A cytochrome P450 terpenoid hydroxylase linked to the suppression of insect juvenile hormone synthesis
(corpora allata/CYP4C7/P450 reconstitution/sesquiterpenoid/NMR)


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ABSTRACT A cDNA encoding a cytochrome P450 enzyme was isolated from a cDNA library of the corpora allata (CA) from reproductively active Diploptera punctata cockroaches. This P450 from the endocrine glands that produce the insect juvenile hormone (JH) is most closely related to P450 proteins of family 4 and was named CYP4C7. The CYP4C7 gene is expressed selectively in the CA; its message could not be detected in the fat body, corpora cardiaca, or brain, but trace levels of expression were found in the midgut and ceca. The levels of CYP4C7 mRNA in the CA, measured by ribonuclease protection assays, were linked to the activity cycle of the glands. In adult females, CYP4C7 expression increased immediately after the peak of JH synthesis, reaching a maximum on day 7, just before oviposition. mRNA levels then declined after oviposition and during pregnancy. The CYP4C7 protein was produced in Escherichia coli as a C-terminal His-tagged recombinant protein. In a reconstituted system with insect NADPH cytochrome P450 reductase, cytochrome b₅₆, and NADPH, the purified CYP4C7 metabolized (2E,6E)-farnesol to a more polar product that was identified by GC-MS and by NMR as (10E)-12-hydroxyfarnesol. CYP4C7 converted JH III to 12-trans-hydroxy JH III and metabolized other JH-like sesquiterpenoids as well. This cis-hydroxylation of sesquiterpenoids appears to be a metabolic pathway in the corpora allata that may play a role in the suppression of JH biosynthesis at the end of the gonotrophic cycle.

Juvenile hormone (JH) plays a central role in insect development, metamorphosis, and reproduction. This sesquiterpenoid epoxide is synthesized in endocrine glands, the corpora allata (CA)(1), and is degraded predominantly by esterases and epoxide hydro- lases (2). The rate of JH synthesis by the CA is a major determinant of the titer of JH in the hemolymph (3), and the regulation of JH synthesis is seen as a potential target for insect control. The biosynthesis of JH has been extensively characterized during the reproductive cycle of the cockroach Diploptera punctata, an insect that serves as a convenient model system. In adult females of this insect, the cycle of JH synthesis is regulated by humoral factors and by innervation from neurosecretory cells in the brain (1). Production of JH by the CA increases 10-fold to levels then declined after oviposition and during pregnancy. The CYP4C7 protein was produced in Escherichia coli as a C-terminal His-tagged recombinant protein. In a reconstituted system with insect NADPH cytochrome P450 reductase, cytochrome b₅₆, and NADPH, the purified CYP4C7 metabolized (2E,6E)-farnesol to a more polar product that was identified by GC-MS and by NMR as (10E)-12-hydroxyfarnesol. CYP4C7 converted JH III to 12-trans-hydroxy JH III and metabolized other JH-like sesquiterpenoids as well. This cis-hydroxylation of sesquiterpenoids appears to be a metabolic pathway in the corpora allata that may play a role in the suppression of JH biosynthesis at the end of the gonotrophic cycle.

MATERIALS AND METHODS

Chemicals and Insects. The cis- and trans-12-hydroxy JH III standards were kindly supplied by F. Couillaud (Centre National de la Recherche Scientifique) and G. D. Prestwich (University of Utah), and other terpenoids were as described in Andersen et al. (22). D. punctata were reared as described (23).

Abbreviations: BSTFA, bis[trimethyl-silyl]trifluoracetamide; CA, corpora allata; JH, juvenile hormone; RT, reverse transcription; THF, tetrahydrofuran.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. AF071072 (CYP4C7), AF071073 (CYP4C4), AF071074 (CYP4C5), AF071075 (CYP4C6)].

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Isolation and Cloning of CYP4C7. The degenerate PCR primer, GA(C/T) AC(T/T) ATG TT(C/T) GA(A/G) GG(A/G/C/T) CA(C/T) GA(C/T) AC and the reverse degenerate primer GC(A/G) AT(C/T) TT(C/T) TG(A/G/C/T) CC(A/G/C/T) AT(A/G) CA(A/G) TT were used to amplify a 452-bp DNA fragment from reverse-transcribed poly(A) RNA isolated from 25 pairs of CA from 5-day-old mated females. This amplon was cloned into the pCR II vector (Invitrogen) and used to isolate an almost-full-length clone from a CA CDNA library. The library was constructed from 240 pairs of CA from 5-day-old mated females by using the Zap-cDNA synthesis kit (Stratagene).

RNase Protection Assays. A riboprobe template for CYP4C7 was prepared by digesting the pBluescript plasmid with Smal. Antisense 32P-labeled RNA fragments were synthesized by runoff transcription from the T7 promoter by using the MAXscript in vitro transcription kit (Ambion), which generated a probe of 175 bp and a protected fragment of 145 bp corresponding to the 3' region of the CYP4C7 message. RNA protection assays were performed by using the Direct Protect lysate ribonuclease protection assay kit from Ambion. The CA were collected in 50 μl of lysis buffer and stored at −80°C until they were assayed. The products of the assay, protected RNA–RNA hybrids, were separated on a denaturing 6% acrylamide gel, and either visualized by autoradiography with the amount of signal quantified by an LKB densitometer or directly quantified by using a PhosphorImager (Molecular Dynamics).

Construction of Expression Vector. The coding region of CYP4C7 was cloned into the expression plasmid pSE380 following modification by PCR mutagenesis. The AT content of the 5' region was increased with PCR mutagenesis by using the primer C ATG GCT GTT GTA TTA TTA CTT ACT TCT CTT GCT ATA GTC. Further PCR mutagenesis with the primer GGG GTA C ATG GCT GTT TTA TTA CTT ACT TCT CTT GCT ATA GTC was performed modification by PCR mutagenesis. The AT content of the 5' region to allow enzyme purification by nickel-chelate chromatography.

Purification of CYP4C7 from Escherichia coli. Flasks containing 500 ml of Terrific Broth (with ampicillin at 50 μg/ml) were inoculated with 500 ml of an overnight culture of E. coli and grown in 1-l of this medium to an absorbance of 0.2 mM concentration immediately before each sonication. The membrane fraction was pelleted by centrifugation at 100,000 × g for 30 min and resuspended in 50 ml of Tris-HCl buffer (0.1 M, pH 7.7) and pelleted a second time. The cell pellet was resuspended in 40 ml of Tris-HCl buffer (0.1 M, pH 7.7) and 10% glycerol, then sonicated. Phenylmethylsulfonyl fluoride (PMSF) was added to achieve a 0.2 mM concentration immediately before each sonication. The membrane fraction was pelleted by centrifugation at 100,000 × g for 30 min and resuspended in 50 ml of Tris-HCl buffer (0.1 M, pH 7.7) and 20% glycerol. CYP4C7 was solubilized from the membranes by adding Emulgen 911 to achieve a 1% concentration, and the product was stored at 4°C for 4 hr. Solubilized proteins were recovered in the supernatant fraction after a further centrifugation at 100,000 × g for 30 min. The supernatant was loaded onto a nickel affinity column equilibrated with Tris-HCl buffer (0.1 M, pH 7.7), 20% glycerol, 0.5 M NaCl, and 5 mM imidazole (equilibration buffer). The column was washed with 50 ml of equilibration buffer with Emulgen 911 to 0.1%, then with equilibration buffer with 0.5% sodium cholate. CYP4C7 was eluted with equilibration buffer with 200 mM imidazole. The P450-containing fractions were pooled and diazomethane when appropriate, buffer exchanged into Tris HCl buffer (0.1 M, pH 7.7), and 5 mM imidazole (equilibration buffer). The column was washed with 50 ml of equilibration buffer with Emulgen 911 to 0.1%, then with equilibration buffer with 0.5% sodium cholate. CYP4C7 was eluted with equilibration buffer with 200 mM imidazole. The P450-containing fractions were pooled and dialyzed against three changes of Tris-HCl buffer (100 mM, pH 7.7), 20% glycerol, and 0.1 mM EDTA.

Spectral Studies. Cytochrome P450 was quantified by measurement of the dithionite-reduced vs. reduced-CO bound-difference spectrum by the method of Omura and Sato (24). Type I difference spectra of ligands (added in ethanol) were obtained from an 800-μl suspension of the membrane fraction of (E. coli + plasmid) in 100 mM Mops containing 0.825 μM cytochrome P450.

Enzyme Assay. Cytochrome P450 enzyme assays were performed in 0.1 M Tris-HCl (pH 7.7) containing 20 μM NADPH, an NADPH-regenerating system (1 mM glucose-6-phosphate, 2 units/ml of glucose-6-phosphate dehydrogenase), 0.2 μM CYP4C7, 0.5 μM recombinant housefly NADPH-dependent cytochrome P450 reductase (ref. 25, and M.B.M., A. Arinó, V. M. Guzman, and R.F., unpublished results), 1 μM cytochrome b5 (26), and 100 μM substrate. The enzymes were reconstituted by adding, in the following order, 0.1 mg/ml dilauroylphosphatidylcholine, 0.015% CHAPS (3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate), CYP4C7, cytochrome b5, and house fly P450 reductase. Buffer was then added to increase the volume to 40 μl, and the mix was incubated on ice for 10 min. Reactions were started by adding 1 vol of protein mix to 9 vol of substrate solution and proceeded for 15–20 min at 30°C. Products were extracted with 2 ml of peroxide-free ethyl ether, methylated with diazomethane when appropriate, buffer exchanged into THF-d8, resuspended in hexane, and analyzed by GC with a flame ionization detector.

NMR Spectroscopy of the Farnesol Metabolite. A large-scale preparation of the principal farnesol metabolite was partially purified by reversed-phase HPLC, and the oil (1–2 mg) was dissolved in tetrahydrofuran (THF-d8) for preliminary evaluation by 500-MHz 1H NMR spectroscopy. The oil consisted of a major component (~60%) and one or more minor components (~40%) that did not appear to be farnesol-derived. Following removal of the THF, the oil was triturated with benzene-d8, and undissolved material was removed by filtration. NMR analysis confirmed that the minor components had been substantially eliminated. As THF-d8 appeared to be a superior solvent for NMR analyses, the benzene was removed, and the oil was redissolved in THF-d6. The spectra were obtained in THF-d6 solutions on a Bruker (Billerica, MA) WM 500 spectrometer. Chemical shifts are reported in δ. The reference signal for the proton spectra (1.73 ppm) was caused by residual protons at C5 and C6 in the THF-d6. The reference signal for the carbon spectra (25.37 ppm) was the result of the signal from C5 and C6 in the THF-d6. 13C NMR 8.5.36 (1, t, H at C9), 5.35 (1, t, H at C2), 5.17 (1, t, H at C4), 4.02 (2, dd, H at C1), 3.84 (2, d, H at C12), 3.52 (1, t, OH at C12), 3.30 (1, t, OH at C2), 2.14 (4, dt, H at C5 and C9), 2.01 (4, t, H at C4 and C6), 1.63 (3, s, CH3), 1.62 (3, s, CH3), 1.61 (3, s, CH3), 1.35 (13, CH3). Microchemical Derivatizations of the JH III Metabolite. Derivatizations were a modification of the procedures of Bergot et al. (27, 28). Fifty microilter of a solution of 1 μl of 60% perchloric acid in 1 ml of methyl-d3 alcohol-d (CD3OD) (99.5 atom %) was added to ~1 μg of CYP4C7 product. After 30 min at room temperature, 0.5 ml of a 2% NaCl solution was added, and the mixture was extracted three times with 100 μl of ethyl acetate. The combined organs were evaporated under N2 until dry. The product was dissolved in 10 μl of ethyl acetate, and 2 μl of this solution was injected into the GC-MS. For trimethylsilylation, 50 μl of bis (trimethylsilyl) trifluoroacetamide (BSTFA) was added to ~1 μg of CYP4C7 product and incubated at 55°C for 1 hr. The reaction mixture was evaporated to dryness under N2. Fifty microliters of ethyl acetate was added and evaporated. The product was dissolved in 20 μl of ethyl acetate, and 2 μl of this solution was injected into the GC-MS.

GC-MS. Metabolites were analyzed with a Hewlett-Packard 5890 GC coupled to an HP 5970 mass selective detector at 70-eV ionization, with total ion detection from 30 to 350 atomic mass units. The GC column was a J & W Scientific (Folsom, CA) DB-5ms, (5%-phenyl)-methylpolysiloxane column, 25 m × 0.32 mm i.d. × 0.52-μm film. The temperature profile was 100°C for 2 min, then 10°C increase per min to 250°C and held for 3 min.
The injection temperature was 250°C and the detector was set at 280°C, with helium carrier gas at a linear velocity of 28.7 cm/sec.

**RESULTS**

Isolation, Cloning, and Sequencing of the CYP4C7 Gene. A reverse transcription–PCR (RT-PCR) approach (29) for amplifying and cloning P450 fragments was used on mRNA from the CA of reproducibly active female *D. punctata* cockroaches. This approach used degenerate primers for the consensus sequence of the helix I and of the heme-binding regions of previously isolated CYP4 P450s. It gave a 452-bp product that when cloned and sequenced revealed high sequence similarity to known P450 proteins. A specific forward primer for this P450 fragment (GCA TCA GGA GTG AGC TG), when used with the degenerate reverse primer, generated an RT-PCR product from CA mRNA but not from abdominal-fat-body mRNA. In positive-control experiments, the combination of degenerate primers produced a signal from abdominal-fat-body mRNA. When cloned and sequenced, the P450 fragment was confirmed to be a CYP4 P450, it gave a 452-bp product that when cloned and sequenced revealed high sequence similarity to known P450 proteins (31). In addition to the highly conserved sequence surrounding the cysteine ligand to the heme (PFSAGPRN-IGQKFA), there is also a highly conserved sequence (IXEEVDTFMLXGHD) that is similar to the conserved helix I and of the heme-binding regions of previously isolated CYP4 proteins (31). In addition to the conserved sequence surrounding the cysteine ligand to the heme (PFSAGPRN-IGQKFA), there is also a highly conserved sequence (IXEEVDTFMLXGHD) in the putative I helix, preceding the threonine residue at the proposed oxygen-binding pocket.

CYP4C7 Expression Is Linked to the Activity Cycle of the CA. We tested whether this P450 was linked to the activity cycle of the CA in adult females (low JH synthesis on day 0, increasing to a peak on day 5 after adult emergence, followed by a rapid decline, then low activity again during pregnancy). Thus we followed the expression of the CYP4C7 gene by ribonuclease protection assays on RNA from the CA, to ensure absolute specificity as well as high sensitivity. The CYP4C7 message is predominantly expressed when the activity of the CA is shut off, starting on day 6 after the peak of JH synthesis and peaking on day 7–8, just before oviposition. The message then gradually declines after oviposition and during pregnancy (Fig. 2). Significant levels of message are found shortly after adult emergence, but the lowest levels are found during vitellogenesis, with undetectable levels of CYP4C7 on day 5. Males and virgin females had 10% and 13%, respectively, of the level of message observed in 7-day-old mated females.

The RNAase protection method confirmed the initial RT-PCR result that CYP4C7 mRNA was not detectable in the fat body. This technique showed no detectable expression in the head or abdominal fat body of 1-, 5-, or 9-day-old insects or the corpora cardiaca or brain of 5-day-old insects. There was also no detectable expression in the postvitellogenic ovary, the thoracic gland from late final-instar larvae, or the tissue remnants of that gland in newly emerged females. Trace levels of expression were observed in the midgut of newly emerged, 4-day-old and 8-day-old mated females and in the gastric caeca from 4-day-old insects. Relative to the fresh weight of the tissues, the mRNA CYP4C7 in the caeca and midgut were 0.20% and 0.10–0.26%, respectively, of the level in a single CA from 7-day-old females. Thus, expression of CYP4C7 was 500-fold higher in the CA than in the midgut or gastric caeca.

**Heterologous Expression of the CYP4C7 Protein.** The cell lysate of *E. coli* cells transformed with the CYP4C7 expression vector contained cytochrome P450 as detected by the characteristic reduced CO/reduced difference spectrum with a peak at 448 nm. The expression construct encoded a 413-His tag that allowed purification of the enzyme on a nickel affinity column. The eluate from the affinity column contained a major protein band of 50–60 kDa after analysis on SDS/PAGE (not shown), with spectra of oxidized, reduced, and CO-bound reduced purified protein typical of P450 proteins (not shown). The level of cytochrome P450 expression was about 140 nmol/liter of culture.

Membrane-bound CYP4C7 gave type I binding spectrum with (2E,6e)-methyl farnesolate and with JH III (spectral binding constant Ks of 0.9 and 2.4 μM, respectively), whereas the affinity for lauric acid, which is not a substrate for CYP4C7 (see below) was lower (74 μM). The purified recombinant CYP4C7 was then reconstituted with the purified recombinant housefly P450 reductase.
ion of farnesol. It was converted by treatment with BSTFA to the spectra of the metabolite, the C12 chemical shift is 68.2 ppm and the C11-methyl chemical shift is 21.3 ppm, respectively. For the 12-hydroxyfarnesol metabolite, the chemical shifts and vicinal C–H spin-coupling constants. In the model compound Z-2-methyl-2-buten-1-ol, the C1-carbon chemical shift is 60.5 ppm and the C2-methyl chemical shift is 21.3 ppm, whereas for E-2-methyl-2-buten-1-ol these values are 68.3 ppm and 13.3 ppm, respectively. For the 12-hydroxyfarnesol metabolite, the C12 chemical shift is 68.2 ppm and the C11-methyl chemical shift is 13.4 ppm, consistent with 10E stereochemistry. Further support for this assignment comes from the similarity of the NMR spectra of the metabolite and 10E)-12-hydroxy-JH III, the C12 chemical shift is 60.5 ppm and the C2-methyl chemical shift is 21.3 ppm, respectively. For the 12-hydroxyfarnesol metabolite, the C12 chemical shift is 68.2 ppm and the C11-methyl chemical shift is 13.4 ppm, consistent with 10E stereochemistry. This functional enzyme system was used to study the metabolism of potential substrates.

**Metabolism of Farnesol by CYP4C7 and Identification of (10E)-12-Hydroxyfarnesol.** (2E,6E)-Farnesol, a precursor of JH III, was metabolized to two more polar compounds. When analyzed by GC-MS, the principal metabolite had diminished abundance of the m/z 69 ion and increased abundance of the m/z 31 ion of farnesol. It was converted by treatment with BSTFA to a compound bearing two trimethylsilyl groups, suggesting the presence of an additional hydroxyl group. The observed 1H NMR spectrum of the principal metabolite was consistent with that of a hydroxylated farnesol. The appearance of three methyl singlets (1.61, 1.62, and 1.63 ppm) and a doublet (coupled to a hydroxyl proton) at 3.84 ppm suggested that hydroxylation had occurred on C10 of the JH III deuteroxyhydrin derivative. The CYP4C7 product had a retention time of 20.02 min, a strong m/z 73(100%) ion indicative of a single trimethylsilyl product, and other major ions at m/z 93(70%) and 55(53%). BSTFA did not react under our conditions to silylate the hydroxyl on C10 of the JH III deuteroxyhydrin derivative. The trimethylsilylation of the deuteroxyhydrin derivative gave a product with a retention time of 22.51 min and gave major ions at m/z 92(100%), 75(63%), and 193(48%). The trimethylsilyl derivative of the CYP4C7 product had a retention time of 19.02 min and major ions at m/z 55(100%), 93(92%), and 81(90%). Its deuteroxyhydrin derivative had a retention time of 22.53 min and gave major ions at m/z 92(100%), 75(63%), and 193(48%). These results (Fig. 4) imply that the major JH III metabolite is a 12- or 12’-hydroxy derivative of JH III. To determine which geometric isomer of 12-hydroxy JH spin-coupling constants. For Z-2-methyl-2-buten-1-ol, the vicinal C–H spin-coupling constant $J_{C1-H3} = 9.3$ Hz, whereas for E-2-methyl-2-buten-1-ol $J_{C1-H3} = 7.6$ Hz. For the 12-hydroxyfarnesol metabolite the vicinal C–H spin-coupling constant $J_{C2-H10} = 7.4$ Hz. These data all concur to identify the principal farnesol metabolite of CYP4C7 as (10E)-12-hydroxyfarnesol.

**Metabolism of JH III by CYP4C7 and Identification of (10E)-12-Hydroxy-JH III.** JH III was metabolized to two more polar products; the major one was identified by a combination of microderivatization and GC-MS analysis (Fig. 4), which indicated the presence of a terminal hydroxyl group as for the farnesol metabolite. JH III has a complex electron-impact fragmentation pattern (33), and under the conditions we used had a retention time of 16.3 min. Major fragment ions were at m/z 43(100%), 81(84%), and 41(70%). Under the same chromatographic conditions major JH III metabolite had a retention time of 19.02 min and major ions at m/z 55(100%), 93(92%), and 81(90%). Its deuteroxyhydrin derivative had a retention time of 22.53 min and gave major ions at m/z 92(100%), 75(63%), and 193(48%). The trimethylsilyl derivative of the CYP4C7 product had a retention time of 20.02 min, a strong m/z 73(100%) ion indicative of a single trimethylsilyl product, and other major ions at m/z 93(70%) and 55(53%). BSTFA did not react under our conditions to silylate the hydroxyl on C10 of the JH III deuteroxyhydrin derivative. The trimethylsilylation of the deuteroxyhydrin derivative gave a product with a retention time of 22.51 min with major ions at m/z 164(100%), 73(71%), and 75(57%). These results (Fig. 4) imply that the major JH III metabolite is a 12- or 12’-hydroxy derivative of JH III. To determine which geometric isomer of 12-hydroxy JH

![Fig. 3. Structure of the 12-hydroxyfarnesol produced from farnesol by CYP4C7.](image)

![Fig. 4. Structure determination of the (10E)-12-hydroxy-JH III, showing the mass fragmentation scheme of JH III, its enzymatic product and derivatives, and abundances of characteristic ions. TMS, trimethylsilyl](image)
Table 1. Substrate selectivity of reconstituted CYP4C7

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Major metabolite</th>
<th>Minor metabolite</th>
</tr>
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<tbody>
<tr>
<td>(2E,6E)-Farnesol</td>
<td>4.12 ± 0.50</td>
<td>0.92 ± 0.12</td>
</tr>
<tr>
<td>(2E,6E)-Farnesal</td>
<td>2.37 ± 0.90*</td>
<td>ND</td>
</tr>
<tr>
<td>(2E,6E)-Farnesoic acid</td>
<td>2.62 ± 0.20</td>
<td>ND</td>
</tr>
<tr>
<td>(2E,6E)-Methyl farnesoate</td>
<td>2.26 ± 0.17</td>
<td>0.71 ± 0.01</td>
</tr>
<tr>
<td>10,11-Epoxy-(2E,6E)-farnesoic acid</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>JH III</td>
<td>0.83 ± 0.27</td>
<td>0.47 ± 0.04</td>
</tr>
<tr>
<td>JH II</td>
<td>0.87 ± 0.17</td>
<td>0.19 ± 0.07</td>
</tr>
<tr>
<td>JH I</td>
<td>0.41 ± 0.03</td>
<td>0.17 ± 0.02</td>
</tr>
<tr>
<td>2,6,10-Trimethylcyclohexanol</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Geraniol</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Geranyl geraniol</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Farnesyl methyl ether</td>
<td>2.10 ± 0.30</td>
<td>0.65 ± 0.10</td>
</tr>
<tr>
<td>Geranyl methyl ether</td>
<td>0.17 ± 0.02</td>
<td>0.16 ± 0.04</td>
</tr>
</tbody>
</table>

Purified CYP4C7 were reconstituted with house fly P450 reductase and cytochrome P450 and an NADPH-regenerating system. Nominal substrate concentration was 100 μM. Values are means ± SD of triplicate assays. ND, no product detected.

*Substrate disappearance, product not identified.

III was produced, authentic (10Z)-and (10E)-12-hydroxy JH III were separated by GC and compared with the major product of CYP4C7 metabolism. The biosynthesized 12-hydroxy JH III had the same retention time as the synthetic (10E)-12-hydroxy JH III standard. The minor metabolite of JH III did not correspond to the 10Z isomer, and its structure has not been elucidated.

**Metabolism of Sesquiterpenoids by CYP4C7.** Table 1 shows that CYP4C7 is active toward sesquiterpenoid compounds. The selectivity toward JH III and its precursors is marked. The enzyme metabolizes unmodified compounds, but rather is involved in metabolizing JH precursors (starting with the opposite end of the molecule (alcohol, aldehyde, carboxylic acid, methyl ester, or methyl ether) appears of less importance. Monoterpenes and diterpenes are not metabolized, nor is the saturated analog of farnesol (2,6,10-trimethylcyclohexadecanol). This selectivity suggests that the enzyme is not involved in biosynthesis, but rather is involved in metabolizing JH precursors (starting with farnesol) to more polar compounds, presumably to facilitate their further catabolism and disposition. CYP4C7 had no activity toward either fatty acids (laurate or palmitate), which are typical substrates of P450s of the CYP4 family in mammals, or cyclodiene insecticides (aldrin or heptachlor).

**Effect of Topically Applied JH III at the End of the Gonotrophic Cycle.** The effect of JH III at the end of the cycle was studied by topical application on day 6 or by repeated applications on days 6, 7, and 8. The high doses and repeated applications were necessary in light of the notoriously rapid in vivo degradation of the natural JH III. The results (Table 2) show that JH had two deleterious effects: inhibition of ovoiposition and abortion of oviposited eggs. There was no resorption of oocytes in nonovulated females, as the oocytes were already chorionated at the time of JH treatment; in these insects, the mature eggs shrunk gradually, releasing the contents and leaving the chorion remains as pigmented spherical bodies. There was no vitellogenesis in the penultimate oocytes in nonovulated and aborted females until 5 wk after JH treatment (at which time the experiment was terminated). These results strongly suggest that JH titers need to decrease rapidly when vitellogenesis and yolk uptake by the oocytes is complete for successful reproduction to occur.

**DISCUSSION**

**Cloning and Characterization of CYP4C7.** Cloning of cytochrome P450 genes by the RT-PCR method has been highly successful in documenting P450 diversity in plants and insects (29). Here it has allowed cloning of a P450 gene that is selectively expressed in the CA. These small endocrine glands are not easily studied by classical biochemical techniques, and reliance on radioenzymological assays has been absolute (34). Heterologous expression of a CA enzyme thus provides a new tool in the study of insect endocrine biochemistry. CYP4C7 contains a region of high sequence similarity to other members of the CYP4 family (Fig. 1), and several CYP4 enzymes of vertebrates have been implicated in ω-hydroxylation of fatty acids as well as leukotriene hydroxylation (30). CYP4C1 of another cockroach, *Blaberus discoidalis*, is a P450 expressed in the fat body that is thought to be involved in lipid mobilization under the control of the hyper-trehalosemic hormone (35). Because high CYP4C7 mRNA levels are found in the CA at the end of vitellogenesis, a time of rapid cell shrinking (but not cell death) in the glands, it was possible that the enzyme was involved in recycling membrane lipids (fatty acids) within the CA. However, we discounted this possibility for several reasons. First, the purified, reconstituted enzyme does not metabolize the fatty acids laurate or palmitate. Second, CYP4C7 is expressed selectively in the CA, with only very limited expression in the midgut and no detectable expression in the postvitellogenic ovary or late-larval prothoracic glands, two tissues where massive cellular degradation is also taking place. Third, CYP4C7 mRNA levels increase in the CA of ovarioctomized insects, yet these glands do not shrink in size and are competent to produce high levels of JH (T.D.S., G.C.U., and R.F., unpublished results). Thus the function of CYP4C7 in the CA is unlikely to be that of a housekeeping enzyme, but is more likely to be directly involved in the JH biosynthetic pathway, as reflected by its substrate selectivity toward sesquiterpenoids.

**Function of the Enzyme.** There is evidence in other invertebrate species that JH-related compounds may act as hormones or prohormones, for instance the production of farnesoyl and methyl farnesolate in crustaceans (36). In insects, the higher homologs of JH III and their respective JH acids are produced by the CA of Lepidoptera (34); other JH-related molecules have been reported as products of the CA as well. These include JH III diol in locusts (37), a very minor product of the CA in this species (38), JH III bisopoxo in higher Diptera (39, 40), and what appears to be a JH acid glucuronide in *Manduca sexta* (41). The JH III bisopoxo is believed to be an authentic JH of higher Diptera, but the role of the other products is less clear. Of direct interest to our present study, a (10Z)-12-hydroxy metabolite of JH III (12'-OH JH III) has recently been identified in *in vitro* incubations of the CA in locusts (42), and several other metabolites have been reported as well, including 8'-OH JH III (38, 42). In the locust, synthesis of the hydroxy-JH metabolites is not increased by farnesol or farnesoyl acid, whereas synthesis of JH is stimulated by these exogenous precursors (38). This suggests either a different level of saturation of the hydroxylase(s) or a physical compartmentation of the pathways of JH III synthesis from endogenous and exogenous substrates. Interestingly, locust CA produced the cis-hydroxy JH III, whereas the cockroach P450 enzyme makes the trans-hydroxy JH III from JH III and trans-hydroxyfarnesol from farnesol. As yet, we do not know whether CYP4C7 is acting primarily to catabolize JH-related compounds.
or whether any of the metabolites are capable of functioning as hormones or prohormones. The 12'-OH JH III produced by locust CA has been reported to have a very slight activity in the enzyme producing 12-ester functionalities that are essential for biological activity. CYP4C7. This highlights the need for a rapid decrease in JH titer once eggs syndrome, inhibition of ovulation and abortion of early embryos. excess at the end of the cycle causes a distinct pathological ctomized insects. We show that in this viviparous cockroach, JH ment therapy, showing that JH can restore egg growth in allate- during the developmental stages, when an intrinsic repression of is involved in the catabolism of sesquiterpenoids within the CA gene expression and its catalytic activity suggests that this enzyme correlated to JH synthesis from the gland. The timing of CYP4C7 to determine which metabolite(s) is the preferred substrate of noids are produced by the CA at the end of the gonotrophic cycle cation of JH III (Table 2).

We still need to identify which if any 12-hydroxy sesquiterpe- noids are produced by the CA at the end of the gonotrophic cycle to determine which metabolite(s) is the preferred substrate of CYP4C7 in vivo. Drosophila Kc cells (which lack squalene syn- thase) as well as rats (treated with farnesol or with a squalene synthase inhibitor) convert farnesol to dicarboxylic acids (45, 46) in a pathway that is initiated by α-hydroxylation of farnesol. By analogy to these known catabolic pathways, it is possible that CYP4C7 is only the first enzyme in the catabolism of allatol sesquiterpenoids. The CYP4C7 gene can now be used as a molecular probe to study the early postvitellogenic events in D. punctata, when signals from the ovary and the brain converge on the CA to shut down JH synthesis.

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