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Determination of the Regiochemistry of Insect Epoxide Hydrolase Catalyzed Epoxide Hydration of Juvenile Hormone by ^{18}O-labeling Studies

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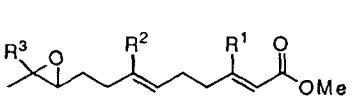
Abstract: The regiochemistry of *Trichoplusia ni* epoxide hydrolase catalyzed epoxide hydration of insect juvenile hormone (JH) III has been determined by GC/MS studies of ^{18}O-label incorporation. Nucleophilic addition occurs at C10 of the C10,11 epoxide of JH III. The identification of isotopically labeled and unlabeled diol from the enzyme catalyzed hydration reaction in $H_2{}^{18}O$ implies that a covalently bound ester intermediate may be involved in the mechanism of the reaction.

New methods for the control of destructive agricultural pests, such as lepidopterous insects, requires the development of highly selective pest management systems as a means to reduce toxicity toward non-target species as well as minimize potential environmental contamination. Insect juvenile hormone (JH), Figure 1, is a regulatory hormone that is unique to arthropods. The regulation of hemolymph JH titer is implicated as a controlling factor in several stages of larval and adult insect development and reproduction. [1-5] Since there is no complement to JH in mammalian systems, a more complete assessment of the mechanisms controlling insect hormonal balance would serve to aid in the design of new methods for selective insect control.



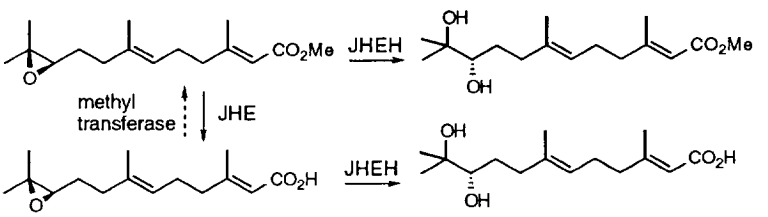
JH 0	R^1, R^2, R^3 = Et
JH I	R^1 = Me; R^2, R^3 = Et
JH II	R^1, R^2 = Me; R^3 = Et
JH III	R^1, R^2, R^3 = Me

Figure 1: Structures of selected juvenile hormones.

JHs are biosynthesized by the corpora allata and other organs and secreted into the hemolymph of the insect. [6] Hemolymph JH binding proteins, both extracellular and intracellular, are thought to protect JH from non-specific degradation and adsorption, while aiding in the transportation of the hormone to various tissues. [7] JH titer in the hemolymph and tissues is regulated by two metabolic degradation enzymes, juvenile hormone esterase (JHE) and juvenile hormone epoxide hydrolase

(JHEH), [3-5] Scheme 1. JH metabolism by either enzyme results in the loss of biological activity. Previous work has focused on JHE metabolic activity: however, the growing importance of JHEH metabolic degradation of JH is noted by several recent publications. Schooley and co-workers reported that in *M. sexta*, a phosphate conjugate of JH III ester-diol was the major product of JH metabolism *in vivo*. [8] The JH III acid-diol conjugate would be the expected product had JHE been the primary metabolic pathway. Unlike JHE, which exhibits highest catalytic activity in the soluble (cytosolic) form, it is not completely clear if JHEH activity is primarily microsomal or cytosolic in nature. The results of earlier studies indicated that JHEH activity was primarily membrane bound. [9,10] More

Scheme 1



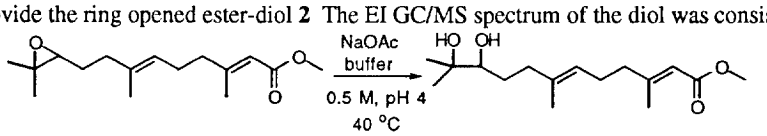
recently, Roe and co-workers reported significant levels of JHEH activity in both cytosolic and microsomal fractions of *M. sexta* tissues using JH III as substrate. [11] Touhara and Prestwich purified insect EH from *M. sexta* eggs and determined the molecular weight of the purified protein to be 50 kDa. [12] In addition, Touhara and Prestwich observed that purified *M. sexta* egg EH metabolized JH four times faster than the corresponding conversion of JH acid to JH acid-diol.

The mechanism of mammalian EH catalyzed epoxide hydration has been extensively studied by several groups. [13-17] These investigations have established that, in general, EH catalyzes the *trans*-addition of water to epoxides and arene oxides with inversion of configuration; however, the actual mechanism of EH catalyzed epoxide hydration was not fully elucidated. One proposed mechanism suggested that a carboxylate residue, possibly an aspartic acid, present in the active site undergoes nucleophilic addition to the epoxide substrate to form an intermediate ester linkage. [17] Subsequent general-base catalyzed addition of water to the ester results in the formation of diol. By this mechanism, oxygen is incorporated into the diol via the carboxylate residue in the first enzymatic turnover. Subsequent enzymatic turnovers would then incorporate oxygen derived from the solvent into the product. Lacourciere and Armstrong published the first report substantiating the involvement of an ester intermediate in the mechanism of epoxide hydration by rat liver microsomal epoxide hydrolase (mEH). [18] Single turnover rat liver mEH catalyzed reactions performed in ^{18}O-labeled water resulted in no incorporation of the ^{18}O-label into the resultant diol while reactions involving multiple catalytic turnovers produced the expected ^{18}O-labeled diol as the product. Hammock, Oesch and co-workers isolated the ester intermediate using JH III as substrate for a soluble recombinant

murine EH by precipitation with acidic acetone. [19] The crude hydroxyacyl enzyme intermediate was reduced with lithium aluminum hydride to provide the triol, 10,11-dihydroxyfarnesol. These data strongly support the formation of an ester intermediate in the mammalian EH catalyzed epoxide hydration reaction.

Many studies have defined the regioselectivity of epoxide hydration catalyzed by mammalian EH using an ^{18}O-label incorporated into the substrate or by carrying out the enzymatic reaction in ^{18}O-labeled aqueous media. [20-24] The position of the label in the product can be simply identified by analysis of the diol GC/MS fragmentation pattern given that the diol fragments during electron impact (EI) MS between the carbons bearing the hydroxyl groups. There are examples of chemical hydration of JH with MS analysis of the diol product. Nakanishi and co-workers hydrolyzed synthetic (±)-C_{18} JH with 0.1N H_2SO_4 in ^{18}O-labeled water. [25] MS analysis revealed that the addition of labeled water occurred nearly exclusively at the C-11 position (97%). Similarly, Toong and co-workers hydrolyzed JH III by reaction with 0.1M H_2SO_4 in $H_2{}^{18}O$. [26] Analysis of the JH III diol produced in the reaction by EI GC/MS determined that 99.5% of the label was present at the more substituted C-11 position (fragment ion of m/z 61), as expected for chemical hydration via an S_N1 mechanism. Interestingly, the regioselectivity of epoxide hydration catalyzed by insect mEH on JH III as a substrate has never been investigated. We report herein our studies on the epoxide hydration of JH III catalyzed by mEH from *Trichoplusia ni*.

Commercially available racemic JH III **1** was treated with an acidic aqueous sodium acetate buffer to provide the ring opened ester-diol **2** The EI GC/MS spectrum of the diol was consistent with



1 **2**

that observed by Toong and co-workers, exhibiting the characteristic m/z 59 and m/z 225 fragment ions for cleavage of the glycol moiety. The GC/MS spectrum of the JH III ester-diol product is shown in Figure 2.

The epoxide hydration reaction of JH III was first carried out in an unlabeled acidic aqueous medium to provide for direct comparison of the enzyme catalyzed hydration product with the product from chemical hydration. The enzymatic hydration of JH III with *T. ni* mEH (crude microsomal preparation) was then investigated.The enzymatic hydration reaction was allowed to proceed to complete conversion (>95% loss of JH III by GC) of the epoxide to the ester-diol **2**. Given the molar quantity of JH III used, we were assured that multiple turnovers of the enzyme catalytic cycle were required for the extent of substrate conversion observed. Additionally, this reaction was performed so as to ensure that the JH III ester-diol metabolite could be observed by GC analysis of the crude reaction mixture. Since the *T. ni* mEH catalyzed reactions were carried out using a crude microsomal

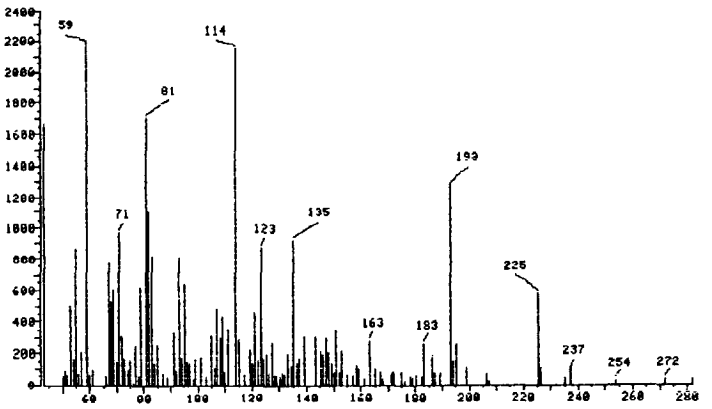
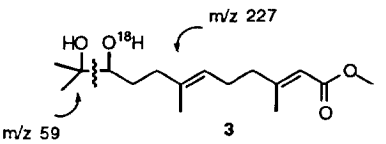


Figure 2: GC/MS spectrum of ester-diol **2**.

protein preparation, 3-octylthio-1,1,1-trifluoropropan-2-one (OTFP) [27] was preincubated with the crude protein homogenate for 10 minutes at 30°C to inhibit any JH esterase activity. A sample of JH III was added to the JH esterase inhibited crude microsomal preparation and the reaction mixture was then incubated for an additional 15 minutes at 30 °C. The ester-diol hydration product was extracted from the reaction mixture with ethyl acetate and subjected to analysis by EI GC/MS. The mass spectrum obtained for the enzymatic hydration ester-diol product was identical to that observed for the ester-diol produced by chemical hydration of JH III. It should be noted that the gas chromatogram of the extract from the enzymatic hydrolysis reaction was quite complex. Two prominent broad signals, possibly derived from protein decomposition products, with retention times of 23 and 25 minutes, respectively, were always present. Fortunately, JH III ester-diol exhibited a retention time of 24 minutes and was fully resolved from the other signals.

The regiochemistry of the enzymatic hydration reaction was then determined by ^{18}O-incorporation experiments. Dilute buffer solutions of microsomal EH preparations were repelleted via ultracentrifugation. The buffer solution was decanted off and the microsomal pellet was resuspended in $H_2{}^{18}O$. In an attempt to remove extraneous non-labeled water, the ultracentrifugation process was repeated prior to carrying out the enzymatic hydration reaction. The mEH catalyzed hydration reaction was then accomplished as described above and the presence of the labeled metabolite **3** was confirmed via EI GC/MS. Only the fragment ion at m/z 227 was observed, indicating regioselective addition of the ^{18}O-label at the C-10 position. No enhancement of the signal for the m/z 61 fragment ion, the possible regioisomeric C-11 addition product, was noted. Interestingly, the GC/MS spectrum of the ^{18}O-labeled product derived from the mEH catalyzed hydration reaction also possessed a significant



signal at m/z 225, Figure 3. As stated previously, recent studies have clearly demonstrated that the mechanism of mammalian epoxide hydrolase involves an ester intermediate. [18,19] The observation of an m/z 225 fragment ion from the ester-diol obtained by insect mEH catalyzed hydration of JH III in

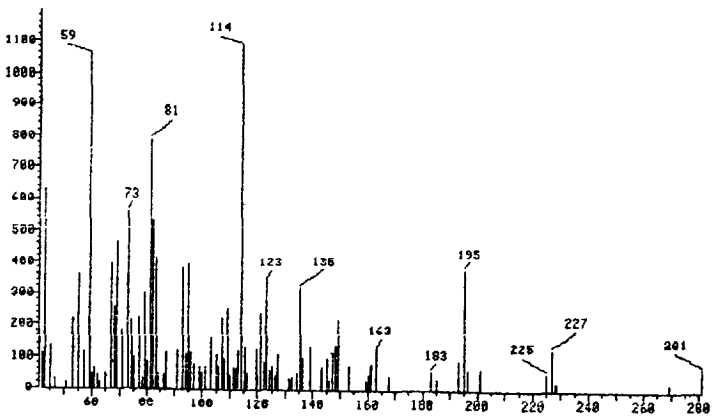


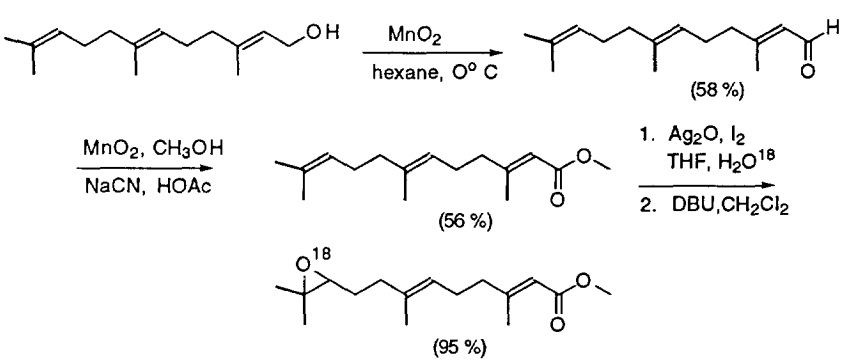
Figure 3. GC/MS spectrum of *T. ni* mEH catalyzed hydration of JH III.

labeled water implies that unlabeled material in this reaction could also arise from an ester intermediate, or could simply be a product of nucleophilic attack by adventitious (unlabeled) water in the reaction medium. In an attempt to more accurately define the origin of the m/z 225 fragment ion, the crude mEH microsomal preparation was lyophilized to remove all residual unlabeled water. One notes that bound water may not be completely removed by this method; however, the amount of unlabeled water remaining should be extremely small. The lyophilized microsomes were reconstituted in ^{18}O-labeled water (97.9% ^{18}O) and the mEH catalyzed hydrolysis reaction of JH III was then repeated as described above. The loss of some activity (10-20%) of the lyophilized mEH microsomal preparation relative to the activity of the centrifuged microsomes was noted. The extent of ^{18}O incorporation in the ester-diol product obtained from hydration reactions using lyophilized microsomes was quantitated using selected ion monitoring (SIM) EI GC/MS. However, the fragment ions at m/z 225 and m/z 227 could not be used to calculate the percent ^{18}O incorporation due to the fact that the peak shape of the signals for these ions varied from the previous GC/MS data of JH III ester-diol. The peak shape

variation indicates interference in the mass channel. As an alternative, the ^{18}O-label was quantitated using the fragment ions observed at m/z 193 and 195 since there appeared to be no interference with other ion fragments in this mass region. The ion at m/z 193 corresponds to JH III ester-diol that has undergone fragmentation at two sites, C-C cleavage of the vicinal diol moiety and α-cleavage of the methoxy group. Analysis of these data determined that the lyophilized enzyme incorporated 91.3% (± 2.7%, 1SD) of the ^{18}O-label at the less substituted C-10 carbon. For comparison, the JH III ester-diol obtained from the ultracentrifuged mEH catalyzed reaction was also monitored for the same ions (m/z 193 and 195) via SIM EI GC/MS. Only 63.8% (± 4.4%) incorporation of the ^{18}O-label was observed. These data indicate that the hydration reaction using the ultracentrifuged mEH microsomal preparation did indeed incorporate unlabeled extraneous water into the ester-diol. Significantly, the observation of unlabeled fragments from the lyophilized mEH catalyzed reaction implies the formation of an ester intermediate in the epoxide hydration reaction, a result in analogy to the mammalian EH catalyzed reaction. [18,19]

Alternatively, the regiochemistry of epoxide enzymatic hydration can also be determined by labeling the substrate and carrying out the hydration reaction in unlabeled water. Given the data from the $H_2{}^{18}O$ labeling experiments, we sought to provide further verification of the regioselectivity of the reaction using ^{18}O-labeled JH III. The JH III ester-diol product obtained by insect mEH catalyzed hydration using labeled substrate would then be expected to possess the ^{18}O-label at C-11. By the early 1970's, fifteen full syntheses were reported for C_{17}- and C_{18}- *Cecropian* derived JHs. [28] A procedure involving conversion of commercially available farnesol to methyl farnesoate via manganese dioxide oxidation [29] was chosen to prepare the labeled substrate, [^{18}O]-JH III, as illustrated in Scheme 2. Crude farnesal was isolated and directly oxidized to the methyl ester via the cyanohydrin

Scheme 2



intermediate. [30,31] Careful control of the concentration of the aldehyde was important for complete conversion of the α,β-unsaturated aldehyde to the methyl ester. A 0.07 M concentration of aldehyde was found to be the most efficient for complete conversion to the ester. The crude unsaturated methyl

ester was epoxidized to [^{18}O]-JH III via formation of an iodohydrin intermediate which collapsed to the oxirane ring under basic conditions. [32] [^{18}O]-JH III was obtained by this method with 83% ^{18}O incorporation at the epoxide as determined by direct comparison of unlabeled and labeled JH III CI GC/MS spectra. *T. ni* mEH catalyzed hydration reactions of [^{18}O]-JH III were then completed using lyophilized and ultracentrifuged microsomal preparations. The ester-diol products from three trials were each analyzed by SIM GC/MS. For each of these reactions, there was no discernible signal at m/z 227, while the signals at m/z 61 and m/z 225 were readily apparent, indicative of nucleophilic addition only at C-10.

Both of the studies described herein, using labeled and unlabeled substrates, clearly indicate that an S_N2-type mechanism is involved in insect mEH catalyzed enzymatic hydration of JH III using a crude microsomal preparation. Nucleophilic addition was found to occur exclusively at the least substituted C-10 position of the epoxide in direct contrast to the chemical hydration reaction which leads to ^{18}O incorporation at the more substituted C-11 carbon. [26] Additionally, the observation of an m/z 225 fragment ion from the mEH (crude microsomal preparation) catalyzed hydration reaction carried out in labeled water is significant in that this result suggests that a carboxylate residue may be involved in the mechanism of insect mEH, similar to that shown to occur in mammalian systems. Mammalian EH has been shown to have a degree of sequence homology with bacterial haloalkane dehalogenase (HAD). [33] HAD is a member of the α,β-hydrolase fold family of enzymes which function via a catalytic triad of nucleophile-base-acid in which aspartate is the presumed nucleophile. An X-ray crystal structure has shown that the mechanism of HAD catalyzed dehalogenation does indeed involve a covalent ester intermediate. Our results support the supposition that insect mEH may be related to mammalian EH with regard to the catalytic mechanism of epoxide hydration. Further studies to define the active site nucleophilic species in insect mEH are underway.

Experimental

General: ^{1}H NMR and ^{13}C NMR spectra were obtained on a GN 300 MHz or 500 MHz spectrometer. Chemical shifts are reported relative to tetramethylsilane. Infrared spectra were obtained on a Perkin Elmer 1600 series FTIR. A Hewlett-Packard 5890 Gas Chromatograph equipped with a FID detector and a J & W Scientific SE-30 fused silica capillary column (ID 0.25 mm) was used for all routine GC analyses. A temperature ramp program (275°C injection port temperature, initial temperature of 100°C for 10 minutes, ramp 10°C/min, 250°C final temperature for 5 minutes) was used for each analysis. EI and CI GC/MS data were obtained on a Hewlett-Packard 5985 B Quadrapole Mass Spectrometer. The EI and CI (methane) analyses were carried out on a 30m DB5 column using a temperature ramp program (injection port temperature 275°C, initial temperature 100°C for 10 minutes, ramp of 8°C/min, 280°C final temperature). Exact mass measurements were

performed on a JOEL HX110HF high resolution mass spectrometer. Elemental analyses were carried out by Atlantic Microlab Inc. (Norcross, Georgia). A Beckman J2-21M/E centrifuge and L8-70M ultracentrifuge were used to prepare the crude *T. ni* microsomal homogenates. A Beckman TL-100 ultracentrifuge was used to remove the buffer from the diluted microsomes. Radiometric assays were accomplished using a Beckman LS 1801 scintillation counter. Unless otherwise stated, all reagents were purchased from Aldrich and purified prior to use. Tetrahydrofuran was distilled from Na/benzophenone. Methylene chloride was distilled from CaH_2. Tritiated racemic JH III (>97% radiopurity, 12 Ci/mmol) was obtained from New England Nuclear Corporation. Unlabeled (cold) JH III was purchased from Sigma Chemical Corporation. Labeled water ($H_2{}^{18}O$, 97.9% ^{18}O) was purchased from Isotec, Inc. Scintillation cocktail, ScintiVerse BD, was obtained from Fisher Scientific. Polygram SILG 25 mm TLC plates were purchased from Brinkmann.

Preparation of *Trichoplusia ni* microsomes. *T. ni* larvae were reared on an artificial diet [35] in a climate controlled environment (30°C, 40% relative humidity with a photoregime of 14L:10D beginning at 6 am). Insects (63 larvae, 0.2 g/each) of stage L_5D_3 (gate 1) larvae were homogenized for 2 minutes in 36 mL of 250 mM sucrose in sodium phosphate buffer (I=0.2, 0.01% 1-phenyl-2-thiourea (PTU), pH 7.5) with a Brinkman polytron (setting of 5). The homogenate was then filtered through glass wool and centrifuged at 4°C for 15 minutes at 10,000g in a Beckman J2-21 M/E centrifuge to remove cellular debris. The cytosol was decanted from the solid pellet and recentrifuged for 1 hour at 100,000g in a Beckman L8-70 M ultracentrifuge. The resulting pellet was washed 2x with buffer and recentrifuged after each wash at 100,000g for 1 hour. The final microsomal pellet was suspended in sodium phosphate buffer. The diluted microsomal preparation was divided into small aliquots and stored at -78°C for later use.

Epoxide hydrolase activity assay. A series of dilutions of microsomes in buffer was prepared to define the linear range of the assay. Diluted microsomes (100 µL) were added to 10 x 75 mm borosilicate tubes in an ice water bath. 3-Octylthio-1,1,1-trifluoro-2-propanone (1 µL, 0.02 M final concentration) was added to each tube, followed by a 10 minute incubation period at 30°C. The tubes were then transferred back to the ice water bath and 1 µL of [^{3}H]-JH III (5×10^{-4} M, 8000 cpm/µL) was added. The mixture was vortexed briefly and reincubated at 30°C for 15 minutes. After transfer to the ice water bath, the reaction mixture was quenched by the addition of 300 µL of 75% methanol in water and 250 µL of isooctane. The two-phase mixture was vigorously vortexed and then centrifuged at 1,000g for 5 minutes. A 100 µL aliquot of each phase was added to 3 mL of scintillation cocktail in a scintillation vial and the amount of radioactivity present was determined. EH activity was then calculated as described in Share and Roe. [36]

Preparation of Methyl 10,11-Dihydroxy-2(E),6(E)-farnesoate by chemical hydration. A 100 μL aliquot of a 5.2 x 10^{-2} M ethanol solution of commercially (Sigma) available juvenile hormone III was placed in a 1 dram vial. The solvent was removed by N_2 purging and 1 mL of sodium acetate buffer (0.5 M, pH 4) was added. The reaction mixture was capped and stirred overnight at 40°C. Solid NaCl was added to the reaction mixture followed by extraction with ethyl acetate (3 x 2 mL). The combined organic layers were dried over Na_2SO_4 and then concentrated under reduced pressure to provide the crude ester-diol. GC R_T 24.5 minutes. ^{1}H NMR ($CDCl_3$) δ 5.6 (s, 1H); 5.2-5.1 (m, 1H); 3.7 (s, 3H); 3.3-3.2 (m, 1H); 2.2-0.7 (m, 22H). EI GC/MS (m/z) 59, 114 (base), 193, 225.

Typical procedure for enzymatic hydration. All enzymatic hydration reactions were run in duplicate. A 100 μL aliquot of diluted microsomes at 0°C was preincubated with 1 μL of 10^{-2} M 3-octylthio-1,1,1-trifluoro-2-propanone for 10 minutes at 30°C. The mixture was then placed back on ice and 1 μL of 5 x 10^{-2} M of cold JH III was added and the solution was then vortexed briefly. The mixture was again incubated at 30°C for 15 minutes. The epoxide hydration reaction was then quenched by the addition of 100 μL of EtOAc, and the mixture was evaporated to dryness. Ethyl acetate (10 μL) was added to the residue and 2 μL of this solution was injected onto the GC and/or GC/MS for analysis.

Enzymatic hydration reactions in ^{18}O-labeled water. A vial of frozen crude microsomes in buffer solution was thawed and a 100 μL sample was ultracentrifuged for 15 minutes at 100,000g. The supernatant was removed via pipet and the pellet was resuspended in ^{18}O-labeled water (100 μL). The process was then repeated to ensure removal of unlabeled water and the microsomal suspension in ^{18}O-labeled water was placed on ice. Alternatively, lyophilized microsomes were resuspended in ^{18}O-labeled water (100 μL) and placed on ice. 3-Octylthio-1,1,1-trifluoro-2-propanone (1 μL, final concentration of 10^{-2} M) was added and the typical procedure for enzymatic hydration given above was then followed.

Preparation of 2(E),6(E)-Farnesal. Manganese dioxide (390 mg, 4.49 mmol) and farnesol (56.8 μL, 0.26 mmol) were combined at 0 °C in dry hexane (8 mL). The slurry was then allowed to warm to room temperature over a period of 30 minutes. The reaction mixture was then filtered through celite. The filtrate was dried over $MgSO_4$ and concentrated to provide the crude product as a yellow oil (58%). GC R_T 21.1 minutes. ^{1}H NMR ($CDCl_3$) δ 10.0 (d, J=8 Hz, 1H); 5.9 (dd, J=1.5, J=8 Hz, 1H); 5.1 (m, 2H), 2.3-1.9 (m, 14H), 1.9 (s, 3H), 1.8 (s, 3H). ^{1}H NMR data corresponded to reported literature values. [37]

Preparation of Methyl 2(E),6(E)-Farnesoate. Farnesal (50 mg, 0.23 mmol) was dissolved in 3.3 mL of dry methanol at room temperature. Acetic acid (20 μL, 0.35 mmol), sodium cyanide (56.36 mg,

1.15 mmol), and manganese dioxide (400 mg, 4.6 mmol) were then added and the mixture stirred overnight under Ar. The solvent was removed under reduced pressure and the residue was washed with ether (3 x 50 mL). The combined ether washings were filtered, dried over $MgSO_4$, and the solvent removed under reduced pressure to provide 32 mg (56%) of a clear oil. GC R_T 21.7 minutes. ^1H NMR ($CDCl_3$) δ 5.7 (s, 1H); 5.1 (m, 2H); 3.7 (s, 3H); 2.2 (s, 3H); 2.2-1.9 (m, 8H); 1.7 (s, 3H); 1.6 (s, 6H). IR (neat) (cm^{-1}): 3396, 2925, 1721, 1649, 1437, 1377, 1224, 1147, 959. ^1H NMR data corresponded to reported literature values. [37]

Preparation of ^{18}O-Methyl 10,11-Epoxy-2(E),6(E)-farnesoate. Methyl farnesoate (20.5 mg, 0.08 mmol) and $H_2{}^{18}O$ (21.6 μL, 0.82 mmol) were combined in dry THF (980 μL) at room temperature under Ar. Silver (I) oxide (28.5 mg, 0.12 mmol) and iodine (31.2 mg, 0.12 mmol) were then added simultaneously. The reaction mixture was allowed to stir for 7 hours at room temperature. 1,8-Diazabicyclo[5.4.0]undec-7-ene (61.3 μL, 0.41 mmol) in 3.6 mL of CH_2Cl_2 was then added and the reaction mixture was allowed to stir at room temperature overnight under Ar. The reaction mixture was then filtered through celite, diluted with 10 mL of CH_2Cl_2, and washed with 10% H_2SO_4 (3 x 10 mL) and saturated aqueous sodium chloride (1 x 10 mL). The combined organics were dried over $MgSO_4$ and concentrated at reduced pressure to give a quantitative yield of an oil (21.9 mg). GC R_T 22.9 minutes. ^1H NMR ($CDCl_3$) δ 5.8 (m, 1H); 5.2 (m, 1H); 3.7 (s, 3H); 2.7 (t, J=6 Hz, 1H); 2.2 (d, J=3 Hz, 3H); 2.2-1.9 (m, 8H); 1.7 (s, 3H); 1.3 (s, 3H); 1.27 (s, 3H). CI GC/MS (m/z) 237 (MH^+ - CH_3OH, base), 297 $(M + C_2H_5)^+$. ^1H NMR data corresponded to reported literature values for non-labeled JH III. [37]

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