **1b** occurs rapidly in hexane, ethanol, or water solutions; a 0.1 mM solution shows > 99% chromophore loss after irradiation for 40 sec (8 × 2537 Å lamps, Rayonet reactor).

Preparation of the diazoketones **3b** and **4b** is also shown in Scheme I. Two-step oxidation[45] of (*E*, *E*)-farnesol to methyl (*E*, *E*)-farnesoate was followed by saponification and subsequent conversion to the acid chloride using oxalyl chloride. Addition of excess ethereal diazomethane[46] at 0° gave diazoketone **3b** containing ≈ 30% of the β,γ-unsaturated isomer **3c**. No pyrazoline formation[46] was observed, pre-

sumably because of the β-substituent on the α,β-unsaturated carbonyl system. Selective epoxidation of the 10,11-double bond was effected with MCPBA to give an 85% yield of **4b** (containing 30% β,γ isomer **4c**) after flash chromatography.

Competition experiments[41] using diazo JHA **1b–4b** with cockroach JHBP and *Drosophila* Kc cell JHRP indicated that the 10,11-epoxides were superior to the 10, 11-alkenes in competing with [^{3}H]-JH III for hormone binding, and that epoxide **2b** competed better than **4b** (Table 1). For this reason, we next undertook the preparation of [10-^{3}H]-**2b** as shown in Scheme 2. Epoxyfarnesyl acetate **2a** was converted to the known[22] diol **8** with dilute acid and subsequently oxidized to keto alcohol **9** using a modified Moffatt oxidation (D. A. Schooley, personal communication). The chromatographed ketol **9** was reduced with tritiated sodium borohydride of very high specific acivity (64 Ci/mmol), and the resulting [10-^{3}H]-diol **8** was converted to the mono-mesylate and thence to the racemic epoxide following the method of Schooley *et al.*[23a,47] Removal of the acetate protecting group occurred during epoxide closure to give [10-^{3}H]-epoxyalcohol **6**. Since attempts at chromatographic purification of epoxyalcohol **6** always resulted in partial decomposition, the crude tritiated epoxide was diazoacetylated as described above and twice chromatographed to afford [10-^{3}H]-**2b**, (^{3}H-EFDA) specific activity 5.4 Ci/mmol.

In vitro *bioassays*. We first examined the details of the interactions of EFDA with ovarian and hemolymph JHBP from mated female cockroaches, *Leucophaea maderae*.[27a] We found that cold EFDA irreversibly reduced [^{3}H]-JH III binding to both JHBPs after less than 20 sec irradiation at 254 nm. No loss in activity was observed after incubation of JHBP and EFDA *without* irradiation. Protection from photoinactivation of JHBP by EFDA could be achieved in the presence equimolar JH III (Figs. 4, 5). Finally, photoaffinity labelled proteins showed loss of binding capacity without alteration of binding affinity. This was the first demonstration of the use of a photoaffinity label in studying JH action on a molecular level.[41a]

Subsequently, we have conducted intensive studies of the nature of the binding site and of the binding proteins using [^{3}H]-EFDA. These results will be presented elsewhere in a full paper in the near future.[41b] Briefly, we (J.K.K. + G.E.K.) have determined that EFDA exhibits saturable finite binding with both *Leucophaea* JHBPs (K_D(EFDA) $\approx 8 \times 10^{-7}$ *M*), comparable to that for JH III (K_D(JH III) $\approx 2 \times 10^{-8}$ *M*). Photolysis with 10^{-8} *M* [^{3}H]-EFDA gives 3–4% binding to protein, of which approximately half is specific binding to the JH binding site (protection by JH III or JH I). We were sufficiently encouraged to extend the study of [^{3}H]-EFDA to other insect JHBP systems.

The photoaffinity labels **1b–4b** were examined for *in vitro* JH esterase inhibition, using hemolymph esterase from fifth instar larval *Trichoplusia ni* (B. Hammock, unpublished data). None of these com-

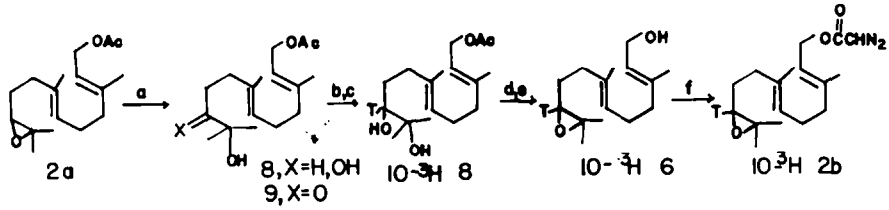
Table 1. *In vitro* assays of diazo JHA with JH binding proteins of three insects

Competitor	Leucophaea[a] Hemolymph	Leucophaea[a] Ovary	Drosophila Kc cell[b] Cytosol	Trichoplusia[c] JHE
1b	1.9×10^{-5}M	4.5×10^{-5}M	88%	$>10^{-4}$M
2a(EFA)	- (3.6×10^{-6})	-	-	-
2b(EFDA)	4.2×10^{-6}(4.75×10^{-6})	6.6×10^{-6}	47%	$>10^{-4}$M
3b	1.0×10^{-5}	1.8×10^{-5}	48%	$>10^{-4}$M
4b	7.3×10^{-6}	1.0×10^{-5}	44%	$>10^{-4}$M
cold JH III	9.0×10^{-8}(8.2×10^{-8})	8.7×10^{-8}	100%	(substrate)

[a]Values show concentration of diazo JHA required to inhibit 50% binding of 1×10^{-8}M [^{3}H]-JH III for the protein indicated. Relative binding affinities (RBA's) and experimental details were presented earlier.[41]

[b]Values indicate the % binding at 10^{-6}M competitor relative to JH III at the same concentration. The assay procedure described in text.

[c]Values show concentration required for 50% inhibition of JH esterase preparation of fifth instar T. ni.



Scheme 2. Synthesis of [10-^{3}H]-10,11-epoxyfarnesyl diazoacetate (^{3}H-EFDA). Reagents: (a) $HClO_4$, H_2O, THF; (b) DCC, C_6H_5N, CF_3CO_2H, C_6H_6, DMSO; (c) [^{3}H]-$NaBH_4$, C_2H_5OH; (d) MsCl, Py, CH_2Cl_2; (e) K_2CO_3, CH_3OH; (f) ClCOCHNNHTs, 2 equiv. Et_3N, CH_2Cl_2.

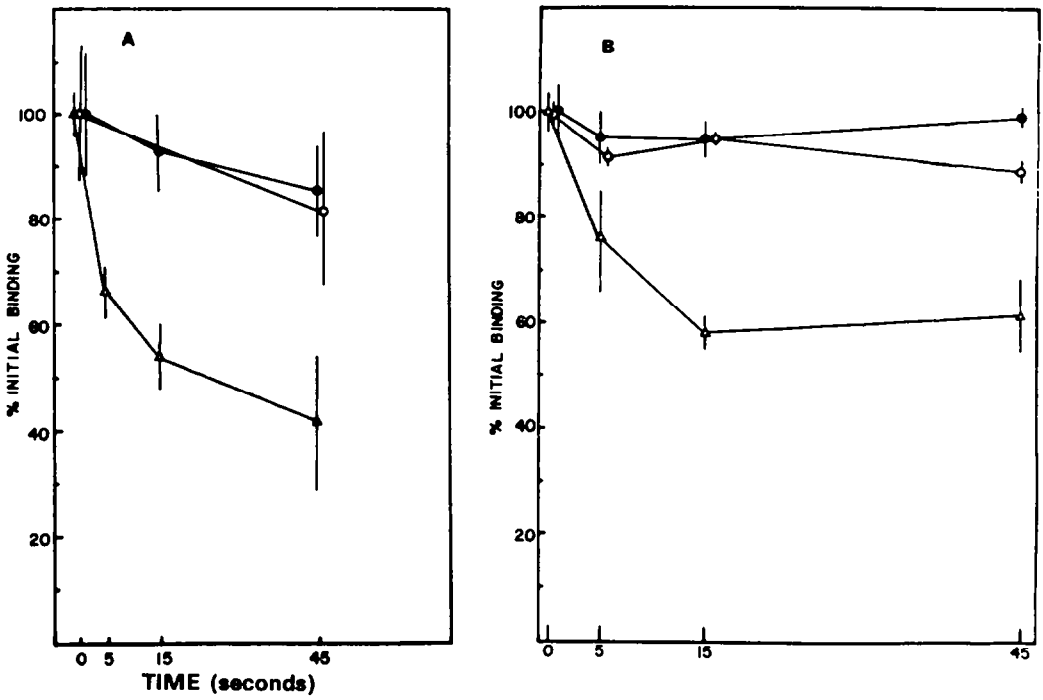


Fig. 5. Comparison of protection experiments for hemolymph JHBP from *Leucophaea* (a) without dextran-coated charcoal to remove excess JH III after photolysis and before [^{3}H]-JH III assay, (b) with dextran-coated charcoal removal of cold JH III.[41] No hormone (open circles), $1.0 \times 10^{-5} M$ EFDA (open triangles), $1.0 \times 10^{-5} M$ EFDA and $1.0 \times 10^{-5} M$ JH III (filled circles). Values are shown as means (n = 3) ± s.d.

pounds caused any detectable inhibition (i.e. < 5% inhibition) at $10^{-4} M$, which is the limit of water solubility of these materials. (Conditions: 10 min preincubation with substrate, followed by assay with $5 \times 10^{-6} M$ [10-^{3}H]-JH III for 15 min/30°). In contrast, trifluoromethyl ketones and EPPAT showed $K_I < 10^{-8} M$ for these preparations. This lack of inhibition renders unlikely a mechanistic possibility previously envisioned based on the FGAR amidotransferase inhibition found for aza-serine[49] (Fig. 6).

It seems probable that the lack of JHE inhibition is due to poor binding. In general, JH esterase from *T.ni* is highly sensitive to steric size, i.e. ethyl and higher esters are hydrolyzed very slowly. The diazoketone and diazoacetate moieties are sterically larger than the corresponding protio compound, and this increased size may account for poor binding to enzyme active site. Support for this hypothesis came from the attempts to inhibit JHE activity through photoattachment of the cold EFDA (R. M. Roe, B. Hammock, G. Prestwich, unpublished observations). Partially purified *T.ni* hemolymph (15–30% PEG 6800 fraction) in a pH 7.5 phosphate buffer containing 25% (w/v) sucrose was preincubated for 1 hr with (a) no inhibitor; (b) $10^{-5} M$ EFDA, and (c) $10^{-6} M$ EFDA. The solutions were irradiated at 254 nm (8 lamps, Rayonet) for 0, 10, 20, 40, 80, 160 sec intervals at 0°, and diluted in phosphate–sucrose buffer. Aliquots were incubated for 30 min with $5 \times 10^{-6} M$ [10^{-3}H]-JH III at 30°, quenched, partitioned (isooctane/aqueous methanol) and counted.[7a] No loss of JHE activity was observed in any samples; indeed, esterase activity increased significantly in the $10^{-5} M$ EFDA treatment for intermediate times. We propose that the inertness of JHE to EFDA would provide a valuable new tool to selectively label nonmetabolic binding proteins in the presence of catabolic JH-binding proteins.

Diazo JHA **1b–4b** were also studied *in vitro* with homogenates of the 7D11 line of cultured *Drosophila melanogaster* Kc cells[28] (E. Chang and M. Bruce, unpublished data.) Competitive studies were conducted by incubation of the Kc cell cytosol for 2.5 hr with

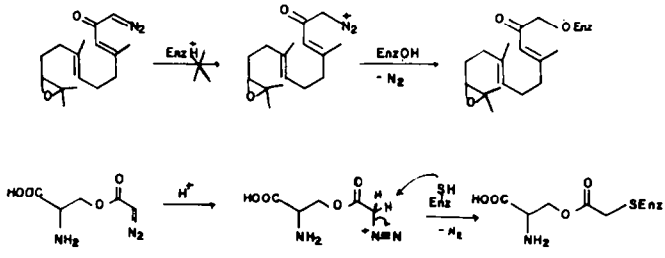


Fig. 6. Role of diazocarbonyls as suicide substrates.

the diazo JHA's at three concentrations, removal of the competitor with DCC (dextran-coated charcoal) for 1 hr, and assay using $1 \times 10^{-8} M$ [^{3}H]-JH III following the DCC method to separate bound and free hormone. The results show that compounds **2b** **3b** and **4b** all are good (50% inhibition) competitors at $10^{-6} M$, but **1b** does not reach this level until $10^{-4} M$.

Again, EFDA (**2b**) was chosen for further studies. Incubation of [^{3}H]-EFDA with intact Kc cells in the absence of esterase inhibitors results in rapid deesterification and epoxide hydrolysis; indeed the preliminary data show more rapid metabolism for EFDA (28% remaining after 30 min) than for JH III (84% remaining after 30 min). This degradation is slow in cytosol but fast in microsomal fractions. The binding of [^{3}H]-EFDA by cytosol showed specific ($K_D \approx 2 \times 10^{-7}$) and nonspecific binding (*ca* 40%). Both JH III and cold EFDA can displace [^{3}H]-EFDA.

Upon photolysis with ^{3}H-EFDA, one can demonstrate both specific and nonspecific binding due to covalent photoattachment to the JH III binding site (Fig. 7). Control cytosol and cytosol which was preincubated with $2.5 \times 10^{-5} M$ cold JH III for 30 min were incubated with $5.0 \times 10^{-7} M$ [^{3}H]-EFDA for 90 min. Photolysis for varying intervals (15 sec to 12 min) was followed by precipitation with 75% ethanol, centrifugation and washing of the pellet, and counting of the solubilized protein. Considerable non-specific binding is observed even when a 100-fold excess of JH III is present. Nonethless, reproducible, specific binding of ^{3}H-EFDA to the JH III binding site is also observed at irradiation times over 2 min. Longer photolysis is required for Kc cell cytosol because the total protein concentration (*ca* 15 mg/ml) is much higher than that used for cockroach JHBP ($\leq$ 1 mg/ml). The absorption of UV light by the protein reduces the intensity of light at the diazocarbonyl chromophores and slows decomposition.

Summary. We have demonstrated the feasibility of photoaffinity labelling as a technique for the study of juvenile hormone binding proteins in insects of three different orders. We urge interested insect biochemists to contact us to help develop this as a routine method for recognizing and characterizing JHBP from insects, and in extending the technique to the labelling of nuclear JHBP. We believe the possibilities have only been hinted at. Efforts are in progress to prepare higher specific activity EFDA analogs, and to design, synthesize, and test photoaffinity labels for JH esterases, JH epoxide hydrolases, and JH analog binding proteins (which often have no affinity for JHBP[27a]).

EXPERIMENTAL

General. Unless otherwise noted, materials were obtained from commercial suppliers and used without further purification. Reagent grade Et_3N was dried by storage over KOH followed by distillation from BaO. CH_2Cl_2 was distilled from $CaCl_2$ and stored over 4 Å molecular sieves. Hexane, EtOAc and MeOH were Fisher HPLC grade and used without further purification. Methanesulfonyl chloride was distilled prior to use. [^{3}H]-Sodium borohydride (64 Ci/mmol) was purchased from New England Nuclear (Boston, Mass.). All reactions were performed under N_2. Bps and mps (Pyrex capillary) are uncorrected. IR spectra were determined with a Perkin–Elmer Model 727 instrument and are reported in wavenumbers (cm^{-1}). 80 MHz ^{1}H NMR spectra were obtained on an CFT-20 spectrometer. Significant ^{1}H NMR data are tabulated in order: number of protons, multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet), coupling constant(s) in Hertz. 20 MHz ^{13}C NMR were recorded using a Varian CFT-20. Chemical shifts are expressed in ppm downfield from internal tetramethylsilane. All NMR spectra were taken in $CDCl_3$ unless otherwise noted.

TLC was performed using MN Polygram Sil G/UV 254 (4 × 8 cm) TLC plates. Flash chromatography on nonradiolabeled material was performed under N_2 pressure on Merck Silica Gel G (400–230 mesh) using hexane-EtOAc mixtures. Chromatography of radiolabeled material was performed using Merck Silica Gel G (400–230 mesh) packed in a disposable Pasteur pipet, eluting with hexane-EtOAc mixtures by applying pressure with a pipet bulb. The chromatograms of nonradiolabeled materials were visualized with an EtOH–vanillin–H_2SO_4 reagent. Visualization of radiolabeled material was accomplished at 254 nm followed by staining with I_2 vapor. Radioactive samples were counted in a Packard Tri Carb liquid scintillation counter using an Omnifluor-toluene cocktail. Counting was 52–57% efficient as determined by quench curves, and all counts were corrected using automatic external standardization.

Experimental details are given only for the synthesis of the [10-^{3}H]-10,11-epoxyfarnesyl diazoacetate. Synthetic details for the non-isotopically labelled photoaffinity labels

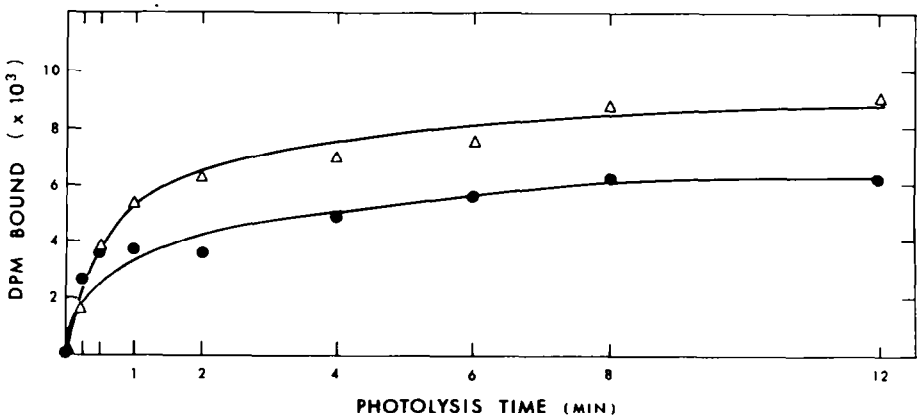


Fig. 7. Photoactivated binding of $5.0 \times 10^{-7} M$[^{3}H]-EFDA (1.2×10^6 dpm) to *Drosophilia* Kc cell cytosolic proteins *in vitro*. Open triangles, no JH III added; filled circles, $2.5 \times 10^{-5} M$ JH III preincubation. Maximum binding efficiency 1%. Values plotted are corrected by subtraction of 0 sec photolysis bound counts.

have been reported previously,[41a,42,44] and experimental details for the *in vitro* bioassays mentioned herein will be published separately.[41a,b]

(E, E)-(10, 11)-*Dihydroxy*-3, 7, 11-*trimethyl*-2, 6-*dodecadienyl acetate* (**8**).[23a] To a stirred soln of **2a** (0.40 g, 1.5 mmoles)[22] in 10 mL THF and 5 mL water was added 5 drops of 70% perchloric acid. After 5 hr, brine was added and the mixture was extracted several times with ether. The organic phase was washed with dil $NaHCO_3$aq and brine, dried (Na_2SO_4), and evaporated to give crude diol which was purified by flash column chromatography. Elution with EtOAc–hexanes (1:2) gave 0.22 g (55% yield) of pure **2**: ^{1}H-NMR ($CDCl_3$) δ 1.15 (3H, s), 1.19 (3H, s), 1.59 (3H, s), 1.68 (3H, s), 2.05 (3H, s), 4.57 (2H, d, 7.1Hz), 5.3 (2H, m).

(E, E)-10-*Keto*-11-*hydroxy*-3, 7, 11-*trimethyl*-2, 6-*dodecadienyl acetate* (**9**). To a stirred soln of dicyclohexylcarbodiimide (0.21 g, 1 mmole) in 4.0 mL dry dimethylsulfoxide was added a soln of **8** (0.1 g, 0.33 mmole) in 2 mL benzene. Pyridine (0.05 mL, 0.6 mmole) and trifluoroacetic acid (0.03 mL, 0.39 mmole) were added and the mixture was stirred 18 hr at ambient temp. EtOAc (1 mL) and water (2 mL) were added and the mixture was extracted with ether. The organic phase was washed several times with water and brine, dried (Na_2SO_4), and evaporated to give crude hydroxyketone which was purified by flash column chromatography. Elution with EtOAc-hexane (1:3) gave 0.03 g (31% yield) of pure **9**: IR ($CHCl_3$) 3486, 1711, 1241 cm^{-1}; ^{1}H-NMR ($CDCl_3$), δ 1.37 (3H, s), 1.60 (3H, s), 1.69 (3H, s), 2.04 (3H, s), 3.76 (1H, s), 4.56 (2H, d, 7Hz), 5.3 (2H, m).

Microscale "cold" reduction of hydroxy ketone **9**. To a stirred soln of **9** (10 mg, 0.03 mmole) in EtOH (2 mL) was added $NaBH_4$ (0.1 mg, 0.003 mmole). After 30 min, 0.25 mL of 1 M AcOH soln was added. Most of the EtOH was evaporated and ether was added to the residue. The organic phase was washed with 10% $NaHCO_3$aq and brine, dried (Na_2SO_4), and evaporated to give crude **8** which was purified by flash column chromatography. Elution with EtOAc/hexanes (1:5) gave 3.5 mg of pure **8** which was identical to the previously prepared sample above.

Microscale conversion of cold **8** *to* 10, 11-*epoxy farnesol* **6**. To a stirred soln of **8** (0.7 mg, 0.002 mmole), Et_3N (0.65 μL, 4 μmmole) in CH_2Cl_2 (1 mL) was added methanesulfonyl chloride (0.18μL, 2 μmole) in CH_2Cl_2 mL) at 0°. The mixture was stirred for 0.5 hr at room temp, solvent was evaporated, and ether was added. The organic phase was washed with 1M HCl, and 10% $NaHCO_3$aq, dried (Na_2SO_4) and evaporated to give crude C-10 monomesylate.

To the mesylate was added 1 mL MeOH and approx. 10 mg K_2CO_3. The mixture was stirred for 1 hr at room temp, solvent was removed and brine was added to dissolve excess K_2CO_3. Ether was added and the organic phase was washed with brine, dried (Na_2SO_4) and evaporated to give a quantitative yield of **6** which was used without further purification (silica gel or alumina chromatography caused decomp): ^{1}H-NMR ($CDCl_3$), δ 1.23 (3H, s), 1.28 (3H, s), 1.60 (3H, s), 1.65 (3H, s), 2.15 (3H, s), 2.65 (1H, t, 6 Hz), 4.07 (2H, br d, 4 Hz), 5.1 (2H, m), 5.3 (1H, brt, 4 Hz).

10,11-*Epoxyfarnesol diazoacetate*[41,42,44] (**2b**). To a cold (0°) soln of 211.7 mg (0.81 mmol) of the *p*-toluenesulfonylhydrazone of glyoxylic acid chloride[43] in 4 mL CH_2Cl_2 was added a soln of 184 mg (0.77 mmol) 10,11-epoxy-farnesol and 81.8 mg (0.81 mmol) Et_3N in 0.5 mL CH_2Cl_2. After the soln had been stirred and allowed to warm to 25° over a 1 hr period, an additional 117.9 mg (1.16 mmol) Et_3N was added and the resulting soln was stirred for 1 hr at 25°. The solvent was removed and the crude product was purified by flash chromatography using hexane-EtOAc (95:5) to yield 74.2 mg clear yellow oil (31%) : IR (neat) 2100, 1685 cm^{-1}; ^{1}H NMR ($CDCl_3$), δ 1.32 (3H, s), 1.36 (3H, s), 1.66 (3H, s), 1.75 (3H, s), 2.15 (8H, br s), 2.72 (1H, t, 6.4 Hz), 4.69 (2H, d, 7.2 Hz), 4.77 (1H, s), 5.27 (2H, m); ^{13}C NMR ($CDCl_3$) δ166.9, 142.4, 134.69, 124.5, 118.50, 64.2, 61.8, 58.3, 46.3, 39.5, 36.4, 27.5, 26.2, 24.9, 18.8, 16.5, 16.1.

Tritiated diol ([10-^{3}H]-**8**). A soln of excess **9** (22.4 mg, 0.076 mmol) in abs EtOH (1 mL) was added to an ampoule containing a stirring bar and 0.30 mg (0.008 mmol) of [^{3}H]-$NaBH_4$ (specific activity, 64.0 Ci/mmol). The flask was washed with EtOH (2 × 1 mL) and the washings were added to the ampoule. After stirring for 45 min, 4 drops of 1 M AcOH was added and the mixture was transferred to a 25 ml round bottom flask, washing the ampoule several times with anhyd ether (1 mL) and adding the washing to the flask. The solvents were removed, 8 mL of anhyd ether was added, and this ether soln was washed with 2 mL of sat $NaHCO_3$aq by stirring. The ether layer was transferred to a test tube and dried over $MgSO_4$, passed through a pipet containing $MgSO_4$, and the solvent was removed to yield 21.1 mg crude material. Pipette flash chromatography of this material using hexane-EtOAc (80:20) afforded 8.1 mg (0.027 mmol) of tritiated **8** (specific activity = 11.1 Ci/mmol).

Tritiated epoxyalcohol ([10-^{3}H]-**6**). To 8.1 mg (0.027 mmol) of tritiated **8** was added 11.2 μL (8.1 mg, 0.08 mmol) Et_3N in 1.5 mL CH_2Cl_2. The flask was cooled to 0° and 3.1 μL of methanesulfonyl chloride was added. After the addition was complete, the ice-bath was removed and the mixture stirred for 0.5 hr, followed by addition of 3.8 μL (2.6 mg, 0.02 mmol) Et_3N in 0.4 mL CH_2Cl_2 and 1 μL (0.013 mmol) methanesulfonyl chloride in 0.4 mL CH_2Cl_2. After stirring for 0.5 hr, the solvent was removed and 10 mL anhyd ether was added. the ether soln was washed with 2 mL of 1 M HCl, 10% $NaHCO_3$ and dried over $MgSO_4$. The ether soln was passed through a pipet containing $MgSO_4$ and the solvent removed. To this crude mesylate was added 2 mL MeOH, and approx. 10 mg K_2CO_3. After stirring for 1 hr at 25° the MeOH was removed, 5 mL ether was added, and 5 mL of dil brine soln was added to dissolve excess K_2CO_3. The ether layer was dried over $MgSO_4$, then passed through a pipet containing $MgSO_4$ to yield 9.0 mg crude tritiated **6** which was directly esterified. The specific activity was approx. 6.2 Ci/mmol for the crude epoxy alcohol.

Tritiated EFDA ([10-^{3}H]-**2b**). To a soln of 9.0 mg (0.038 mmol) of tritiated **6** and 6.1 mg (0.060 mmol) Et_3N in 2 mL CH_2Cl_2 was added 14.8 mg (0.057 mmol) *p*-toluenesulfonylhydrazone of glyoxylic acid chloride at 0°. After the soln was stirred and allowed to warm to 25° over a 1.5 hr period an additional 11.4 mg (0.113 mmol) Et_3N was added and the resulting soln stirred for 1.5 hr at 25°. The solvent was evaporated and the yellow residue was taken up in 1 mL EtOAc:CH_2Cl_2 (4:96) and filtered through a pipet containing silica gel, rinsing the flask several times, to remove polar impurities. Chromatography of the crude product using hexane–EtOAc (95:5) afforded 4.7 mg of tritiated diazo acetete ([10-^{3}H]-**2b**)) (57% from tritiated diol [10-^{3}H]-**8**, specific activity = 5.4 Ci/mmol. Several additional fractions containing the difficult-to-remove tosyl containing by-product[48] were reserved for future use.

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