Substrate Requirements for Lepidopteran Farnesol Dehydrogenase

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Farnesol dehydrogenase of the lepidopteran *Manduca sexta* shows surprisingly high substrate specificity, as inferred from the binding of substrate analogs and (potential) alternative substrates. The enzyme is not a simple alcohol dehydrogenase, as ethanol and octanol are not substrates for this enzyme. The enzyme also does not appear to be related to *Drosophila* alcohol dehydrogenase since secondary alcohols are much poorer inhibitors. Several farnesol analogs with modified carbon skeletons have been tested for their ability to function as inhibitors of farnesol dehydrogenase. Substrate competition studies indicate that the enzyme is highly specific for alcohols with Δ-2,3 unsaturation, *trans* allylic olefin geometry, and alkyl chain hydrophobicity corresponding to at least three isoprene units. These results suggest that farnesol dehydrogenase is a unique dehydrogenase that should be further examined as a potential target for anti juvenoid development.

Keywords: *Farnesol dehydrogenase; juvenile hormone biosynthesis; Manduca sexta; substrate analogs*

INTRODUCTION

The final steps of juvenile hormone (JH) biosynthesis involve functionalization of the isoprenoid skeleton obtained from farnesyl diphosphate (FPP) synthase (Scheme 1). Among these transformations is the sequential oxidation of farnesol (**1**) to farnesal (**2**) and farnesoic acid (FA, **3**), mediated by alcohol and aldehyde dehydrogenases. To date, few structural studies have been performed on either of these two enzyme systems. Initial attempts to monitor the dehydrogenases were made by selective gel electrophoresis staining techniques (L'Hélias, 1979; Madhavan et al., 1973). Results obtained suggested that oxidative activity relating to farnesol metabolism was due to an octanol dehydrogenase (ODH), while protein bands corresponding to the conversion of farnesal to farnesoic acid were characterized as aldehyde oxidases (AO). Because much higher activity was observed with octanol, ethanol, and benzaldehyde as substrates, the metabolism of farnesol was initially attributed to nonspecific oxidative enzymes.

Evidence for the presence of distinct dehydrogenases in the biosynthesis of farnesoic acid was obtained by direct *in vitro* assays using homogenized corpora allata of adult female *Manduca sexta* (Baker et al., 1983). While farnesol dehydrogenase activity showed little enhancement with the addition of cofactors, conversion of the corresponding aldehyde to acid showed a marked increase with the addition of 2 mM NAD. Several related terpenoids were also tested for enzyme turnover—geraniol was found to be an excellent substrate for farnesol dehydrogenase and was readily oxidized to the corresponding acid, while nerol showed poor enzymatic conversion.

Few examples of farnesol metabolism have been seen in other species. Redox-mediated isomerization of farnesol occurs in plants and is believed to be an important terpene biosynthetic pathway for several natural products (Overton and Roberts, 1974; Ikeda et al., 1991). Farnesol was also found to be readily metabolized to farnesoic acid in rat liver homogenates by NAD-dependent dehydrogenases; however, the *in vivo* significance of this chemistry is elusive as this does not represent a normal biosynthetic pathway (Christophe and Popják, 1961). Whether these oxidative pathways are related to insect farnesol metabolism has yet to be established.

In this study, the substrate specificity of lepidopteran farnesol dehydrogenase, inferred from the binding of substrate analogs, was examined to ascertain whether nonspecific endogenous dehydrogenases were responsible for farnesol metabolism. Using *M. sexta* larvae, a high degree of specificity can be seen for the conversion of farnesol to farnesal in corpora allata–corpora cardiaca homogenates. These results suggest that farnesol dehydrogenase is indeed a selective enzyme in lepidopteran JH biosynthesis.

METHODS AND MATERIALS

Chemical Sources. Tween 80, NAD, bovine serum albumin (BSA), and geranylgeraniol were obtained from Sigma Chemical Co. Solvents (with the exception of DMSO) and tris(hydroxymethyl)aminomethane were obtained from Fisher Scientific Co. Unless otherwise stated, all other chemicals were obtained from Aldrich Chemical Co.

Insects and Enzyme Source. *M. sexta* larvae were reared on an artificial diet and maintained under constant humidity (70%) and photoperiod (18 h light/6 h dark), using previously described procedures (Bell and Joachim, 1976). Animals were staged prior to molting at third and fourth stadia by observing head capsule detachment (Vince and Gilbert, 1977). Corpora cardiaca–corpora allata (cc–ca) complexes from day 1, fifth stadium larvae (V/1) were removed following previously established procedures (Bhaskaran and Jones, 1980).

Enzyme Assays. Four cc–ca pairs were placed in 450 μL of 100 mM Tris-HCl buffer (pH 7.4) and homogenized on ice using a Duall glass homogenizer (Kontes). After removal of cellular debris by centrifugation at 3000*g* for 10 min, 45 μL aliquots of supernatant were placed in microcentrifuge tubes. Tween 80 and NAD were added as stock solutions (5 μL each) to give final concentrations of 0.05% and 5 mM, respectively. Substitution of 1% BSA for Tween 80 resulted in lower enzymatic activity but had no effect on the relative inhibitory potencies of farnesol analogs.

Farnesol and farnesal dehydrogenase activity were determined using 10-[^{3}H]farnesol (specific activity 30 mCi/mmol),

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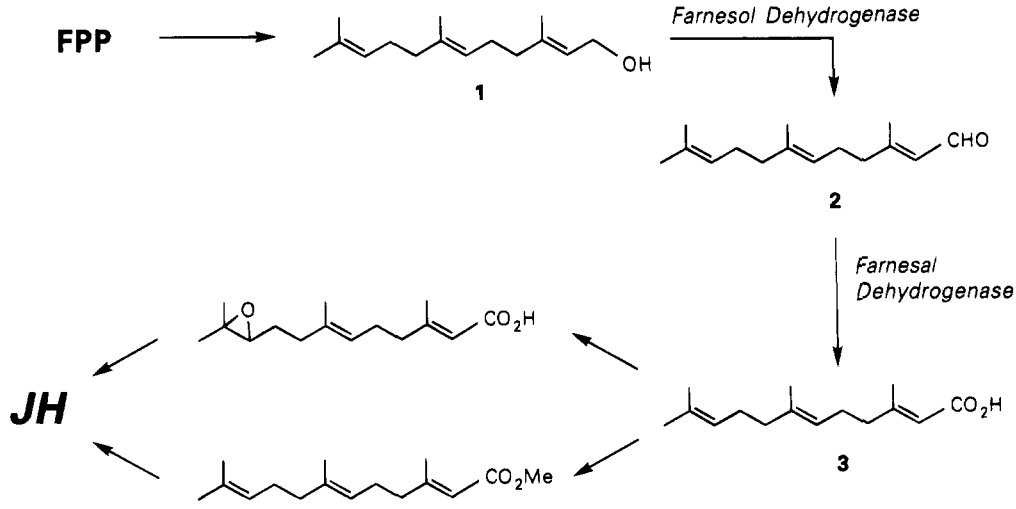
Scheme 1. Enzymatic Conversion of Farnesyl Diphosphate (FPP) to JH



obtained by reaction of the corresponding radioactively labeled *tert*-butyl diphenylsilyl protected trisnoraldehyde with isopropylphosphorane (Sen and Garvin, 1995). Radioactively labeled substrate was added in DMSO (1 μL) to the prepared enzyme solution (55 μL, see above) to give a final concentration of 4 μM. The solution was incubated at 28 °C on a rotary shaker for 60 min and then quenched by the addition of acetonitrile (60 μL) containing farnesol, farnesal, and farnesoic acid standards. The reaction mixture was extracted twice with CH_2Cl_2 (60 μL each), and the concentrated organic extract was placed on a plastic backed normal phase TLC plate (40 × 80 mm, Machery-Nagel Polygram SilG/UV_{254}). Double elution with 10% ethyl acetate/hexane, containing 5% triethylamine, gave clean separation of starting material and products (R_f farnesol = 0.43, R_f farnesal = 0.88, R_f farnesoic acid = 0.09). TLC plates were cut into several zones following visualization of the aldehyde and acid by UV fluorescence and the alcohol by water staining (Jork et al., 1990) and then analyzed by liquid scintillation counting (Beckman LS1801, using Scintiverse BD). Under these assay conditions, further metabolism to epoxyfarnesoate, methyl farnesoate, or JH was not observed, as determined by TLC and HPLC analysis. Typical conversions were 30–35% for farnesal and 15% for farnesoic acid.

Substrate Analog Studies. The substrate specificity of farnesol dehydrogenase was determined by examining the effect of each of the analogs on farnesol metabolism Thus, assays were performed by first preincubating cc–ca homogenates with substrate analogs at increasing concentrations

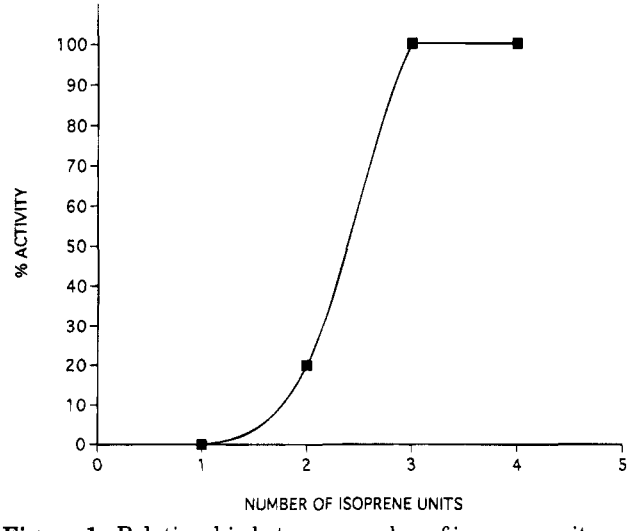


Figure 1. Relationship between number of isoprene units and substrate activity for several long-chain terpenols.

(0, 10, 50, 100, 500 μM) for 30 min and then adding radioactively labeled farnesol and monitoring the effects on farnesal and farnesoic acid formation. Their effectiveness as substrates is expressed as the amount of substrate analog required to decrease farnesal and farnesoic acid formation by 50%.

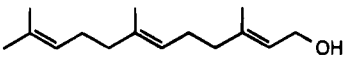
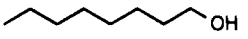
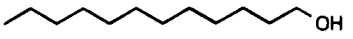
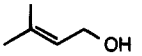
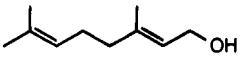
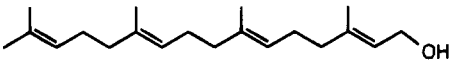
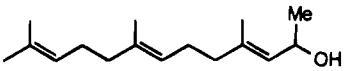
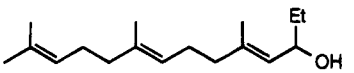
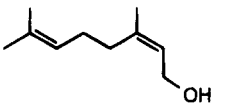
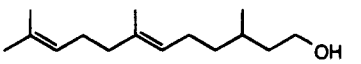
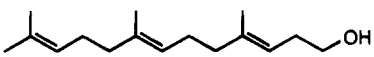
Synthesis of Farnesol Analogs. *all-trans*-Farnesal was synthesized from farnesol by allylic oxidation using activated manganese dioxide (Xiao and Prestwich, 1990). Farnesoic acid was prepared as a mixture of 2*E* and 2*Z* isomers (70/30) from farnesal by reaction with phosphate-buffered sodium chlorite (Bal et al., 1981). Flash chromatography was performed using previously established procedures (Still et al., 1978). "Standard workup" refers to extraction with hexanes, washing of the organic extracts with brine, drying of the combined organic extracts with anhydrous $MgSO_4$, filtering of the sample under vaccum, and concentration of the resulting eluent by rotary evaporation.

*(3E,7E)-4,8,12-Trimethyl-3,7,11-tridecatrien-2-ol (**4**).* To a solution of farnesal (50 mg, 0.23 mmol) in dry THF (10 mL) at −78 °C was added dropwise methyllithium (1.4 M in diethyl ether, 0.16 mL, 1 equiv). The reaction mixture was warmed to room temperature (rt) over 3 h and then quenched by the addition of brine. Standard workup followed by flash chromatography [10% ethyl acetate/hexane (EA/H)] afforded pure farnesol analog **4** (46 mg, 87%): IR (neat) 3341 (broad), 1661 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.23 (d, J = 6.2 Hz, 3H, C-1 CH_3), 1.60 (s, 6H, C-14, C-15 CH_3) 1.68 (s, 6H, C-13, C-16 CH_3), 2.05 (m, 8H, C=CCH_2), 4.60 (dq, 1H, J = 8.5, 6.2 Hz, CHOH), 5.09 (m, 2H, C-7, C-11 C=CH), 5.21 (d, 1H, J = 8.5 Hz, C-3 C=CH). Anal. Calcd for $C_{16}H_{28}O$: C, 81.29; H, 11.93. Found: C, 81.39; H, 11.78.

*(4E,8E)-5,9,13-Trimethyl-4,8,12-tetradecatrien-3-ol (**5**).* Ethylmagnesium bromide (0.16 M in THF, 3 mL) was added dropwise to a solution of farnesal (50 mg, 0.22 mmol) and dry THF (5 mL) at 0 °C. The reaction mixture was stirred for an additional 15 min and then quenched by the addition of water and subjected to standard workup conditions. Flash chromatography using 10% EA/H afforded pure farnesol analog **5** (49 mg, 89%): IR (neat) 3240 (broad), 1675 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.88 (t, 3H, J = 7.4 Hz, CH_2CH_3), 1.45 (m, 2H, CH_2CH_3), 1.60 (s, 6H, C-15, C-16 CH_3), 1.68 (s, 6H, C-14, C-17 CH_3), 2.04 (m, 8H, C=CCH_2), 4.29 (dt, 1H, J = 8.6, 6.6 Hz, CHOH), 5.09 (m, 2H, C-8, C-12 C=CH), 5.16 (d, 1H, J = 8.6 Hz, C-4 C=CH). Anal. Calcd for $C_{17}H_{30}O$: C, 81.54; H, 12.08. Found: C, 81.54; H, 12.27.

*(6E)-3,7,11-Trimethyl-6,10-dodecadien-1-ol (**6**).* Dihydrofarnesol was prepared by homogeneous hydrogenation of farnesol using bis(triphenylphosphine)rhodium(I). The catalyst was first prepared *in situ* by the addition of triphenylphosphine (47 mg, 4 equiv, recrystallized from 90% EtOH) to a solution of chloronorbornadiene rhodium dimer (20 mg, 10 mol %, Strem Chemical Co.) in dry THF (5 mL). After

Table 1. Substrate Specificity of Farnesol Dehydrogenase using CA Homogenates of Larval *M. sexta* (V/1)

Entry	Substrate		$K_{M(\mu M)}$	IC_{50} (μM)
1		**(1)**	10	
2				na*
3				na
4				>500
5				na
6				50
7				10
8		**(4)**		100
9		**(5)**		500
10				500
11		**(6)**		500
12		**(7)**		>500

* na indicates no activity at 500 μM.

stirring under argon for 1 h, the catalyst was saturated with H_2 at 1 atm of pressure for 0.5 h. A solution of farnesol in dry THF (5 mL) was added dropwise and the reaction mixture was stirred at rt overnight, under an H_2 atmosphere. The entire solution was then passed through a short silica column to remove the catalyst. Flash chromatography with 10% silver nitrate impregnated silica (0–5% EA/H gradient) of the concentrated eluent afforded pure dihydrofarnesol (36 mg, 36%): ^{1}H NMR ($CDCl_3$) δ 0.90 (d, 3H, J = 6.4 Hz, $CHCH_3$), 1.37 (m, 5H, C-2, C-4 CH_2, C-3 CH), 1.60 (s, 6H, C-13, C-14 CH_3), 1.68 (s, 3H, C-12 CH_3), 2.00 (m, 6H, C=CCH_2), 3.67 (dd, 2H, J = 5.8, 5.8 Hz, CH_2OH, 5.09 (m, 2H, C=CH).

(3E,7E)-4,8,12-Trimethyl-3,7,11-tridecatrien-1-ol (Homofarnesol, 7). Homofarnesol was synthesized in two steps, using a previously described method for the synthesis of homogeraniol (Leopold, 1986). To a suspension of methyltriphenylphosphonium bromide (243 mg, 0.68 mmol) in dry THF (5 mL) at −78 °C was added *n*-BuLi (1.6 M in hexane, 0.43 mL, 1 equiv). The solution was warmed to rt over 1 h to ensure complete ylide formation and then cooled to 0 °C, and farnesal (100 mg, 0.45 mmol) in THF (5 mL) was added dropwise. The reaction mixture was immediately warmed to rt, and stirring was continued for an additional 2 h. Brine was added, and the mixture was subjected to standard workup conditions. Flash chromatography using hexane afforded pure tetraene (86 mg, 87%): ^{1}H NMR ($CDCl_3$) δ 1.60 (s, 6H, C-14, C-15 CH_3), 1.68 (s, 3H, C-13 CH_3), 1.77 (s, 3H, C-16 CH_3), 2.05 (m, 8H, C=CCH_2), 4.98 (d, J = 10.8 Hz, 1H, *trans* CH=CH_2), 5.09 (unresolved ddd, J = 8.1, 1.1 Hz, 1H, *cis* CH=CH_2), 5.12 (m,

Table 2. Calculated Partition Coefficients for Several Long-Chain Alcohols Tested for Farnesol Dehydrogenase Activity

alcohol	calcd π	K_m (μM)	IC_{50} (μM)
octanol	2.84[a] (3.15)[b]		na[c]
geraniol	2.84		50
farnesol	4.24	10	
dodecanol	4.84 (5.13)		>500
geranylgeraniol	6.24		10

[a] Calculated values were determined using the additive method described in Leo et al. (1971). [b] Experimental value. [c] No activity at 500 μM.

2H, C-7, C-11 C=C*H*) 5.86 (d, J = 10.8 Hz, 1H, C*H*CH=CH_2), 6.58 (ddd, J = 16.9, 10.8, 10.8 Hz, 1H, C*H*=CH_2).

The resulting tetraene was immediately subjected to selective hydroboration using disiamylborane. A stock solution of the reagent was first prepared by adding 2-methyl-2-butene (2 M in THF, 3.3 mL) to a cooled (−30 °C) solution of diborane (1 M in THF, 3 mL). After stirring at 0 °C for 2 h, a portion of this solution (0.63 mL) was added dropwise to the tetraene (60 mg, 0.27 mmol, in 5 mL of dry THF) at 0 °C. The reaction was sluggish and required stirring for 2 days at 4 °C for complete consumption of starting material. At that time, the excess disiamylborane was destroyed by the addition of ethanol (6 μL), and the organoborane was oxidized by the sequential addition of 3 M sodium hydroxide (0.1 mL) and hydrogen peroxide (30%, 0.1 mL) at −30 °C. After warming to rt over 3 h, the mixture was extracted with ether, and the combined organic layers were washed with brine and dried ($MgSO_4$). Flash chromatography (2−5% EA/H gradient) afforded pure homofarnesol (37 mg, 55%): ^{1}H NMR ($CDCl_3$) δ 1.65 (s, 6H, C-14, C-15 CH_3), 1.68 (s, 6H, C-13, C-16 CH_3), 2.04 (m, 8H, C=CC*H*$_2$), 2.29 (dt, J = 6.4 Hz, 2H, C*H*$_2$$CH_2OH$), 3.62 (t, J = 6.4 Hz, 2H, C*H*$_2$OH), 5.11 (m, 3H, C=C*H*).

RESULTS AND DISCUSSION

Several alcohols were tested for their ability to bind to lepidopteran farnesol dehydrogenase. Results, expressed as IC_{50} values, are listed in Table 1. In contrast with the implications of previous studies (L'Hélias, 1979; Madhavan et al., 1973), we were surprised to find that straight-chain aliphatic alcohols do not inhibit the oxidative metabolism of farnesol. We have recently performed similar gel electrophoresis studies on lepidopteran farnesol dehydrogenase from *M. sexta* larvae and have found that a protein band of approximately 240 000 MW stains selectively for farnesol metabolism. Staining of this band with either ethanol or octanol as substrate does not occur (S. E. Sen, L. Flannigan, G. M. Garvin, unpublished results). Since both ethanol and octanol are excellent substrates for alcohol and octanol dehydrogenase, respectively, these results are in accord with the fact that farnesol oxidation within the corpora allata is mediated by an oxidative enzyme of relatively high substrate specificity.

Carbon Skeleton Requirements. Alcohols possessing modified terpene skeletons were also tested for their ability to inhibit lepidopteran farnesol dehydrogenase. As can be seen in Figure 1, substrate analog binding was found to require a minimum of three isoprenoid units for optimum activity. Thus, geraniol (entry 6, Table 1) showed a 5-fold reduction in activity, while geranylgeraniol (entry 7) was as effective as farnesol. Although this trend in substrate reactivity appears to be directly related to increasing hydrophobicity, an examination of the calculated octanol/water partition coefficients (π; Leo et al., 1971) of all long-chain alcohols tested shows poor correlation with observed inhibitory potency (Table 2). The observation that dodecanol binds poorly to farnesol dehydrogenase, despite its similar hydrophobicity to farnesol, would indicate that activity is largely dependent on the presence of the sesquiterpenoid carbon skeleton.

Alcohol Substitution. At present, only one insect alcohol dehydrogenase has been characterized. Unlike the corresponding enzyme found in yeast and vertebrate liver, *Drosophila* ADH is a short-chain dehydrogenase that is devoid of zinc and has a preference for secondary alcohols (Chambers, 1984; Winberg and McKinley-McKee, 1992). An examination of C-1 methyl and ethyl analogs of farnesol (**4** and **5**, entries 8 and 9, Table 1, respectively) shows that secondary analogs bind much more poorly to the lepidopteran dehydrogenase. Increased steric bulk, as seen in the ethyl analog, results in almost complete loss of activity. These results suggest structural differences between *Drosophila* ADH and lepidopteran farnesol dehydrogenase.

Allylic Alcohol Modifications. In view of the enzyme's high specificity for a terpenol skeleton of at least three isoprenoid units, a systematic examination of the allylic portion of the molecule was undertaken. As has been previously noted by others (Baker et al., 1983), modification of the *trans* allylic olefin geometry leads to decreased substrate turnover. Under our assay conditions, nerol (entry 10, Table 1) displayed 10-fold less activity than the corresponding 2*E* monoterpene, geraniol. Because this drop in activity could be due to misorientation of the hydroxyl moiety within the active site cavity, C-2,3 dihydrofarnesol (**6**, entry 11) was examined for potential substrate activity. This compound is also a poor inhibitor of farnesol dehydrogenase and is of comparable activity to nerol. Finally, homofarnesol (**7**, entry 12) was tested to determine the effect of adding an additional methylene unit between olefin and hydroxyl functionalities. Unlike the previous analogs, this molecule shows very little inhibition of farnesol metabolism, even at 500 μM concentrations.

On the basis of these results, molecular modeling studies were performed to examine the structural differences between farnesol and substrate analogs with modified allylic alcohol moieties. Superimposition of energy-minimized structures (MM2, using MacroModel version 4.0; Mohamadi et al., 1990) shows that substrate modification of the Δ-2,3 olefin can lead to either misorientation of the hydroxyl functicnality or significant movement of the C-3 alkyl substitutents (Figure 2). Thus, for nerol, proper orientation of the hydroxyl functionality (superimposition A) leads to incorrect positioning of the terpene chain (denoted R) and C-3 methyl substituent, while correct orientation of the alkyl substituents (superimposition B) causes significant perturbation from the normal hydroxyl group orientation. Since catalysis of all previously studied alcohol dehydrogenases requires hydroxyl activation prior to C-1 hydride elimination (Fersht, 1985), it is unlikely that this type of gross deviation in hydroxyl orientation would be tolerated within the enzyme's active site. For dihydrofarnesol, saturation of the Δ-2,3 olefin results in significant reorientation of both the C-3 alkyl substituents and C-2 hydrogen (superimposition C). Whether loss of activity of this racemic analog is due to steric or electronic changes of the terminal allylic moiety cannot yet be determined without further analog studies. However, it appears that lepidopteran farnesol dehydrogenase may rely on π−π interactions between Δ-2,3 olefin and active site residues for improved substrate binding, as well as hydrophobic interactions

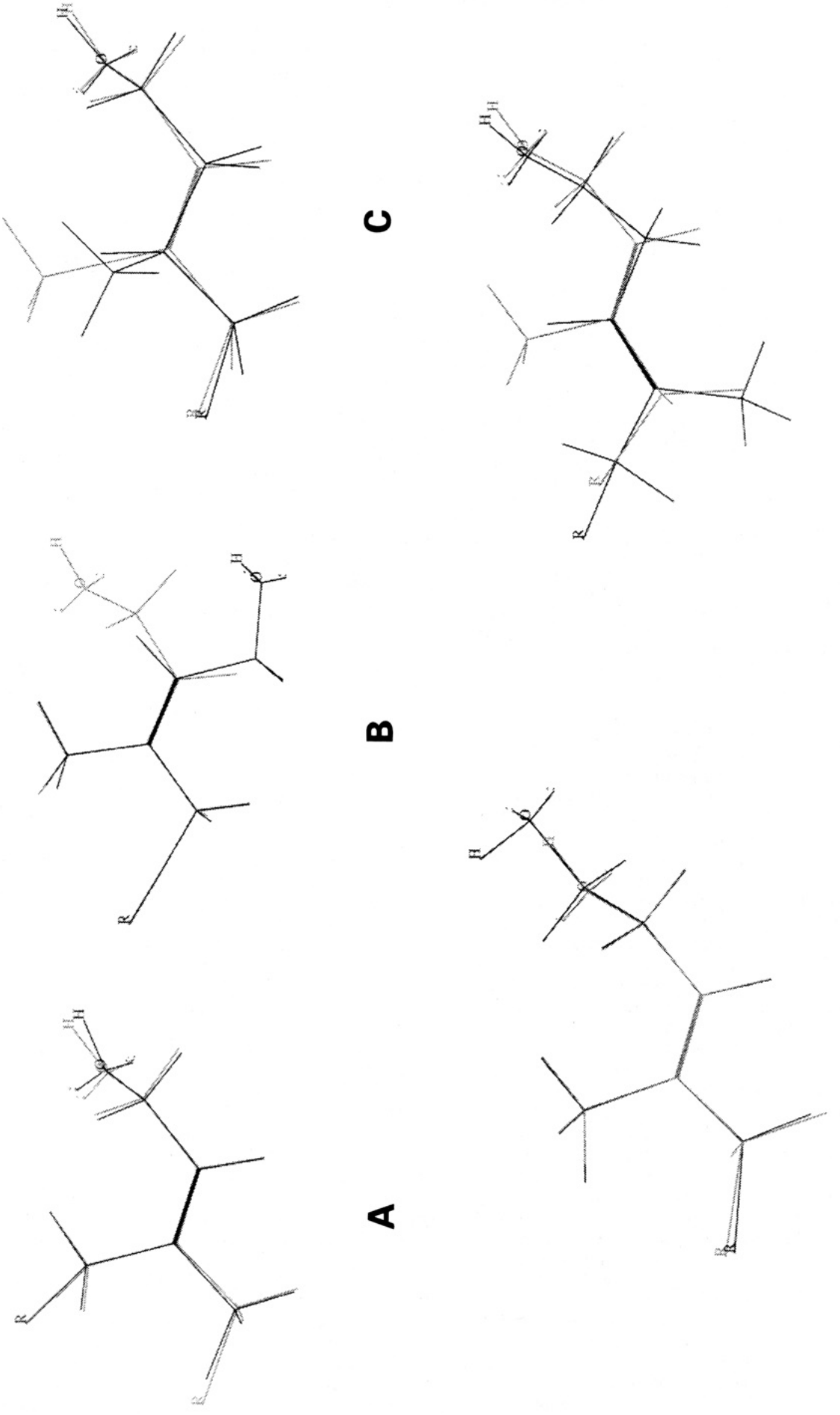


Figure 2. Superimposition of energy-minimized terminal allylic alcohol portions of farnesol (in gray), nerol, dihydrofarnesol, and homofarnesol.

which allow the enzyme to discriminate between C-3 alkyl modified skeletons. The importance of these results, in relation to JH homolog biosynthesis, has not yet been determined; however, intact cc–ca pairs from adult female *M. sexta* have been shown to oxidize (4*R*)- or (4*S*)-3,4,11-trimethyl-7-ethyl-2,6,10-tridecatrien-1-ol (Schooley and Baker, 1985). Finally, we attribute the lack of any appreciable activity of homofarnesol to the fact that the hydroxyl group and the C-4 substituents of this molecule cannot simultaneously adopt the same orientation as farnesol (superimpositions D and E, Figure 2).

CONCLUSIONS

The enzymatic conversion of farnesol to farnesal within insect corpora allata is an important step in juvenile hormone (JH) biosynthesis. Results from this study, using a substrate competition assay, indicate that lepidopteran farnesol dehydrogenase from *M. sexta* shows optimum activity with primary allylic alcohols, of at least three isoprene units. The observation that neither octanol nor secondary allylic farnesol analogs are good inhibitors of the dehydrogenase also suggests that it is not a simple octanol dehydrogenase, nor does it show any similarity with a previously characterized alcohol dehydrogenase from *Drosophila*. The disruption of normal endogenous juvenile hormone levels has been shown to be an effective method for affecting insect growth and development. Methods employed include the use of JH analogs and inhibitors of JH biosynthesis and degradation. Results from this study suggest that farnesol dehydrogenase is a highly specific enzyme that should be further examined as a new avenue for the development of JH biosynthesis inhibitors.

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