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Binding Specificity of the Juvenile Hormone Carrier Protein from the Hemolymph of the Tobacco Hornworm *Manduca sexta* Johannson (Lepidoptera: Sphingidae)[†]

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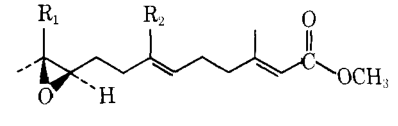
ABSTRACT: A series of analogues of insect juvenile hormone (four geometric isomers of methyl epoxyfarnesenate, several para-substituted epoxygeranyl phenyl ethers, and epoxyfarnesol and its acetate and haloacetate derivatives) was prepared to investigate the binding specificity of the hemolymph juvenile hormone binding protein from the tobacco hornworm *Manduca sexta.* The relative binding affinities were determined by a competition assay against radiolabeled methyl (*E,E*)-3,11-dimethyl-7-ethyl-*cis*-10,11-epoxytrideca-2,6-dienoate (JH I). The ratio of dissociation constants was estimated by plotting competitor data according to a linear transformation of the dissociation equations describing competition of two ligands for a binding protein. The importance of the geometry of the sesquiterpene hydrocarbon chain is indicated by the fact that the binding affinity is decreased as *Z* (cis) double bonds are substituted for *E* (trans) double bonds in the methyl epoxyfarnesenate series; the unepoxidized analogues do not bind. A carboxylic ester function is important although its orientation can be reversed, as indicated by the good binding of epoxyfarnesyl acetate. In the monoterpene series, methyl epoxygeranoate shows no affinity for the binding protein, but substitution of a phenyl or *p*-carbomethoxyphenyl ether for the ester function imparts a low, but significant affinity. These data taken together with earlier results indicate that the binding site for juvenile hormone in the hemolymph binding protein is characterized by a sterically defined hydrophobic region with polar sites that recognize the epoxide and the ester functions.

Insects undergo extensive postembryonic development and thus provide intriguing experimental systems for exploring control of developmental events. The regulation of these processes by two insect hormones, juvenile hormone (JH[1]) and ecdysone, the molting hormone, has been the focus of much attention.

The structures of the three known insect juvenile hormones are shown below. Juvenile hormone acts during larval stages by preventing the growth and differentiation of those cells destined to form typical adult structures. Hormone titer remains high during early larval stages but decreases dramatically in the last larval stage. The absence of JH at this time allows expression of genetic information leading to metamorphosis from the larva to the pupa.



JHI, $R_1 = CH_2CH_3$; $R_2 = CH_2CH_3$ (Röller et al., 1967)
JHII, $R_1 = CH_2CH_3$; $R_2 = CH_3$ (Meyer et al., 1968)
JHIII, $R_1 = CH_3$; $R_2 = CH_3$ (Judy et al., 1973)

In the hemolymph of the tobacco hornworm, *Manduca sexta,* a specific binding protein carries JH from the site of synthesis, the corpus allatum, to target tissues throughout the

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[1] Abbreviations used are: JH, juvenile hormone; JH I, methyl (*E,E*)-3,11-dimethyl-7-ethyl-*cis*-10,11-epoxytrideca-2,6-dienoate; JH II, methyl (*E,E*)-3,7,11-trimethyl-*cis*-10,11-epoxytrideca-2,6-dienoate; JH III, methyl (*E,E*)-10,11-epoxyfarnesenate; DEAE, diethylaminoethyl; THF, tetrahydrofuran; NMR, nuclear magnetic resonance; IR, infrared; GLPC, gas–liquid partition chromatography; TLC, thin-layer chromatography; PLC, preparative thin-layer chromatography; Tris, tris(hydroxymethyl)-aminomethane.

insect (Kramer et al., 1974). This carrier protein protects the hormone from degradative enzymes that are present in hemolymph during early larval stages of the insect (Sanburg et al., 1975a,b; Hammock et al., 1975). In addition, the carrier protein enhances the effect of JH on the deposition of cuticle in wing imaginal disks in tissue culture (Sanburg et al., 1975b). During the last larval stage in *Manduca*, a specific esterase appears that is able to hydrolyze the carrier protein-bound juvenile hormone (Sanburg, et al., 1975a). The action of the esterase is sufficient to account for the decline in JH titers observed during this stage of larval life (Akamatsu et al., 1975).

In order to examine the specificity of the interaction of juvenile hormone with the hormone binding site on the carrier protein, we have synthesized a number of juvenile hormone analogues and measured their relative dissociation constants with the *Manduca* carrier protein using a competition assay. By comparing the relative affinities of these synthetic hormone analogues, the structural features of the juvenile hormone molecule that are important for interaction with the carrier protein have been delineated.

Experimental Procedure

Materials

Pure synthetic methyl (*E*,*E*)-3,11-dimethyl-7-ethyl-*cis*-10,11-epoxytrideca-2,6-dienoate (JH I) was purchased from Eco Control and Regis Chemical Co. Methyl (*E*,*E*)-3,11-dimethyl-7-[1,2-^{3}H]ethyl-*cis*-10,11-epoxytrideca-2,6-dienoate and methyl (*E*,*E*)-3,11-dimethyl-7-ethyl-*cis*-10,11-epoxy-[10-^{3}H]trideca-2,6-dienoate (11.7 and 13.5 Ci/mmol, respectively) were from New England Nuclear. Triton X-100 was from Research Products International and DEAE-filter paper was obtained from Reeve Angel. Hydroxylapatite was from Clarkson Chemical Co. and SP-Sephadex C-50 from Pharmacia. Silica gel for column chromatography was obtained from Brinkmann.

The following compounds were obtained from the sources indicated: farnesol (four isomer mixture, Givaudan); PBr_3 (Aldrich, Fisher); methanesulfonyl chloride (Aldrich, distilled from P_2O_5); LiCl (Baker, dried at 120 °C for 2 days); *s*-collidine (Eastman, distilled); chloroacetic acid (Matheson Coleman and Bell); bromoacetic acid (Aldrich, 2× recrystallized from hexane); iodoacetic acid (Eastman, 2× recrystallized from hexane); dicyclohexylcarbodiimide (Eastman, Aldrich); geraniol (Aldrich Gold Label, 99+%); *m*-chloroperbenzoic acid (Aldrich, 85%); phenol (Merck); *p*-ethylphenol (Aldrich, distilled); *p*-iodophenol (Aldrich); *p*-methylmercaptophenol (Aldrich); methyl *p*-hydroxybenzoate (Aldrich); triphenylphosphine (Aldrich); chloroacetone (Aldrich, distilled); nerol (Fluka, 98%); BuLi, 2.4 M in hexane (Ventron); trimethyl phosphonoacetate (Aldrich).

Dry solvents were prepared in the following manner: ether and dimethoxyethane, distilled from CaH_2; pyridine and *s*-collidine, distilled from KOH; methanol, Fisher Spectrograde over Linde 4-Å molecular sieves; dimethylformamide, stirred with BaO, $MgSO_4$, and passed through alumina prior to use; THF, distilled from Na/naphthalene.

Manduca sexta eggs were a gift from Drs. R. A. Bell and J. P. Reinecke, U.S. Department of Agriculture, Fargo, N.D., and larvae were reared as previously described (Kramer et al., 1974). Juvenile hormone carrier protein was prepared as described previously (Kramer et al., 1976b) or by substituting chromatography on hydroxylapatite or SP-Sephadex C-50 (P. E. Dunn and R. C. Peterson, unpublished results) for the isoelectric focusing step. The product derived from these procedures was identical with the α form of the binding protein previously reported (Kramer et al., 1976b). Other materials used were the same as described previously (Kramer et al., 1974, 1976a) or of the highest purity commercially available.

Methods

Nuclear magnetic resonance (NMR) spectra were obtained on a Varian A60A or HA100 spectrometer and are expressed as parts per million downfield from tetramethylsilane as internal standard (δ scale). Infrared (IR) spectra were taken on a Beckman IR-12 spectrophotometer. Gas-liquid partition chromatography (GLPC) was done on a Hewlett-Packard Model 5750 Research Chromatograph using a 0.125 in. × 5 ft column of 3% OV 17 on 100/120 Supelcoport in either the isothermal or temperature programmed mode as indicated. Melting points were determined on a Fisher-Johns apparatus and are uncorrected.

Analytical thin-layer chromatography (TLC) was carried out on Merck silica gel GF-254 glass-backed plates. Preparative thin-layer chromatography (PLC) was done on 20 × 20 cm or 100 × 20 cm glass plates coated to a thickness of about 2 mm with Merck silica gel 60 PF-254 + 366. The smaller plates were heat activated prior to use. Development was with ether/hexane mixtures and visualization was by use of phosphomolybdic acid spray reagent, iodine, or UV light.

Air- and water-sensitive reactions were conducted under an atmosphere of dry nitrogen in pre-dried solvents with reagents being added via syringe through rubber septa. Organic extracts were dried over powdered anhydrous magnesium sulfate. All farnesol derivatives prepared were of the 2*E*,6*E* (i.e., trans,trans or "natural") configuration unless otherwise noted. All compounds reported under the Experimental Procedure section gave satisfactory elemental analyses and/or spectral properties consistent with their assigned structures.

Synthesis

Acetonyltriphenylphosphonium Chloride and Triphenylphosphineacetylmethylene (***1***). The procedure of Ramirez and Dershowitz (1957) was used with modification.

Geranyl Chloride (***2***). The compound was prepared according to Collington and Meyers (1971). The yield was 83% after short-path distillation (bp 44–56 °C (0.20 mm)).

Neryl chloride (***3***) was prepared in 83% yield as described for geranyl chloride.

Geranyl Acetone (***4***). The method of Cooke (1973) was used. The crude product was distilled, and the fraction boiling between 68 and 75 °C (0.20 mm) was collected. This material contained some geraniol as well as other minor impurities but was used directly for the preparation of methyl farnesenate; the impurities were removed at a later stage. Pure geranyl acetone may be obtained by column chromatography (silica gel, 10% ether in hexane).

Neryl acetone (***5***) was prepared as described for geranyl acetone.

Methyl (*2E,6E*)*- and* (*2Z,6E*)*-farnesenates* (***6*** *and* ***7***) were prepared according to Anderson et al. (1972). The yield was 77% of a product whose isomer composition (GLPC, 180 °C) was 64% 2*E*,6*E*, 33% 2*Z*,6*E*, and 3% of an impurity. The isomers were separated by PLC on 100 × 20 cm silica gel plates (5% ether in hexane, 4 developments). The *E* isomer was found at R_f 0.463–0.540 and the *Z* isomer at R_f 0.570–0.640: NMR (CCl_4), 2*E*,6*E* isomer, δ 1.59 (s, 6 H, C-7, C-11 CH_3's), 1.66 (s, 3 H, C-11 CH_3), 1.98 (s, 4 H, 2 × H-9, 2 × H-8), 2.14 (d, 3 H, *J* = 1 Hz, C-3 CH_3), 2.14 (m, 4 H, 2 × H-5, 2 × H-4), 3.60 (s, 3 H, CO_2CH_3), 5.04 (t, br, 2 H, H-10, H-6), 5.58 (s, 1 H, H-2). Diagnostic NMR peaks for the 2*Z*,6*E* isomer were δ 1.87 (d, 3 H, *J* = 2 Hz, C-3 CH_3), 2.57 (t, 2 H, *J* = 8 Hz, 2 × H-4).

*Methyl (2E,6Z)- and (2Z,6Z)-Farnesenates (**8** and **9**).* The C-2 isomers of methyl (6*Z*)-farnesenate were prepared from neryl acetone in the manner described previously for the 6*E* series. Two solvent systems were used for PLC: 6% ether in hexane (4 developments), $R_{f(2E)}$ 0.520–0.620, $R_{f(2Z)}$ 0.645–0.715; 5% ether in hexane (4 developments), $R_{f(2E)}$ 0.420–0.585, $R_{f(2Z)}$ 0.630–0.695. By GLPC (190 °C) the isomeric composition of the crude product was 68% 2*E*,6*Z*, 30% 2*Z*,6*Z*, and 2% impurity.

Diagnostic NMR peaks for the 2*E*,6*Z* isomer were δ 1.59 (s, 3 H, C-11 CH_3), 1.67 (s, 6 H, C-11, C-7 CH_3), 2.09 (m, 4 H, 2 × H-5, 2 × H-4), 2.12 (d, 3 H, *J* = 2 Hz, C-3 CH_3).

Diagnostic NMR peaks for the 2*Z*,6*Z* isomer were δ 1.85 (d, 3 H, *J* = 2 Hz, C-3 CH_3), 2.58 (t, 2 H, *J* = 8 Hz, 2 × H-4).

*Methyl 10,11-epoxy-(2E,6Z)-farnesenate (**12**)* was prepared according to Anderson et al. (1972). A 53% yield was obtained after PLC (ether/hexane, 1:4; 4 developments), followed by charcoal treatment, passage through a short silica gel column (ether/hexane, 1:1), and solvent removal: NMR (CCl_4) δ 1.19, 1.22 (2 s, 6 H, C-11 CH_3's), 1.53 (m, 2 H, 2 × H-9), 1.68 (s, 3 H, C-7 CH_3), 2.12 (m, 9 H, 2 × H-8, 2 × H-5, 2 × H-4, C-3 CH_3), 2.48 (t, 1 H, *j* , 6 Hz, H-10), 3.58 (s, 3 H, CO_2CH_3), 5.08 (t, br, 1 H, H-6), 5.55 (s, 1 H, H-2).

*Methyl 10,11-epoxy-(2Z,6Z)-farnesenate (**13**)* was prepared as described above. Diagnostic NMR peaks were δ 1.87 (d, 3 H, *J* = 1 Hz, C-3 CH_3), 2.57 (m, 3 H, H-10, 2 × H-4).

*Methyl 10,11-epoxy-(2Z,6E)-farnesenate (**11**)* was prepared as described above. Diagnostic NMR peaks were δ 1.86 (d, 3 H, *J* = 1 Hz, C-3 CH_3), 2.55 (m, 3 H, H-10, 2 × H-4).

*Methyl 10,11-Epoxy-(2E,6E)-farnesenate (JH I, **10**).* This compound has been previously described (Reibstein et al., 1976).

Methyl epoxygeranoate was prepared by oxidation of geraniol to methyl geranoate by the method of Corey et al. (1968), followed by epoxidation with *m*-chloroperbenzoic acid.

*Geranyl bromide (**14**)* was prepared by the method of Osbond (1961) at 0 °C under nitrogen. Distillation (70 °C (3 mm)) from $CaCO_3$ through base-washed glassware gave an 84% yield.

*6,7-Epoxygeranyl bromide (**15**)* was prepared according to Bowers (1969). Peaks characteristic of the terminal epoxide were observed in the NMR (CCl_4): δ 1.18, 1.22 (2 s, 6 H, C-7 CH_3's), 1.60 (m, 2 H, 2 × H-5), 2.49 (t, 1 H, *J* = 6 Hz, H-6).

*Epoxygeranyl p-ethylphenyl ether (**17**)* was prepared as described by Bowers (1969). Purification was by PLC (silica gel, 15% ether in hexane, 3 developments), followed by bulb-to-bulb distillation ($CaCO_3$, base-washed glassware, *T*(bath) 145 °C (0.2 mm)). The yield was 73%.

The phenyl (**16**), *p*-tolyl, *p*-isopropylphenyl, *p*-iodophenyl (**18**), *p*-carbomethoxyphenyl (**19**), and *p*-methylmercaptophenyl (**20**) ethers of epoxygeraniol were similarly prepared from epoxygeranyl bromide and the appropriate phenol. Yields were about 70%.

*Farnesyl acetate (**21**), 10-bromo-11-hydroxy-10,11-dihydrofarnesyl acetate (**22**), 10,11-epoxyfarnesol (**23**), and 10,11-epoxyfarnesyl acetate (**24**)* were prepared according to Hanzlik (1973).

*10,11-Epoxyfarnesyl chloride (**25**)* was prepared by the method of Collington and Meyers (1971).

Diagnostic peaks in the NMR spectrum (CCl_4) were δ 1.18, 1.22 (2 s, 6 H, C-11 CH_3's), 1.52 (m, 2 H, 2 × H-9), 2.48 (t, 1 H, *J* = 6 Hz, H-10), 3.98 (d, 2 H, *J* = 8 Hz, 2 × H-1).

Farnesyl Chloroacetate. To a solution of 666 mg (3 mmol) of farnesol and 284 mg (3 mmol) of chloroacetic acid in 15 mL of dry ether at 0 °C was added 618 mg (3 mmol) of dicyclohexylcarbodiimide. After stirring for 15 min the mixture was placed in a refrigerator for 24 h. It was then filtered; the precipitate was washed with ether, and the filtrate and wash were combined. The ether solution was washed with 10% $NaHCO_3$ (2×) and dried, and the solvent removed to give a pale yellow oil. Column chromatography of this material on deactivated silica gel (12% w/w water) with 5% benzene in hexane yielded 634 mg (71%) of clear oil. An analytical sample was obtained by bulb-to-bulb distillation (*T*(bath) 139 °C (0.35 mm)): NMR (CCl_4) δ 1.57 (s, 6 H, C-11 and C-7 CH_3's), 1.64 (s, 3 H, C-11 CH_3), 1.72 (s, 3 H, C-3 CH_3), 2.00 (m, 8 H, CH_2's), 3.90 (s, 2 H, CH_2Cl), 4.61 (d, 2 H, *J* = 7 Hz, 2 × H-1), 5.02 (t, br, 2 H, H-6, H-10), 5.30 (t, 1 H, *J* = 7 Hz, H-2); IR (neat) 1770 cm^{-1} (C=O). Anal. Calcd for $C_{17}H_{27}ClO_2$: C, 68.32; H, 9.11; Cl, 11.86. Found: C, 68.44; H, 9.13; Cl, 11.81.

In a similar manner, farnesyl bromoacetate and iodoacetate were prepared in 74 and 65% yields, respectively, by condensing farnesol with the appropriate haloacetic acid. Column chromatography was done with 8 or 10% benzene in hexane. Formation and purification of the iodoacetate were done in the dark. This compound is unstable, but storage at −20 °C minimizes decomposition. The products were dried in vacuo at 0 °C for several hours; no distillation was attempted. Spectral properties and analyses are given below.

Farnesyl bromoacetate displayed significant spectral data: NMR (CCl_4) δ 3.68 (s, 2 H, CH_2Br); IR (neat) 1745 cm^{-1} (C=O). Anal. Calcd for $C_{17}H_{27}BrO_2$: C, 59.48; H, 7.93; Br, 23.27. Found: C, 59.68; H, 7.92; Br, 22.94.

Farnesyl iodoacetate displayed significant spectral data: NMR (CCl_4) δ 3.55 (s, 2 H, CH_2I); IR (neat) 1735 cm^{-1} (C=O). Anal. Calcd for $C_{17}H_{27}IO_2$: C, 52.31; H, 6.97; I, 32.51. Found: C, 52.55; H, 7.19; I, 33.04.

*Epoxyfarnesyl chloroacetate (**29**)* was prepared from epoxyfarnesol and chloroacetic acid as described for farnesyl chloroacetate. The crude product was purified on deactivated silica gel (12% w/w water) with 2% ether in benzene to give a 78% yield. An analytical sample was obtained by bulb-to-bulb distillation (*T*(bath) 140 °C (0.2 mm)): NMR (CCl_4) δ 1.18, 1.20 (2 s, 6 H, C-11 CH_3's), 1.48 (m, 2 H, 2 × H-9), 1.60 (s, 3 H, C-7 CH_3), 1.72 (s, 3 H, C-3 CH_3), 2.05 (m, 6 H, CH_2's), 2.46 (t, 1 H, *J* = 6 Hz, H-10), 3.91 (s, 2 H, CH_2Cl), 4.60 (d, 2 H, *J* = 8 Hz, 2 × H-1), 5.08 (t, br, 1 H, H-6), 5.30 (t, 1 H, *J* = 8 Hz, H-2); IR (neat) 1760 cm^{-1} (C=O). Anal. Calcd for $C_{17}H_{27}ClO_3$: C, 64.85; H, 8.64; Cl, 11.26. Found: C, 64.87; H, 8.47; Cl, 10.86.

Epoxyfarnesyl bromoacetate and iodoacetate were similarly prepared in 84 and 60% yields, respectively, by condensation of epoxyfarnesol and a haloacetic acid. Formation and purification of the iodoacetate were done in the dark. An analytical sample of this compound could not be obtained due to its instability. The products were dried in vacuo at 0 °C for several hours; no distillation was attempted.

*Epoxyfarnesyl bromoacetate (**30**)* displayed significant spectral data: NMR (CCl_4) δ 3.69 (s, 2 H, CH_2Br); IR (neat) 1745 cm^{-1} (C=O). Anal. Calcd for $C_{17}H_{27}BrO_3$: C, 56.83;

H, 7.57; Br, 22.24. Found: C, 56.97; H, 7.61; Br, 22.57.

Epoxyfarnesyl iodoacetate (***31***) displayed significant spectral data: NMR (CCl_4) δ 3.57 (s, 2 H, CH_2I); IR (neat) 1733 cm^{-1} (C=O). Anal. Calcd for $C_{17}H_{27}IO_3$: C, 50.25; H, 6.70; I, 31.23. Found: C, 51.19; H, 6.46; I, 30.37.

Competition Assay

The affinity of the carrier protein for hormone analogues was determined by means of a competition assay. A series of incubation mixtures was prepared that contained varying concentrations of unlabeled competitor compound, a constant amount of carrier protein (1.2–1.7 × 10^{-7} M), and a constant amount of tritiated juvenile hormone (JH I, 0.5–1.0 × 10^{-6} M). These mixtures were prepared by combining the competitor and juvenile hormone dissolved in benzene–hexane (4:1) or toluene in centrifuge tubes and removing the solvent under a stream of nitrogen. Buffer [40 μL, 5 mM Tris-HCl, pH 8.3 (23 °C)] was added and the tubes were mixed intermittently for at least 4 h at room temperature to allow the hormone and competitor to dissolve. Tubes were cooled to 0 °C; carrier protein (20 μL) was then added, and the mixture was allowed to equilibrate for 4 h. Radioactive complex was determined by the DEAE filter assay (see Results).

The derivation of the equation used to calculate the ratio of dissociation constants is described below. The partitioning of the components in the competition assay is described by the two dissociation equations:

$$K_D{}^H = \frac{HP}{C_H} \tag{1}$$

$$K_D{}^A = \frac{AP}{C_A} \tag{2}$$

where $K_D{}^H$ and $K_D{}^A$ are the dissociation constants for juvenile hormone and competitor, respectively, H is the unbound juvenile hormone concentration, A is the unbound competitor concentration, P is the uncomplexed carrier protein concentration, and C_H and C_A are juvenile hormone–carrier protein complex and competitor–carrier protein complex concentrations, respectively.

Equations 3, 4, and 5 describe the conservation of hormone, competitor, and protein:

$$H_0 = H + C_H \tag{3}$$

$$A_0 = A + C_A \tag{4}$$

$$P_0 = C_H + C_A + P \tag{5}$$

where H_0, A_0, and P_0 are the total concentrations (bound and free) of juvenile hormone, competitor, and carrier protein. Under the conditions of the assay, free hormone is much greater than bound hormone (>sevenfold excess); the same holds true for free and bound competitor. Thus, using the approximations $JH_0 \simeq JH$ and $A_0 \simeq A$ and eq 1, 2, and 5, expressions for the hormone–carrier protein complex in the absence (C_H) and presence ($C_{H'}$) of competitor can be derived:

$$C_H = \frac{H_0P_0}{K_D{}^H + H_0} \tag{6}$$

$$C_{H'} = \frac{H_0P_0K_D{}^A}{K_D{}^A(K_D{}^H + H_0) + K_D{}^HA_0} \tag{7}$$

The ratio of the complex in the absence to that in the presence of competitor (eq 8) is obtained by dividing eq 6 by eq 7:

$$\frac{C_H}{C_{H'}} = \frac{H_0P_0/(K_D{}^H + H_0)}{H_0P_0K_D{}^A/[K_D{}^A(K_D{}^H + H_0) + K_D{}^HA_0]} \tag{8}$$

Algebraic manipulation of eq 8 leads to the final equation (9):

$$\frac{C_H}{C_{H'}} = 1 + \frac{K_D{}^H}{(K_D{}^H + H_0)K_D{}^A}A_0 \tag{9}$$

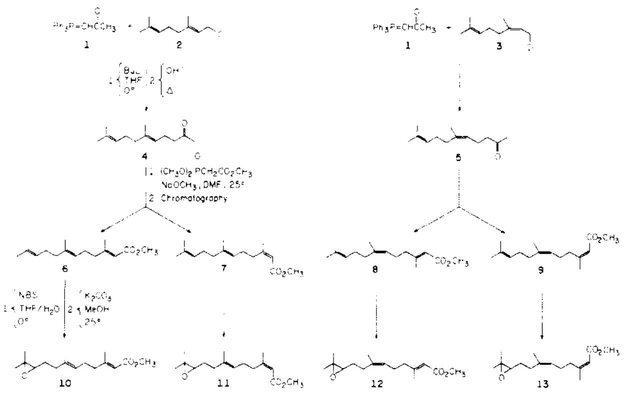
When the ratio of complex in the absence and presence of competitor ($C_H/C_{H'}$) is plotted against the concentration of competitor (A_0) present in the assay mixture, the slope of the resulting line will be inversely proportional to the dissociation constant of the complex of the competitor with the carrier protein. Since $K_D{}^H$ is known and H_0 can be measured, $K_D{}^H/K_D{}^A$ can be calculated from the slope.

Results and Discussion

Synthesis of Juvenile Hormone Analogues

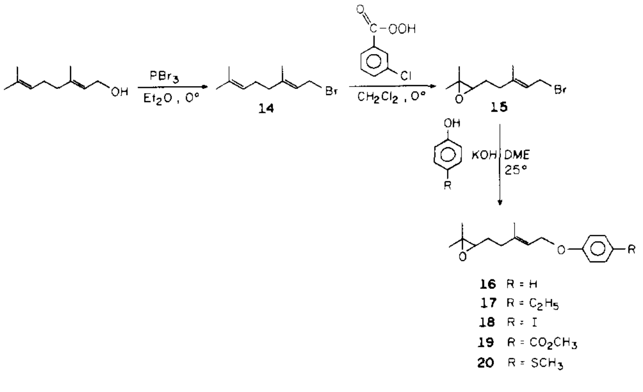
Isomers of Juvenile Hormone III (*Scheme I*). Geranyl acetone (**4**) and neryl acetone (**5**) were prepared by alkylation of the lithium anion of acetylmethylenetriphenylphosphorane (**1**) with geranyl chloride (**2**) and neryl chloride (**3**), respectively, followed by hydrolysis of the phosphorus-containing moiety (Cooke, 1973). By use of the Wadsworth–Emmons reaction with trimethyl phosphonoacetate, a mixture of methyl (2*Z*,6*E*)- and (2*E*,6*E*)-farnesenate (**6** and **7**) could be prepared from geranyl acetone and methyl (2*Z*,6*Z*)- and (2*E*,6*Z*)-farnesenate (**8** and **9**) from neryl acetone. The two C-2 isomers could be separated by PLC, and the four isomeric methyl farnesenates were selectively epoxidized at the 10,11-double bond by the *N*-bromosuccinimide procedure (see Scheme III). The products must be stored under argon.

SCHEME I: Synthetic Route to the Double Bond Isomers of Juvenile Hormone III.



Geraniol-Based Compounds (*Scheme II*). Geraniol was

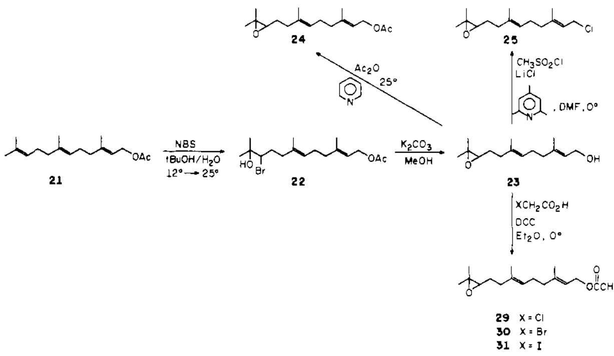
SCHEME II: Preparation of Epoxygeranyl Phenyl Ethers.



converted to the allylic chloride (**2**) by the general procedure of Collington and Meyers (1971). Geranyl bromide (**14**) was prepared by treatment of geraniol with PBr_3 (Osbond, 1961). Reaction of geranyl bromide with *m*-chloroperbenzoic acid at 0 °C gave the terminal epoxide and further reaction with various para-substituted phenols afforded epoxygeranyl phenyl ethers (**16–20**) in good yield.

Farnesol and Epoxyfarnesol Derivatives (*Scheme III*). Farnesol was purchased as a four-isomer mixture; the 2*E*,6*E* isomer was obtained by careful distillation through an annular Teflon spinning band column (Katzenellenbogen, 1969). Farnesol was converted to the acetate (**21**) in nearly quantitative yield with acetic anhydride/pyridine. The terminal double bond of farnesyl acetate was selectively converted to the bromohydrin (**22**) by *N*-bromosuccinimide in an aqueous-organic solvent mixture (Hanzlik, 1973). Base treatment of 10-bromo-11-hydroxy-10,11-dihydrofarnesyl acetate resulted in simultaneous ring closure to the epoxide and deacetylation to give 10,11-epoxyfarnesol (**23**) in quantitative yield. 10,11-Epoxyfarnesol reacted with acetic anhydride/pyridine (Hanzlik, 1973) to give the desired acetate (**24**). Epoxyfarnesyl chloride (**25**) was prepared by reaction of the alcohol with methanesulfonyl chloride, LiCl, and *s*-collidine in dimethylformamide at 0 °C (Collington and Meyers, 1971).

SCHEME III: Preparation of Epoxyfarnesol Derivatives.



Haloacetate Esters. The haloacetates of farnesol were prepared by dicyclohexylcarbodiimide-mediated condensation of alcohol and acid for 24 h at 0 °C. Chromatography was necessary to separate the ester from unreacted farnesol to prevent hydrolysis; this must be done on deactivated silica gel (12% w/w water). The epoxyfarnesyl haloacetates (**29–31**) (Scheme III) were prepared in an analogous manner. The iodoacetates were much less stable than the corresponding chloro- and bromoacetates. The epoxides must be stored under argon as they can absorb nitrogen.

Methods for Assaying the Binding of Juvenile Hormone to the Hemolymph Carrier Protein

Several methods for measuring the concentration of the complex between radiolabeled hormone and carrier protein were explored. Early studies employed a charcoal–dextran assay similar to that successfully used in steroid binding experiments (King and Mainwaring, 1974). In this procedure, the free ligand becomes tightly adsorbed to charcoal, while ligand complexed to protein does not. Rapid centrifugation or filtration can be used to remove the charcoal, and the ligand remaining in the supernatant represents complexed hormone. Control experiments indicated that radiolabeled juvenile hormone is indeed rapidly and quantitatively adsorbed to charcoal while complexed hormone is only slowly adsorbed. However, problems were encountered because of the binding of juvenile hormone to nonpolar surfaces, including glass (Kramer et al., 1976b), and the slow stripping of the hormone from the carrier protein by charcoal. Thus, high blank values and moderate variation in reproducibility were encountered, rendering the method unsuitable for quantitative studies. However, the charcoal adsorption assay remains extremely convenient for rapid, semiquantitative measurement of carrier protein during purification steps (Kramer et al., 1976a).

We also explored chromatography on minicolumns of Sephadex G-25 (King and Mainwaring, 1974) or LH-20 (Ginsburg et al., 1974), which has been used successfully in steroid binding studies. The latter method was found acceptable for measurement of juvenile hormone–binding protein complex, but it was slow and cumbersome. We turned, therefore, to a filter disk method (Kramer et al., 1976b) in which carrier protein could be retained on an ion exchange medium (Whatman DE 81 paper), while the uncharged free hormone passed through the filter. In our first experiments with this technique, high blank values were obtained, and in common with the other methods described so far, the stoichiometry of hormone to carrier ranged from 1.2 to 1.8 mol of hormone per mol of protein. We adopted the modification of Brumbaugh et al. (1974), and added the detergent Triton X-100 to the wash buffer. This lowered the blank values to acceptable levels and yielded stoichiometry of 1.0 mol of hormone per mol of protein (Kramer et al., 1976b). It should be noted that this method is applicable only to the carrier protein from *M. sexta.* For other species, it may well be important to modify assay conditions to ensure that the protein–hormone complex is retained by the ion exchange medium and not dissociated during the wash. One of the other methods discussed above might also be employed.

Competition Experiments

For treatment of data from competition experiments we have derived a linear equation (eq 9) that relates the ratio of complex in the absence and presence of competitor to the concentration of competitor. The slope of this line can be used to determine the ratio of dissociation constants, $K_D{}^H/K_D{}^A$. A low affinity of the competitor for the carrier protein (high value for dissociation constant) will result in a low value for the ratio of dissociation constants.

The linear equation described above offers several advantages over the usual data treatment for competition assays. The most common method of data presentation (percent competition vs. log of competitor concentration) results in a sigmoid curve (Korenman, 1970), and the concentrations at which 50% inhibition of binding is observed are used to determine the relative binding affinities. The ratio of association (or dissociation) constants can be calculated from the relative binding affinities. Using the data treatment described here, the slope of a straight line is used to determine the ratio of dissociation constants and simple statistical methods (such as least-squares linear regression) can be used to evaluate the best slope and intercept.

Data for the competition of JH III geometric isomers against radiolabeled JH I for carrier protein were plotted according to eq 9 (Figure 1). As shown, the data do indeed lie on a straight line with an ordinate intercept of 1.0. We have employed this treatment for the determination of the ratio of dissociation constants for all competitors examined.

The naturally occurring juvenile hormones have the *E* (trans) configuration at the double bonds in positions 2 and 6. Table I summarizes data for the geometric isomers of JH III

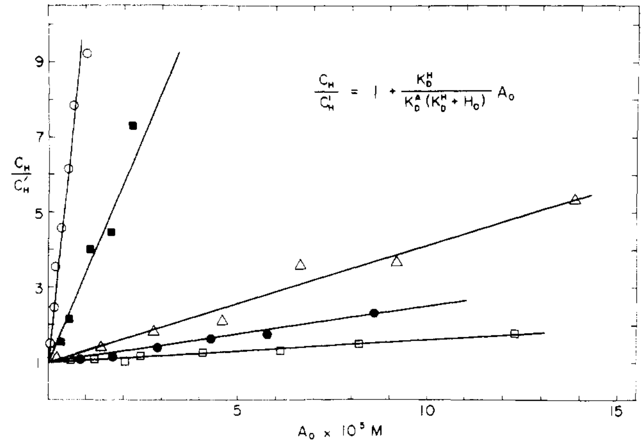
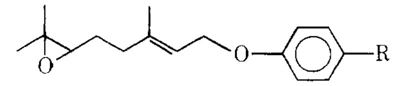


FIGURE 1: Plot of competition data of JH III geometric isomers against radiolabeled JH I according to eq 9. Competition mixtures were prepared as described under Experimental Procedure. Competitor compounds used were JH I (○); JH III (2*E*,6*E*) (■); JH III (2*E*,6*Z*) (△); JH III (2*Z*,6*E*) (●); JH III (2*Z*,6*Z*) (□).

Table II: Relative Affinities of Epoxygeranyl Phenyl Ethers and Methyl Epoxygeranoate.



Substituent, R	RDC[a]	r[b]	Solubility (M) ($\times 10^5$)[c]
H (16)	0.005	0.960	24
CH_3	<0.001		9.2
CH_2CH_3 (17)	<0.004		3.1
$CH(CH_3)_2$	<0.008		1.5
I (18)	<0.010		1.0
SCH_3 (20)	<0.006		3.2
$COOCH_3$ (19)	0.010	0.974	12
Methyl epoxygeranoate	<0.0005[d]		

[a] Ratio of dissociation constants, JHI = 1.0. [b] Correlation coefficient. [c] Solubility in 5 mM Tris-HCl, pH 8.3. [d] Limit was calculated from the finding that no competition occurred with the highest concentration of competitor tested, which was below the limiting solubility of the compound.

TABLE I: Relative Affinities of JH Geometric Isomers for the *Manduca sexta* Carrier Protein.

	RDC[a]	r[b]
JH I	1.0	0.986
JH III (2*E*,6*E*) (**10**)	0.29	0.984
JH III (2*E*,6*Z*) (**12**)	0.030	0.989
JH III (2*Z*,6*E*) (**11**)	0.014	0.962
JH III (2*Z*,6*Z*) (**13**)	0.007	0.984

[a] Ratio of dissociation constants, JH I = 1.0. [b] Correlation coefficient.

TABLE III: Relative Affinities of Farnesol Derivatives.

	RDC[a]	r[b]
Epoxyfarnesyl acetate (**24**)	0.11	0.975
Epoxyfarnesyl chloroacetate (**29**)	0.049	0.989
Epoxyfarnesyl bromoacetate (**30**)	0.051	0.984
Epoxyfarnesyl iodoacetate (**31**)	0.046	0.979
Farnesyl chloroacetate	<0.01	
Farnesyl iodoacetate	<0.01	
Epoxyfarnesol (**23**)	0.006	0.942
Epoxyfarnesyl chloride (**25**)	<0.001	
Methyl farnesenate (2*E*,6*E*) (**6**)	<0.001	

[a] Ratio of dissociation constants, JH I = 1.0. [b] Correlation coefficient.

shown in Figure 1. When an *E* double bond is replaced by a *Z* (cis) double bond, the relative affinity decreases. When both double bonds have the *Z* configuration, the relative affinity is reduced further. It appears that the *E* configuration at the 2,3 position has a greater influence on binding to the carrier protein than the *E* configuration at the 6,7 position. This correlates well with the biological activity of the geometric isomers of juvenile hormones in a number of different insects representing several insect orders (Wigglesworth, 1969; Schwieter-Peyer, 1973).

A series of compounds based upon epoxygeranyl phenyl ether has been tested for hormonal activity (Jacobson et al., 1972). Some compounds in this series were prepared and tested as competitors. In order to evaluate the competitive binding results accurately, it is important that the limit of solubility not be exceeded for these compounds. Table II lists the solubilities of epoxygeranyl phenyl ethers as determined by a spectrophotometric method (Kramer et al., 1976a). An upper limit on the ratio of dissociation constants was set when no competition was observed for the compound at its limit of solubility. The only compounds in this group that competed with JH I for the hormone binding site were the unsubstituted parent compound and the *p*-carbomethoxy derivative.

The ratios of dissociation constants with respect to JH I for a number of farnesol derivatives are shown in Table III. Epoxyfarnesol competes very poorly and epoxyfarnesyl chloride does not compete with JH I for the binding site of carrier protein. It has previously been reported that the acids of JH I and III do not bind to the carrier protein (Kramer et al., 1976a; Goodman et al., 1976). This indicates that the carboxyl ester function is important for complex formation. Several compounds have been prepared in which the ester function is reversed, an acid esterified to epoxyfarnesol. These compounds do show competition against JH I. The data indicate that epoxyfarnesyl acetate has a lower dissociation constant (greater affinity) for carrier protein than do the epoxyfarnesyl haloacetates. The higher dissociation constants calculated for the haloacetate esters compared to the unsubstituted ester may be due to the increased bulk of the halogen derivatives. It should also be noted that the haloacetates may alkylate amino acid residues in the carrier protein and thus restrict labeled hormone from the active site by an irreversible rather than competitive process.

On the other hand, the unepoxidized farnesyl haloacetates and farnesyl acetate do not compete. The solubilities of these compounds are no doubt lower than the corresponding epoxidized compounds. For example, the contribution of the epoxide to the solubility of this type of compound can be seen by comparing methyl epoxyfarnesenate (JH III, limit of solubility 3.9×10^{-4} M, determined as described previously; Kramer et al., 1974) and methyl farnesenate (limit of solubility 6×10^{-6} M; Kramer et al., 1976a); the epoxidized compound is 65 times more soluble in aqueous buffer than the nonepoxidized ester. Thus, it is not possible to use high concentrations of the farnesyl acetates to determine accurately a low ratio of dissociation constants. The importance of the epoxide function for interaction with the carrier protein is indicated by the lack of competition of the unepoxidized compounds and also by the previous observation that the 10,11-diol of JH III shows no competition (Kramer et al., 1976a).

From the results reported here and those reported previously (Kramer et al., 1976a; Goodman et al., 1976) it is possible to draw conclusions about the specificity of binding between the *Manduca* carrier protein and juvenile hormone. Both the ester and epoxide functions are important for this interaction. Elimination of the epoxide abolishes any detectable binding to the carrier protein. The orientation of the ester function can be reversed with only a tenfold decrease in binding relative to JH I (or a threefold decrease relative to JH III). A very low level of competition can also be observed for some of the epoxygeranyl phenyl ethers. An increase in the length of the branches from the main carbon chain enhances binding as previously reported (Kramer et al., 1976a; Goodman et al., 1976). The order of the binding affinities of the three naturally occurring hormones is JH I > JH II > JH III. This indicates that hydrophobic residues at the binding site of the protein play a role in the interaction of the hormone and protein. The site also has definite geometrical requirements since exchange of *Z* for *E* double bonds greatly diminishes the strength of binding.

In *Manduca* the juvenile hormone carrier protein is present in larvae in sufficient concentration to ensure that most of the hormone is in the form of the complex. In this way the hormone is protected from degradation by esterolytic enzymes. The high affinity and restricted specificity of the carrier protein for the molecular architecture of the known natural hormones confirm that this molecule is indeed the hormone transport vehicle, and not merely a general lipid carrier. Thus, it is clearly distinguished from lipoproteins which are reported to be hormone carriers in *Hyalophora cecropia* (Whitmore and Gilbert, 1972). Similar specific carrier proteins have been reported in a number of other insect species (Kramer et al., 1976a; Ferkovich et al., 1975). It is likely that at life stages when juvenile hormone is necessary for control of development, oogenesis, or other functions, many insects will be found to have specific carrier proteins, rather than relying upon nonspecific lipoproteins as transport vehicles. The specific carrier protein complex may also be the form of juvenile hormone that interacts with target tissues.

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