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Supplementary Information for

Terpene synthases in stink bugs: De novo formation of an aggregation pheromone precursor in the harlequin bug *Murgantia histrionica*

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Supplementary Information

SI Results

Chemical Identification of Sesquipiperitol. The identification of sesquipiperitol by synthesis and chemical transformations is shown in Fig. S2*A*. Hagiwara et al. (1) reported oxidation of zingiberenols to sesquipiperitones using mildly acidic pyridinium chlorochromate (PCC) by virtue of an allylic isomerization of the tertiary alcohol to a secondary alcohol prior to the oxidation. Application of this procedure to a ~1:1 mixture of *SSR*-zingiberenol and *RRR*-zingiberenol led to the production of *RR*- and *SR*-sesquipiperitones baseline separated on an HP-MS GC column (Fig. S2*B*). Oxidation of *SSR*-zingiberenol **4** of 95:5 diastereomeric ratio resulted in the isolation of *SR*-sesquipiperitone **5** of approximately the same stereoisomeric purity (Fig. S2*B*), thus confirming that during allylic isomerization-oxidation, carbons at positions 6 and 7 were unaffected.

PCC oxidation with the hexane extract from recombinant *Mh*IDS-1 (*Mh*TPS) activity assays resulted in the formation of a compound that matched 6*S*,7*R*-sesquipiperitone **5** but not 6*R*,7*R* -sesquipiperitone based on GC retention time and mass spectral fragmentation (Fig. S2*C*). Because the compound of interest in the *Mh*TPS assay was not zingiberenol, an oxidative introduction of an oxo-group at position 1 would arise from a corresponding secondary alcohol, which is sesquipiperitol.

Reduction of the carbonyl group in **5** with lithium aluminum hydride provided a final proof for sesquipiperitol being the major *Mh*IDS-1 (*Mh*TPS) product. Treatment of ketone **5** with lithium aluminum hydride in ether resulted in the formation of epimeric alcohols **3** and **6** (Figs. S2*A* and S2*D*), separated by chromatography on SiO_2. GC retention times of these alcohols were almost indistinguishable on the HP-5MS column, yet matching that of the main *Mh*IDS-1 (*Mh*TPS) product (Fig. S2*D*). The mass spectrum of this compound was nearly identical to those of alcohols **3** and **6** thus proving its structure as sesquipiperitol (Fig. S2*D*).

Absolute Configuration of Sesquipiperitol. As shown in Fig. S2*C*, oxidation of sesquipiperitol produced by *Mh*TPS led to 6*S*,7*R*-sesquipiperitone **5** but not 6*R*,7*R*–

sesquipiperitone. However, the oxidation product was indistinguishable by GC retention time and mass-spectrum from 6*R*,7*S*-sesquipiperitone prepared analogously from 7*S*-zingiberenol. 6*S*,7*R*- and 6*R*,7*S*-Sesquipiperitones were also inseparable on chiral Hydrodex-β-6-TBDMS and Lypodex-G columns. To determine the absolute configuration of enzymatically produced sesquipiperitol at C-7, it was converted to bisabolane (2). Dehydration of sesquipiperitol with phosphorous oxychloride formed bisabolatrienes, which were hydrogenated in the presence of Adams (PtO_2) catalyst to bisabolane **7** (Figs. S2*A* and S5*A*). The same dehydration/hydrogenation sequence was conducted with (7*R*)- and (7*S*)-zingiberenols (3) to make bisabolane standards (Fig. S5*A*). Chiral GC analysis showed a partial separation of (7*R*)- and (7*S*)-bisabolane standards on a Hydrodex β-6TBDM column (dehydration/hydrogenation eliminated chiralities at C-1 and C-6, but creates 1,4-substituted *cis*/*trans*-bisabolanes) (Fig. S5*A*). Co-injection of samples resulted in an alignment of bisabolanes derived from enzymatically produced sesquipiperitol with the (7*R*)-bisabolane standards (Fig. S5*A*). This result proved a 7*R* configuration of the *Mh*TPS sesquipiperitol product. Since the relative configuration of this compound was found to be either 6*S*,7*R*, or 6*R*,7*S*, the absolute configuration at C-6 has to be (*S*).

To determine the configuration of enzymatically derived sesquipiperitol at C-1, we examined other chiral GC columns for the separation of epimeric alcohols formed by reduction of the carbonyl group in ketone **5** (Fig. S2*A*). Retention times of epimeric sesquipiperitol **3** and **6** were substantially different on Hydrodex β-6TBDM (Fig. S5*B*), and the late eluting peak matched the enzymatically produced sesquipiperitol. The stereochemistry of this sesquipiperitol as epimer **3** was proven by NMR recordings (Table S1, Fig. S5*C*). NOESY (Fig. S5*C*) showed *cis* orientation of H-1 and H-6 in **6** and *trans* orientation of these protons in alcohol **3**. This proved 1*S* configuration of alcohol **3** and, hence, 1*S*,6*S*,7*R* configuration for synthetic **3** and *Mh*TPS produced sesquipiperitol (Fig. S5*C*). Thus, the enzymatically produced sesquipiperitol was identified as (-)-(1*S*,6*S*,7*R*)-2,10-bisaboladien-1-ol. ^{1}H and ^{13}C NMR data of sesquipiperitols **3** and **6** were in accord with those of monoterpene analogs, *cis*- and *trans*-piperitols (4) providing additional proof for stereochemical assignments.

SI Materials and Methods

Chemicals and Reagents. Unless otherwise specified, all reagents and solvents were purchased from Aldrich Chemical Co. [1-3H]-FPP (20 Ci/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO). Preparation of terpene standards for mass spectral comparisons was performed in the following way: β-Sesquiphellandrene was prepared following McBrien et al. (5). Zingiberene was isolated from ginger oil (6). 4-*epi*-β-Bisabolol was prepared according to Fráter and Müller (7). γ-Curcumene was prepared by dehydration of 4-*epi*-β-bisabolol as described (8) and was isolated by argentation chromatography on 15% $AgNO_3$-SiO_2 eluting with hexane/ether, 20:1. GC-MS (*m/z*, %): 204 (38), 161 (12), 134 (17), 121 (71), 119 (100), 105 (42), 93 (55), 91 (39), 79 (22), 77 (22), 55 (17), 41 (25). Mass spectral data were in close agreement with those described (9).

Insects. To establish a *M. histrionica* colony for RNAi treatment, late instar nymphs were collected from insecticide-free vegetable plots in Beltsville, MD. Insects were held in cages (Bioquip) holding potted 4-8 week old collard (*Brassica oleracea* 'Champion') and mustard (*Brassica juncea* 'Southern Curled Giant') plants in climate controlled greenhouses (25 +/- 5°C, 16:8 h L:D, 65% RH). Newly eclosed adults were removed from cages three times weekly and moved to new cages holding potted plants, isolating males and females. Insects for tissue-specific qRT-PCR analysis were reared on 6-8 week old collard in mesh cages (Bioquip) in the greenhouse (Virginia Tech) under LD photoperiod at 25°C. Insects were separated by sex and kept until the immature (2-3 day post molt) or mature (14-15 day post molt) adult stage.

Crude Protein Extracts of *M. histrionica*. Fifteen-day old virgin male and female *M. histrionica* were used to prepare crude protein extracts. Extracts were prepared separately for sexes and tissues. Insects were fixed with hexane and dissected in phosphate buffered saline, pH 7. Tissues (head and thorax, cuticle, midgut, and fatbody) were frozen in liquid nitrogen, pulverized with a mortar and pestle, and suspended in 25 mM HEPES, 5 mM $MgCl_2$, 10% glycerol, pH 7. Protein concentration was determined with a Bradford Assay (Bio-rad) according to the manufacturer's instructions.

Identification and Cloning of *IDS* type Genes. Two putative isoprenyl diphosphate synthase-like genes (*MhIDS1 – MhTPS*, *MhIDS2 – MhFPPS*) were identified with tblastn searches of transcriptome data retrieved at the USDA, Beltsville (10) using functionally characterized insect *trans*-IDS or bifunctional IDS/TPS (*I. pini*) query sequences (Table S2). Primers were designed using Geneious (v. 7.1.9) to clone full-length *MhTPS* (*Mh*TPS_1F/*Mh*TPS_1158R) and *MhFPPS* (*Mh*FPPS_1F/*Mh*FPPS_1218R) (Table S3). Primers were also designed to clone *MhFPPS* without a predicted transit peptide sequence (*tMhFPPS*) (primers t*Mh*FPPS_1F/*Mh*FPPS_1218R). cDNAs were generated from total RNA using GoScript reverse transcriptase (Promega). Target sequences were amplified using Q5 proofreading DNA polymerase (New England Biolabs) and purified by gel extraction (New England Biolabs). Purified products were A-tailed using *Taq* DNA polymerase (New England Biolabs) and ligated into the pGEM-T Easy vector (Promega). Sequences were verified before cloning into expression vectors.

Heterologous Expression of Recombinant *MhFPPS*. Full-length and truncated *MhFPPS* were amplified from pGEM-T Easy constructs with Q5 DNA polymerase and cloned into the pEXP5-NT/TOPO expression vector containing an N-terminal 6x histidine tag (Invitrogen). pEXP5-NT/TOPO constructs were transformed into *Escherichia coli* strain BL21(DE3)pLysS (Life Technologies). *E. coli* cultures (50 mL) were grown at 37°C and 220 rpm and induced with 1 mM IPTG after reaching an OD_{600} of 0.6. Upon induction, the cells were cultivated at 18°C for another 18 h prior to collection by centrifugation for 15 min at 5,000 *g* at 4°C. Pellets were resuspended in 2 mL chilled extraction buffer (50 mM Tris HCl [pH 7.5], 20 mM imidazole, 300 mM NaCl, 10% glycerol (v/v), 5 mM $MgCl_2$, 2 mM DTT) supplemented with 0.3 mg/mL lysozyme (AppliChem), 2.5 U/mL benzonase (Novagen) and proteinase inhibitors (Protease Inhibitor Mix HP, SERVA) and incubated at 4°C for 30 min on ice. Cells were disrupted by a 4 x 30 s treatment with a sonicator (Bandelin UW2070, Berlin, Germany; 50%) and lysates centrifuged at 4°C for 30 min at 15000xg to obtain soluble fractions. Recombinant proteins were purified using Ni-NTA Spin Columns (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For enzymatic assays, the buffer was exchanged with 25 mM 3-(N-morpholino)-2-

hydroxypropanesulfonic acid (MOPSO, pH 7.2, 10% [v/v] glycerol, 1 mM DTT, 5 mM $MgCl_2$) using PD-10 Desalting Columns (GE Healthcare Life Sciences).

Heterologous Expression of Recombinant *MhTPS*. For bacterial expression, the full-length *MhTPS* cDNA was amplified from the pGEM-T Easy construct (primers *Mh*TPS_1F/*Mh*TPS_1158R) and cloned into the pEXP5-NT/TOPO (Invitrogen) expression vector containing an N-terminal 6x histidine tag. A truncated form of *MhTPS* (*tMhTPS*, bp 136-1158) was designed and synthesized by GenScript (Piscataway, NJ) with codon optimization for *E. coli* and cloned into pET19b. Following transformation into *E. coli* BL21(DE3)pLysS cells (Invitrogen), single colonies were selected at 18°C on LB with ampicillin (100 µg/mL) and chloramphenicol (34 µg/mL). Expression cultures were started in 5 ml LB plus antibiotics prior to inoculation of 200 mL of the same medium and cultivation at 18°C for 4-8 h. Expression was induced at an OD_{600} of 0.50 with 0.5 mM IPTG. Following cultivation of 48 h, cells were washed in 100 mL wash buffer (20 mM Tris-HCl, 50 mM KCl, pH 7), pelleted and resuspended in 15 mL cell lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 5 mM imidazole, 0.5 mM PMSF, 2 mM DTT, pH 8). Cells were lysed on ice for 2 x 30 s (1 min interval) at 20% amplitude (Branson Digital Sonifier) and the supernatant was partially purified with Ni-NTA agarose (Qiagen) using three washes of 30 mM imidazole. The target protein was eluted in a single 1 mL fraction with 250 mM imidazole and desalted into TPS assay buffer (25 mM HEPES, 10 mM $MgCl_2$, 10% glycerol, pH 7) using PD MiniTrap G-25 desalting columns (GE Healthcare).

For expression in insect cells (*Sf*9), a cDNA encoding *Mh*TPS protein (without an N-terminal His-tag) was produced for cloning into the pENTR4^{NcoI-} (11) vector by Gibson assembly using the In-Fusion HD Cloning Kit (Clontech). Briefly, the ORF was amplified from a pBLUNT vector construct using *Mh*TPS_FusF1 and *Mh*TPS_FusR1 primers. pENTR4^{NcoI-} was amplified using pENTRF4 and pENTRR5 primers as described previously (12). Recombinant plasmids were transformed into *E. coli* Stellar^{TM} DH5α and confirmed by sequencing.

Recombinant *Mh*TPS was produced in *Sf*9 cells using the BaculoDirect Expression System (Invitrogen). Briefly, the coding region was transferred from pENTR4^{NcoI-} into the linearized BaculoDirect vector by LR recombination. High-titer P3 viral stocks were

prepared by successive amplifications of P1 and P2 stocks. Serum-adapted cells were infected with P3 viral stock and incubated for 72 h in 50 mL cultures in Sf900 II serum-free media (Invitrogen) supplemented with 10% (vol/vol) FBS (Atlas Biologicals). Optimal viral doses and infection times were determined by monitoring recombinant protein production by SDS-PAGE. Cells were harvested in 100 mM sodium phosphate buffer, pH 7.6, and resuspended in assay buffer (25 mM HEPES, 5 mM $MgCl_2$, 5 mM KCl, 10% glycerol, pH 7) with 500 µM PMSF and Protease Inhibitor Cocktail (Sigma). Lysates were prepared as previously described (11).

IDS Activity Assay and Analysis. For enzyme assays, 96 µl of partially purified protein were mixed with 2 µl 50 µM isopentenyl diphosphate (IPP; Sigma) and 2 µl 50 µM dimethylallyl diphosphate (DMAPP; Sigma) and incubated at 30°C for 2 h. Analysis of IDS enzyme products was done using an Agilent 1260 HPLC system (Agilent Technologies) coupled to an API 5000 triple-quadrupole mass spectrometer (AB Sciex Instruments) according to the protocol described by Beran et al. (13).

TPS Activity Assay. Enzyme activity was assessed in crude protein extracts from whole male and female bugs and from the cuticle, head and thorax, midgut, and fatbody tissues of male bugs. Enzyme assays were performed by administering 50 µM (*E*,*E*)-FPP to protein extracts (50 µg total protein) in 25 mM HEPES, 5 mM $MgCl_2$, 5 mM KCl, 10% glycerol, pH 7 at a final volume of 200 µL. An equal volume of hexane was overlaid on each preparation to collect volatiles using a 6 h incubation period at 30°C. Products were extracted by vigorously mixing preparations for 15 sec and the organic phase was separated by centrifugation at 4000xg for 10 min.

Assays with heterologously expressed protein were performed in assay buffer (see above) in a total volume of 250 µl with partially purified protein, 1 mM DTT, and 50 µM allylic substrate [(*E*,*E*)-, (*Z*,*E*)- or (*Z*,*Z*)-FPP] and incubated at 30°C for 1 h with a 250 µL hexane overlay. Assays were stopped on ice and compounds extracted by mixing (vortex) at maximum speed for 15 sec. Phases were separated by centrifugation at 4000xg for 10 min and the hexane phase was removed and dried over $MgSO_4$. One µl of liquid sample was injected in splitless mode and analyzed by GC-MS. For identification of the *Mh*TPS

product, assays were scaled up by incubating 1 mL protein in a 7 mL screw top test tube and the obtained hexane extract was used for further analysis.

To determine enzyme kinetic parameters, assays were performed in a final volume of 50 µL with 0.3 µg partially purified, *E. coli* expressed *Mh*TPS enzyme and increasing concentrations of [1-^{3}H]-(*E*,*E*)-FPP (64 µCi mmol^{-1}). Assays were incubated in triplicate at 30°C for 5 min prior to extraction of the reaction product with 250 µl hexane. Quantification of the radioactive product were as described by Tholl et al. (14). Calculation of *K*m and *V*max values was performed by rectangular hyperbolic regression analysis using the Hyperbolic Regression Analysis (HYPER 1.01) software (J.S. Easterby, University of Liverpool).

Gas Chromatography-Mass Spectrometry Analysis of *Mh*TPS Products and Pheromone Emissions. GC-MS analysis was performed by liquid injection at 240°C running in split 5 mode and separated on a GC-2010 gas chromatograph (Shimadzu, Kyoto, Japan) using a 30 m x 0.25 mm i.d. x 0.25 µm film Zebron ZB-XLB column coupled with a QP2010S mass spectrometer (Shimadzu). The GC program was as follows: 40°C with 2 min hold, then raised to 220°C at 5°C/min, then raised to 240°C at 70°C/min followed by a 2 min hold time. Mass spectrometry was performed with an ion source temperature of 240°C, interface temperature of 280°C, electron ionization (EI) potential of 70 eV, and scan range of 50 to 400 atomic mass units. Helium was used as a carrier gas at 1.9 mL/min. Terpene olefin products were identified by comparison of retention times and mass spectra with those of authentic standards.

Comparative hot injection and cool-on-column GC-MS analyses of the TPS assay products were performed at the USDA, Beltsville, in electron impact (EI) ionization mode at 70 eV with an Agilent Technologies 5973 mass selective detector interfaced with a 6890 N GC system equipped with a 30 m x 0.25 mm i.d. x 0.25 µm film HP-5MS Agilent J&W column. The column temperature was maintained at 40ºC for 5 min, and then raised to 240ºC at 7ºC/min, then to 270 at 15ºC/min. Helium was used as a carrier gas at 1 ml/min. Injections of 1 µl hexane extract were done either splitless at 260ºC, or cool-on-column at 70ºC.

An HP-5MS column was also used for GC-MS analysis (see above) of pheromone emissions from the RNAi-mediated knockdown experiment. Injections were done splitless at 260ºC and the column temperature was maintained at 40ºC for 5 min, and then raised to 270ºC at 10ºC/min. Helium was used as a carrier gas at 1 ml/min.

GC Analyses of Chemical Modification Products. GC analyses were performed on an Agilent Technologies 6890N instrument equipped with a flame ionization detector and a 25 m x 0.25 mm ID Hydrodex β-6TBDM capillary column (Macherey-Nagel GmbH & Co. KG, Düren, Germany). Hydrogen was used as a carrier gas at 1.5 ml/min. The temperature program for bisabolanes was: 50 (5) to 90 (111) at 10ºC/min. The temperature program for sesquipiperitols was: 50 (5) to 130 (67) at 10 ºC, then to 160 ºC at 15ºC/min. Splitless injections were conducted at the injector temperature 260ºC and detector temperature 270ºC. GC-HRMS analysis was performed in TOF EI mode on a Waters GCT Premier instrument equipped with a DB5-MS column.

Identification of the *Mh*TPS Product Sesquipiperitol. Various stereoisomers for chemical correlations were produced according to Fig. S2*A*. For these procedures, flash chromatography was performed with 230–400 mesh silica gel (Fisher Scientific). TLC analyses were conducted on Whatman AL SIL G/UV plates using 20% ethanol solution of phosphomolybdic acid, and/or UV for visualization of spots. Optical rotations were obtained using a Perkin-Elmer 241 polarimeter with a 1.0 mL cell.

(3*S*,6*S*,7*R*)-1,10-bisaboladien-1-ol (*SSR*-zingiberenol) was prepared as described in Khrimian et al. (15). An approximate 1:1 mixture of *cis*-(7*R*)-1,10-bisaboladien-3-ols (*SSR*- zingiberenol and *RRR*-zingiberenol) was synthesized as described in Leskey et. al. (16).

A mixture of (6*R*,7*R*)-2,10-bisaboladien-1-one and (6*S*,7*R*)-2,10-bisaboladien-1-one (*RR*- and *SR*-sesquipiperitones) was prepared following Hagiwara et al. (1). Briefly, an approximately 1:1 mixture of *SSR*-zingiberenol and *RRR*-zingiberenol (107 mg, 0.48 mol), pyridinium chlorochromate (PCC, 310 mg, 1.40 mmol), and dichloromethane (2 ml) was stirred at 0ºC for 15 min, then at 25ºC for 2 h. The mixture was poured into ether (10 ml) and washed consecutively with 5% NaOH, 5% HCl, 0.1 M $NaHCO_3$, brine, then dried with

sodium sulfate. Flash chromatography with hexane/ethyl acetate, 10:1, afforded ~ a 1:1 mixture of sesquipiperitones (73 mg, 69%) almost baseline separated on a HP-5MS GC column. The late eluting peak was identified as *SR*-sesquipiperitone **5** based on oxidation of *SSR*-zingiberenol.

(6*S*,7*R*)-2,10-Bisaboladien-1-one (*SR*-sesquipiperitone **5**) was prepared analogously to the above experiment from *SSR*-zingiberenol **4** (154 mg, 0.69 mol, 95:5 dr), PCC (446 mg, 2 mmol) in CH_2Cl_2 (3 ml). GC-MS analysis showed 93:7 and NMR 95:5 ratios of (6*S*,7*R*)- and (6*R*,7*R*)-2,10-bisaboladien-1-ones. $[\alpha]_D^{20}$ +43.8 (*c* 2.65, $CHCl_3$). Lit. (1) $[\alpha]_D^{20}$ +37.1 (*c* 3.7, $CHCl_3$). ^{1}H NMR (600 *MHz*, $CDCl_3$): 0.78 (d, *J* = 6.6 Hz, 3H), 1.26 (m, 2H), 1.57 (s, 3H), 1.66 (s, 3H), 1.74 (m, 1H), 1.91 (s, 3H), 1.92 (m, 2H), 1.99 (m, 1H), 2.13 (dt, *J* = 12.6, 4.2 Hz, 1H), 2.28 (m, 2H), 2.32 (m, 1H), 5.09 (tm, *J* = 7.2 Hz, 1H), 5.84 (m, 1H). 95:5 Diastereomeric ratio was found by integration of methyl groups at 0.78 (6*S*,7*R*) and 0.91 (6*R*,7*R*). ^{13}C NMR (151 *MHz*, $CDCl_3$): 15.6, 17.6, 22.4, 24.1, 25.7, 26.0, 30.3, 30.9, 34.7, 49.8, 124.5, 127.2, 131.4, 161.1, 201.1. ^{13}C NMR data were in a close agreement with those reported (1).

For the oxidation of the *Mh*TPS assay product with PCC, a hexane solution (50 µl) of the *Mh*TPS assay extract was stirred in an open conic vial till dryness. Dichloromethane (70 µl) was added followed by PCC (1-2 mg) and the mixture was stirred for 2 h. Ether (1 ml) was added and the content of the vial was filtered through a short pad of SiO_2. The solution was analyzed by GC-MS by splitless injection vs. *SR*-sesquipiperitone **5**.

The reduction of *SR*-sesquipiperitone **5** was performed by adding lithium aluminum hydride (210 µl of 1.0 M in ether; 0.21 mmol) at -25^{o}C to a solution of the ketone (42 mg, 0.19 mmol) in ether (2.0 ml). The temperature was allowed to rise to -10^{o}C, and the mixture was stirred for 2 h. Water (8 µl) was added to the mixture, followed by 15% NaOH (8 µl), and again water (24 µl). The crystalline residue was filtered, the ether solution was concentrated and flash chromatographed with hexane/ethyl acetate, 11:1. *RSR*-Sesquipiperitol **6** (5 mg, 12%) was isolated as a faster-eluting product, $[\alpha]_D^{20}$ +168.4 (*c* 0.38, $CHCl_3$), followed by a mixture of *SSR*-sesquipiperitol **3** and *RSR*-sesquipiperitol **6** (9 mg, 21%), then *SSR*-sesquipiperitol **3** (19 mg, 45%), $[\alpha]_D^{20}$ -15.4 (*c* 0.91, $CHCl_3$). ^{1}H and ^{13}C NMR data of the alcohols are presented in Table S1. ^{1}H NMR data of **3** and **6** were in agreement with those published for their enantiomers (17). ^{1}H and ^{13}C NMR data of **3** were

in good agreement with those reported by Sy and Brown (18) and ^{13}C NMR data of **3** matched those reported by Cool (19).

Dehydration/hydrogenation of sesquipiperitol was done in the following way: An aliquot of the *Mh*TPS assay extract (50 µl) containing ~10 µg sesquipiperitol was concentrated with a gentle stream of N_2 and taken into 50 µl dichloromethane. The solution was cooled to 0ºC and treated with 2 µl of a premixed solution of $POCl_3$ (1.2 µl) and pyridine (20 µl). The mixture was stirred at 25ºC for 16 h, diluted with 200 µl CH_2Cl_2 and treated with 30 µl water. The organic layer was separated and washed with water (30 µl) and 5% sodium bicarbonate (10 µl). The dichloromethane solution was made up to 300 µl volume by adding fresh solvent and filtered through a small cartridge of Na_2SO_4 before analyzing by GC to confirm the presence of zingiberene, β-sesquiphellandrene and other dehydration products. This solution was hydrogenated in the presence of a PtO_2 (Adams) catalyst until the complete hydrogenation of bisabolatrienes to bisabolane **7,** which appears on a HP-5MS GC column as a mixture of well-separated *cis* and *trans* isomers (at *para* position of the cyclohexane ring) on the background of piperidine formed by co-hydrogenation of pyridine. The mixture was washed with 0.05 M HCl (2 x 10 ul), water (2 x 20 ul), 5% $NaHCO_3$ and dried with Na_2SO_4. The mixture was analyzed on a Hydrodex-β-6-TBDMS GC column against standards of 7*S*- and 7*R*-bisabolanes prepared analogously by dehydration/hydrogenation of 7*S*- and 7*R*-zingiberenols (3).

NMR Analysis. ^1H NMR spectra were obtained at 600 *MHz* and ^{13}C spectrum at 151 *MHz* on a Bruker AVIII-600 *MHz* spectrometer**.** Chemical shifts are reported in δ units and referenced to the residual $CDCl_3$ solvent signal; coupling constants are reported in Hz. COSY, ^{13}C-DEPT, HSQC, and NOESY spectra were also recorded to assign protons and carbons and stereochemistries of compounds **3** and **6** (Fig. S5*C*).

Gene Expression Analysis. Insects used for tissue and sex-specific gene expression analysis were killed with hexane vapor in a screw top jar and dissected in PBS (137 mM NaCl, 8.1 mM Na_2HPO_4, 1.5 mM NaH_2PO_4, 2.7 mM KCl, pH 7.2). Insects were dissected in groups of five and tissues frozen in liquid nitrogen. Three rounds of dissections were performed for a total of three replicates for five tissue groups (head, thorax, midgut, soft

tissue minus midgut and abdominal cuticle including attached epithelial cells. All samples were kept at -80°C before RNA extraction. cDNAs were generated from total RNA as described above. Relative transcript abundance was measured by quantitative (Real Time)-Reverse Transcription PCR (qRT-PCR) using the ddCt method and normalized to 18S rRNA (20). Primers were designed using Geneious (v. 7.1.9) to amplify a fragment of approximately 100 bp (Table S3) and tested for non-specific binding using the *M. histrionica* transcriptome data. Primer efficiencies were measured to be between 90-105%. Reaction plates contained 1 µL cDNA (25 ng), 0.3 µM of each primer, 7.8 µL dH_2O and 10 µL *Power*SYBR Green PCR Master Mix (Applied Biosystems) per well. The samples were analyzed on an Applied Biosystems 7300 using 40 cycles of 50°C 2 min, 95°C 20 min, 95°C 15 sec, 60°C 1 min. Primers were tested for non-specific amplification by analyzing the dissociation curve after PCR. Significance was measured using one-way analysis of variance (ANOVA) and means grouped by Tukey's HSD.

RNAi Treatment. dsRNA was prepared using the MEGAscript RNAi kit (Ambion) per manufacturer's instructions. Adult males were injected 3-5 d post eclosion ($n = 6$ per group) with 400 ng dsRNA in PBS (16) (pH 7) using a 31 ga Hamilton syringe (10 µL 1701SN, 2 inch, pt 2; Hamilton Company, Reno NV) inserted 3 mm between the pygophore and connexivum. Changes in transcript abundance were measured using qRT-PCR at 12 days post injection as above.

Volatile Collection from RNAi Treated Insects. Treated insects were allowed to recover on potted collards (Brassica oleracea 'Champion') for 3 days before cohorts of 3 males were moved to each volatile collection chamber. Volatile collection chambers were 1 L glass jars (Wide Mouth Quart Mason Jars; Ball Jar Company, Muncie, Indiana) with Teflon lids (Savilex, Minneapolis, MN). Insects were fed with organic cauliflower florets, which were replaced every other day (16L:8D photoperiod). Headspace from each cohort was collected by drawing humidified air into the container through 6−14 mesh activated charcoal (Fisher Scientific) and out of the container by vacuum (~1 L/min), through traps (15 cm × 4 mm i.d.) containing HeyeSep Q (200 mg each; Hayes Seperations Inc., Bandera, TX). After 48 h of collection, the adsorbents were eluted with CH_2Cl_2 (1 mL/sample). The

solutions were stored at 10°C before analyses. Three cohorts of each treatment were sampled for each experimental bout, and this experiment was repeated three times so that collections were taken from 9 cohorts of each treatment total. Because the data from headspace collections was non-normal and did not respond to transformation, we compared the amount of murgantiol detected in treated and control cohorts by conducting a generalized log-linear analysis assuming a Poisson distribution, followed by contrast tests for mean separation (alpha = 0.05).

Amino Acid Sequence Analysis of *Mh*TPS and *Mh*FPPS. Amino acid sequence alignment of *Mh*TPS and *Mh*FPPS with other insect TPS and IDS proteins was performed in Geneious (v. 7.1.9) using MUSCLE with full end gap penalty.

Sequence Analysis and Tree Reconstruction. Multiple sequence alignments were computed using MAFFT and default parameters (https://www.ebi.ac.uk/Tools/msa/mafft/). Based on these alignments, trees were reconstructed with MEGA6 (21) using a maximum likelihood algorithm. All positions with <80% site coverage were eliminated. A bootstrap resampling analysis with 1000 replicates was performed to evaluate the topology of the generated trees. A substitution model test was performed with MEGA6 to identify the best-fit substitution model for each dataset. The substitution models used for tree reconstructions are described in the respective figure legend.

Homology modeling and substrate docking. Homology models of *Mh*TPS and *Mh*FPPS were generated using the SWISS-MODEL server (https://swissmodel.expasy.org/) and the crystal structure of avian FPP synthase (PDB-ID: 1FPS) (22) as a template. The generated model was subject to energy minimization with the YASARA force field (www.yasara.org/minimizationserver.html) and assessed for stereochemical correctness on the basis of Ramachandran plots using ProCheck (23). Ligand docking of FPP and DMAPP was performed using the Molegro Virtual Docker software (www.clcbio.com) with ligand structures obtained from available co-crystallized structures of avian FPP synthase (PDB-ID: 1UBX and 1UBY) (24) and subsequent visualization in PyMOL.

Site directed mutagenesis. To generate selected mutants, whole plasmid PCR amplification was performed using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs) according to the manufacturer's instructions. Amplifications were performed with the *Mh*TPS- pEXP5-NT/TOPO and t*Mh*FPPS- pEXP5-NT/TOPO constructs and site-specific sense and anti-sense primers containing the desired mutations (Table S3). Mutagenized constructs were sequence verified and protein variants were expressed in *E. coli* and purified as described above prior to the analysis of TPS and IDS activity. TPS enzyme assays were performed with 2 ml of purified protein for 20 h at 30°C. SDS-PAGE was applied to verify successful expression of each protein variant.

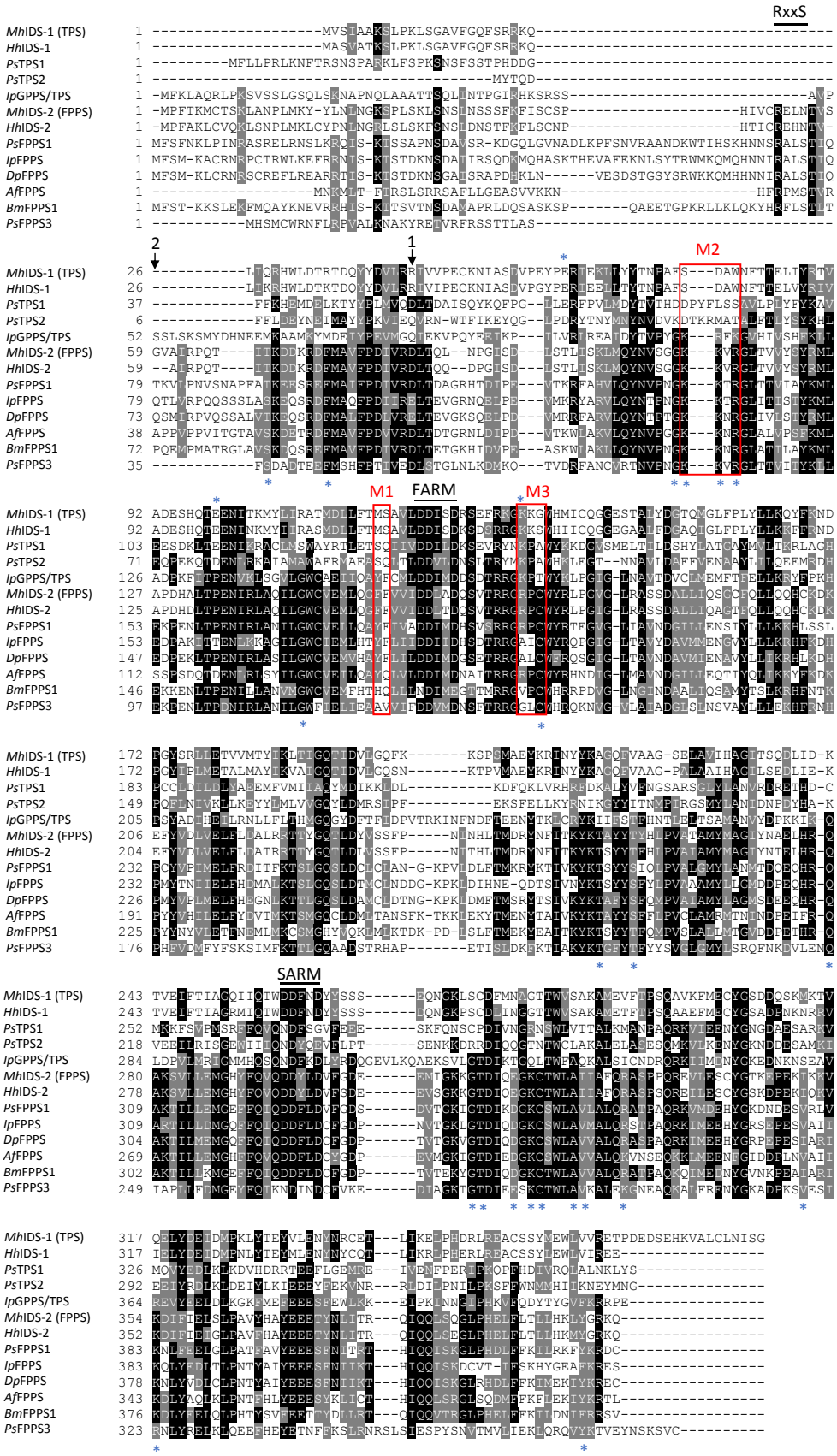


Fig. S1. Amino acid sequence alignment of *Mh*IDS-1 (*Mh*TPS) and *Mh*IDS-2 (*Mh*FPPS) with other functionally confirmed or putative Hemipteran, Lepidopteran, and Coleopteran IDS and TPS proteins. The RxxS motif (labeled) indicates a putative mitochondrial targeting sequence cleavage site. The first aspartate rich motif (FARM) and second aspartate rich motif (SARM) are also labeled. Blue asterisks mark identical or similar residues that are conserved only in IDS proteins including *Ip*GPPS/TPS (bottom) or TPS proteins (top). Red boxes mark sequences selected for site directed mutagenesis as described in the text. Arrows 1 and 2 represent truncation sites for *Mh*-IDS1 and *Mh*-IDS2, respectively. *Af*, *Aphis fabae*; *Bm*, *Bombyx mori*; *Dp*, *Dendroctonus ponderosae*; *Hh*, *Halyomorpha halys*; *Ip*, *Ips pini*; *Mh*, *Murgantia histrionica*; *Ps*, *Phyllotreta striolata*.

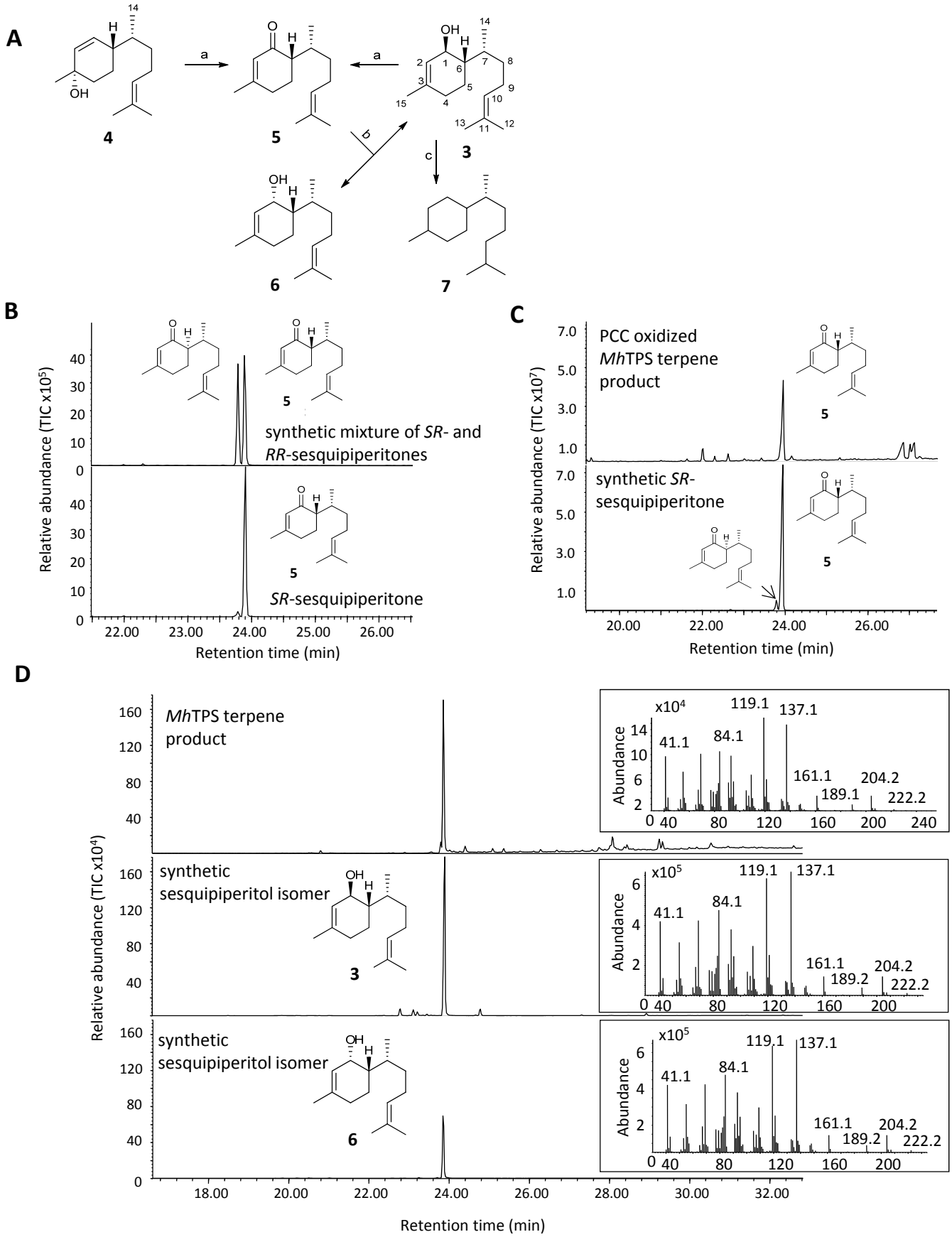


Fig. S2. Non-stereospecific identification of sesquipiperitol. (*A*) Synthesis and chemical transformations of sesquipiperitols. Reagents: a) pyridinium chlorochromate; b) $LiAlH_4$; c) 1. $POCl_3$/Py 2. H_2/PtO_2. **3**, *SSR*-sesquipiperitol; **4**, *SSR*-zingiberenol; **5**, *SR*-sesquipiperitone; **6**, *RSR*-sesquipiperitol; **7**, bisabolane. (*B*) GC-MS TIC of a synthetic mixture of *SR*-sesquipiperitone **5** and *RR*-sesquipiperitone (top) and individual **5** (bottom). (*C*) GC-MS TIC of the *Mh*IDS-1/TPS terpene alcohol product upon oxidation with PCC (top) and synthetic *SR*-sesquipiperitone **5** (bottom); on HP-5MS column. (*D*) GC-MS TIC of the *Mh*IDS-1 (*Mh*TPS) terpene alcohol product (top) and synthetic sesquipiperitol isomers **3** (middle) and **6** (bottom); cool-on-column injection on HP-5MS column; insets represent mass spectra of the corresponding compounds.

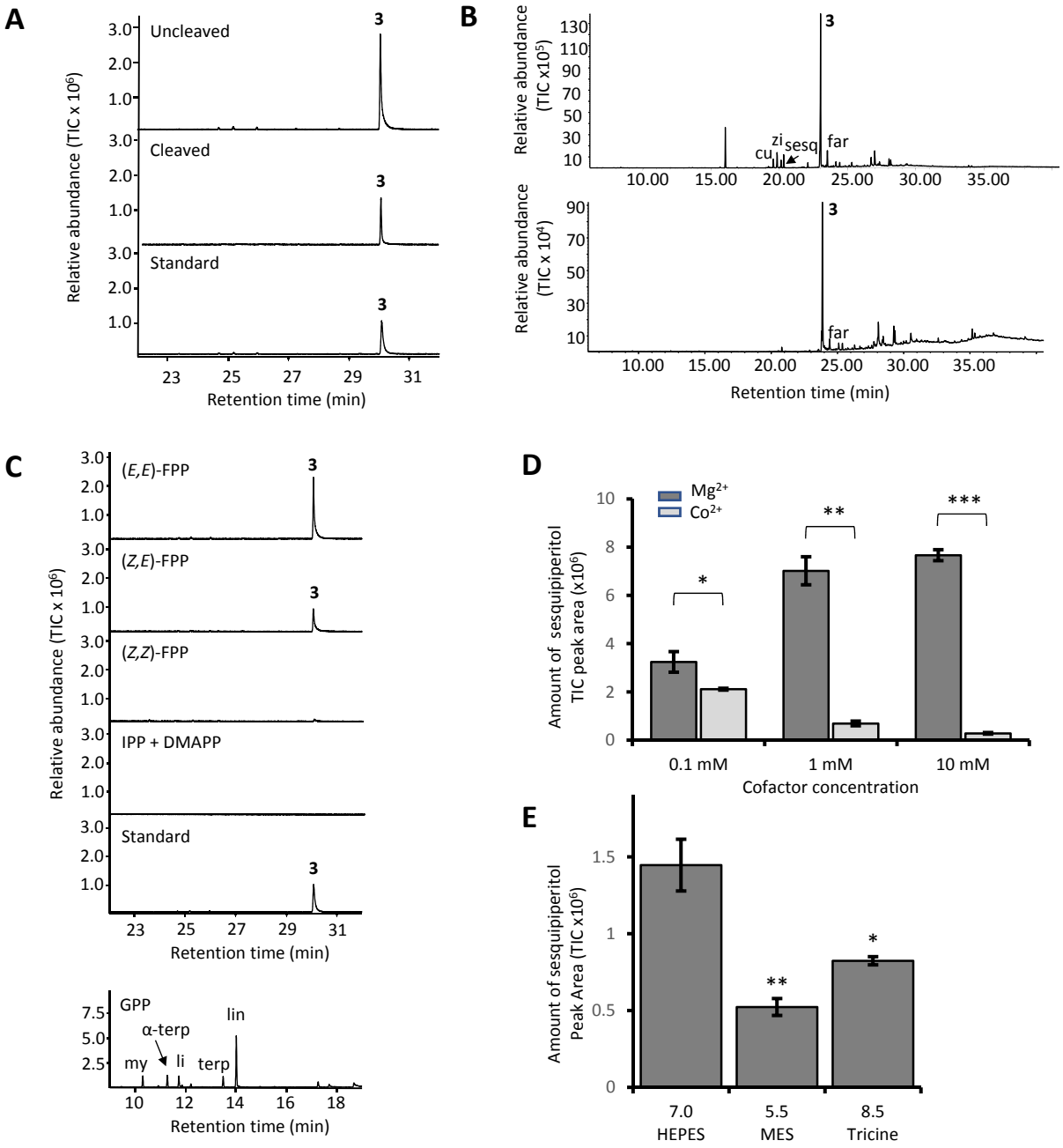


Fig. S3. Functional assays of *Mh*TPS. (*A*) TPS assay products before and after cleaving the N-terminal histidine tag using (*E*,*E*)-FPP as the substrate. (*B*) GC-MS analysis of hexane extracts from *Mh*TPS activity assays (protein with N-terminal His-tag) at different injection temperatures. Splitless injection at 260°C (top). Cool-on-column injection at 70°C (bottom). (*C*) Terpene synthase assay products with (*E*,*E*)-FPP, (*Z*,*E*)-FPP, (*Z*,*Z*)-FPP, IPP and DMAPP. Substrates were used at 50 µM except for IPP (100 µM). All assays were overlaid with hexane and extracts were analyzed by GC-MS. **3**, Sesquipiperitol; cu, γ-curcumene; far, (*E*,*E*)-farnesol; li, limonene; lin, linalool; my, myrcene; sesq, β–sesquiphellandrene; α-terp; α-terpinene; terp, terpinolene; zi, zingiberene. (*D*) Sesquipiperitol produced by *Mh*TPS under different cofactor conditions and (*E*) pH conditions using (*E,E*)-FPP as the substrate. Student's t-test, (*A*) $*P < 0.05$, $**P < 0.005$, $***P < .0005$, (*B*) $*P < 0.05$, $**P < 0.01$.

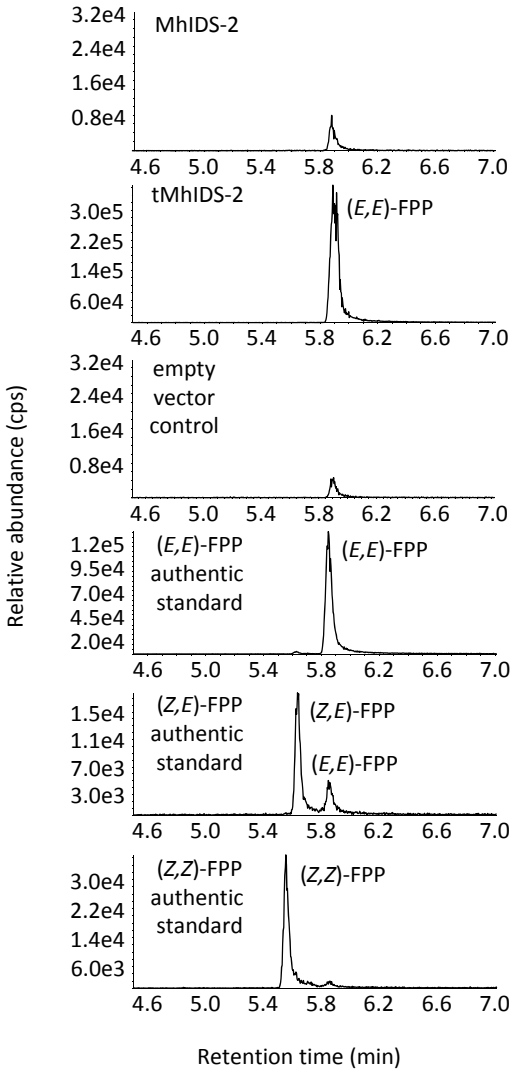


Fig. S4. LC-MS chromatograms after functional assays of *Mh*IDS-2 and t*Mh*IDS-2. 50 µM IPP and 50 µM DMAPP were provided as substrates.

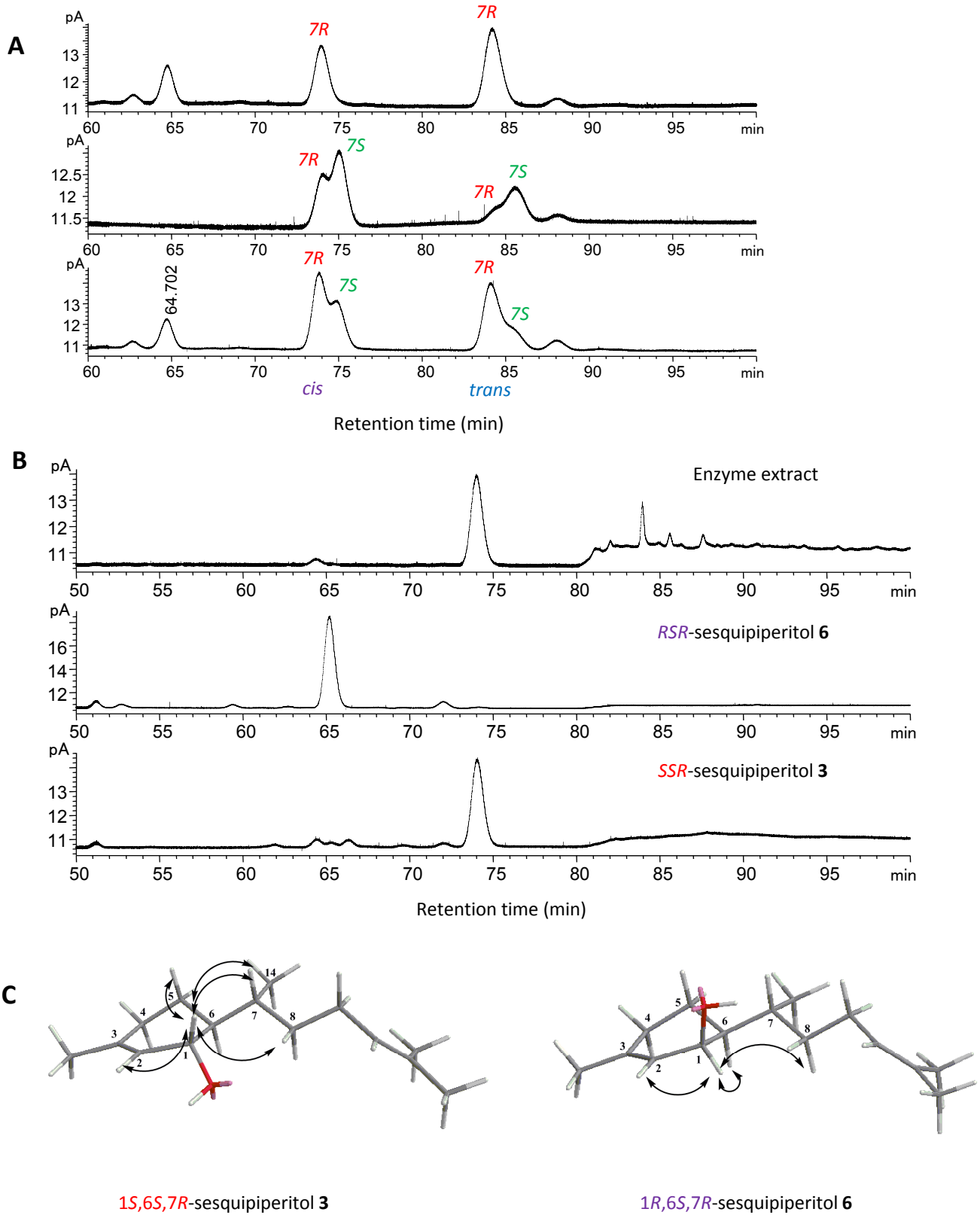


Fig. S5. Determination of the absolute configuration of sesquipiperitol. (*A*) 7*R* stereo configuration of enzymatically produced sesquipiperitol. Chiral GC analysis of (top) bisabolanes derived from enzymatically produced sesquipiperitol by dehydration/hydrogenation, (middle) bisabolane standard mixture made by dehydration/hydrogenation of (7*S*)- and (7*R*)-zingiberenols, (bottom) co-injection of both. Assignment of *cis*- and *trans*-isomers is arbitrary. (*B*) Determination of the configuration of enzymatically derived sesquipiperitol at C-1. Chiral GC analysis of (top) enzymatically produced sesquipiperitol, (middle) *RSR*-sesquipiperitol **6**, (bottom) *SSR*-sesquipiperitol **3**. Separation in (*A*) and (*B*) was performed on a on Hydrodex β-6TBDM column. (*C*) NOESY correlations of H-1 protons in sesquipiperitols **3** and **6** in MM2 minimized energy conformations in ChemBio3D Ultra 11.0. Enhancement between H-1 and H-6 was found for compound **6** but not **3**.

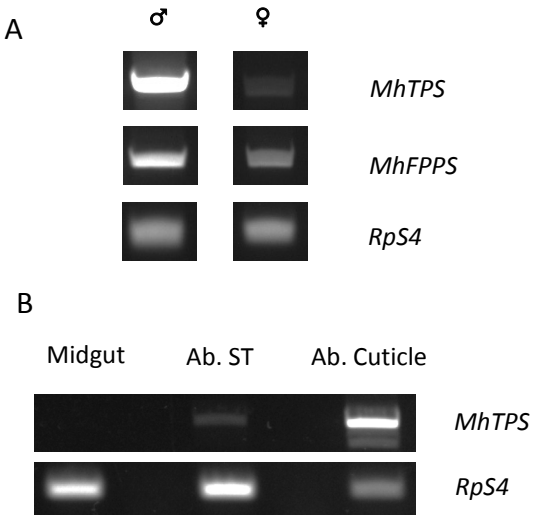


Fig. S6. Transcript abundance of *MhTPS* and *MhFPPS* in mature *M. histrionica* males and females determined by RT-PCR. (*A*) Transcript abundance by sex. (*B*) Transcript abundance in mature males by tissue. Ab. ST = Abdominal soft tissue, Ab. Cuticle = abdominal cuticle including tissue lining cuticle without soft tissue and midgut, *RpS4* = ribosomal subunit protein S4.

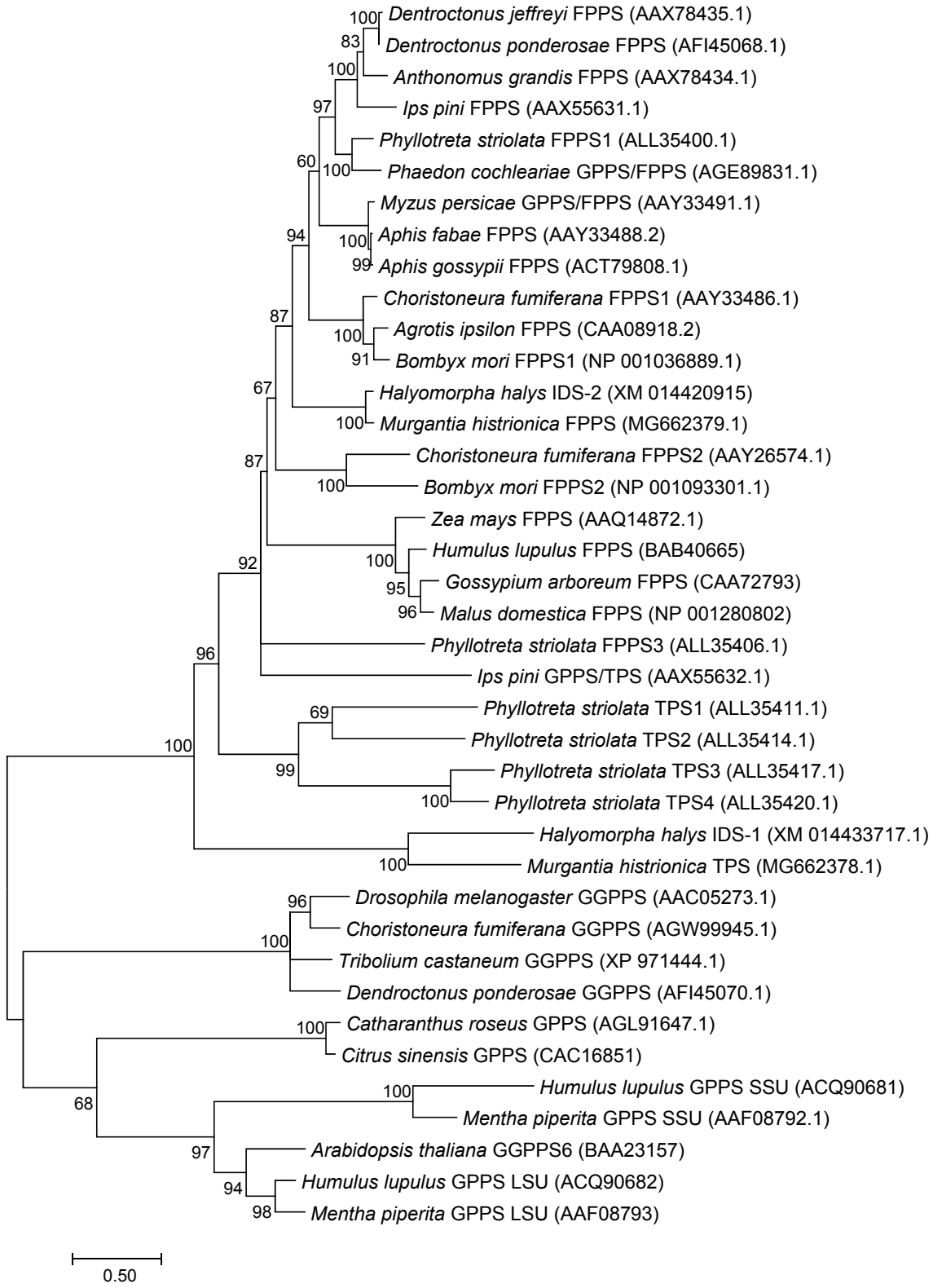


Fig. S7. Majority-rule phylogram inferred from maximum-likelihood analysis of GPPS, FPPS, GGPPS and TPS enzymes of insects and plants. The Maximum Likelihood method was based on the Le and Gascuel (25) (LG G+I) model. Bootstrap values (n = 1000 replicates) are shown next to each node. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

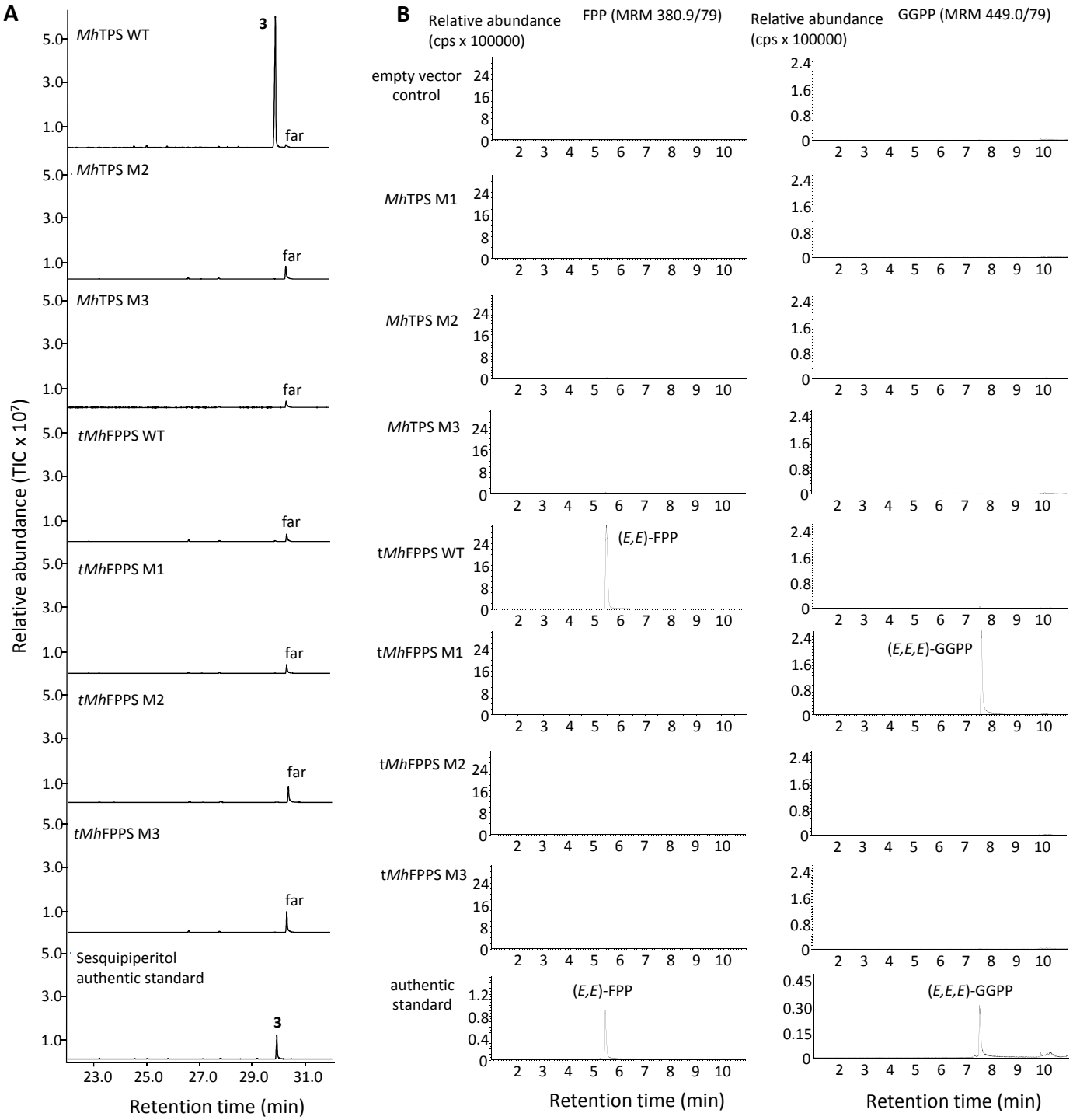


Fig. S8. TPS and IDS activity of *Mh*TPS and t*Mh*FPPS mutants. Genes were expressed in *E. coli* and affinity-purified recombinant proteins were incubated with (*E*,*E*)-FPP (*A*) or IPP and DMAPP (*B*) in the presence of the cofactor magnesium. TPS enzyme products were analyzed by GC-MS (*A*) and IDS enzyme products were analyzed using LC-MS/MS (*B*). (*E*,*E*)-Farnesol (far) occurred as an unspecific hydrolysis product of (*E*,*E*)-FPP in all TPS assays due to an extended incubation time. WT, wild type; site specific mutations: *Mh*TPS M1 - $M_{118}S_{119}$ to $F_{118}F_{119}$, *Mh*TPS M2 - $S_{77}DAW_{80}$ to $K_{77}KVR_{80}$, *Mh*TPS M3 - $K_{135}KG_{137}$ to $R_{135}PC_{137}$, t*Mh*FPPS M1 – $F_{95}F_{96}$ to $M_{95}S_{96}$, t*Mh*FPPS M2 – $K_{54}KVR_{57}$ to $S_{54}DAW_{57}$, t*Mh*FPPS M3 –$R_{112}PC_{114}$ to $K_{112}KG_{114}$. **3**, Sesquipiperitol.

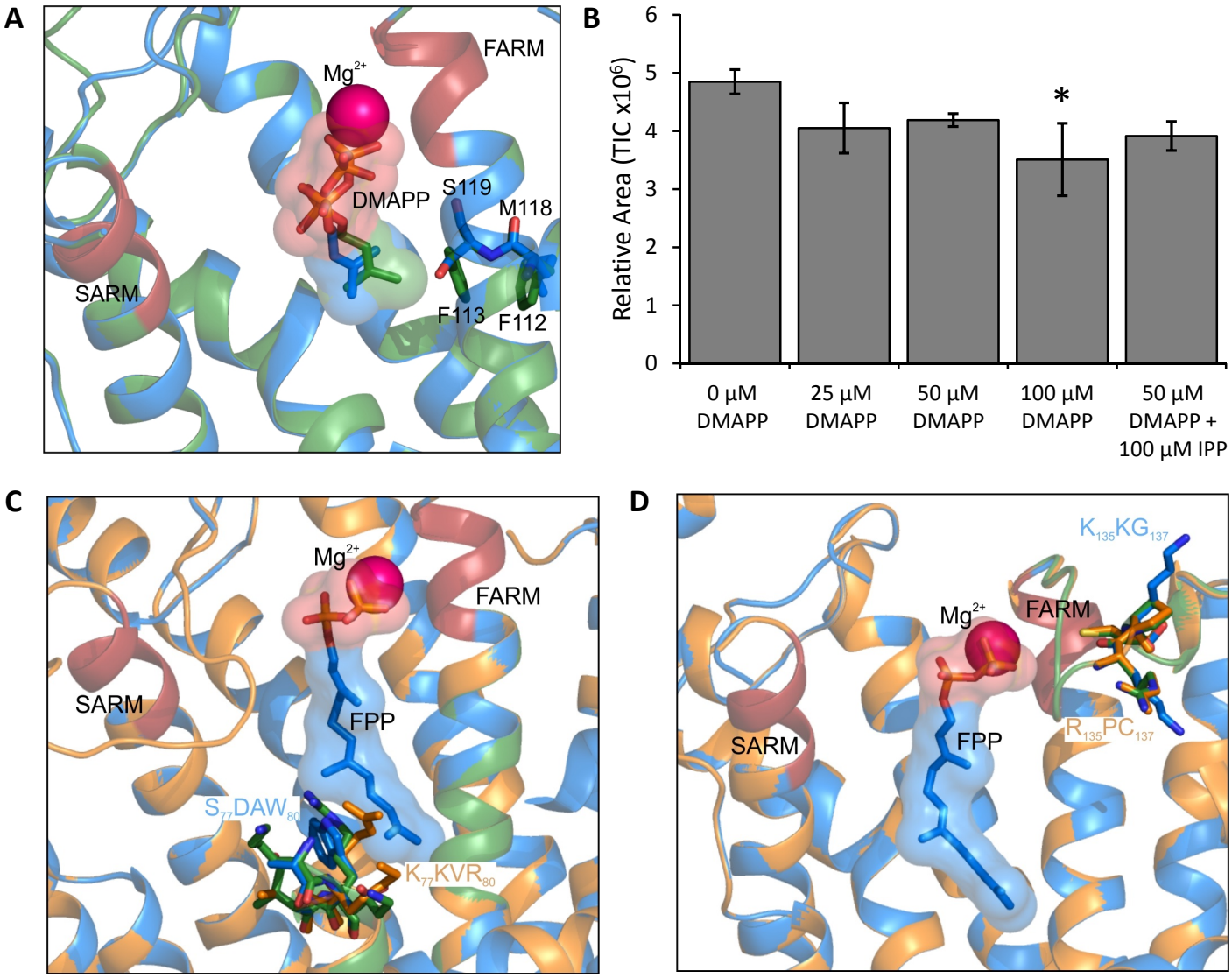
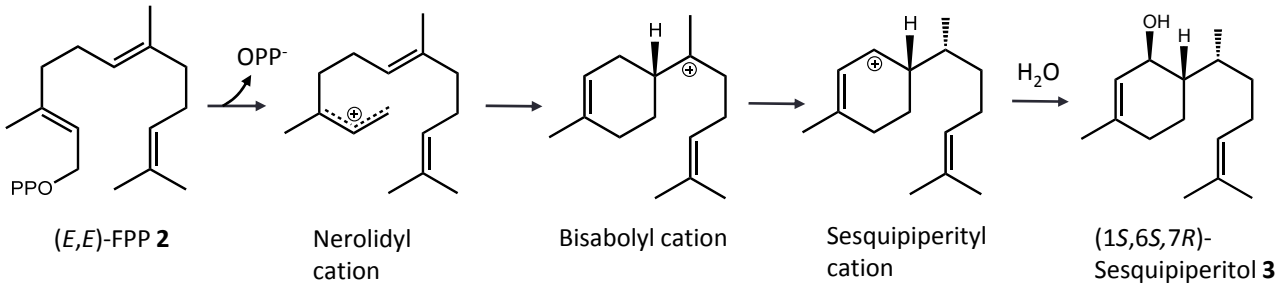


Fig. S9. (*A*) Position of DMAPP in the active site of *G. gallus* FPPS (green) and the *M. histrionica* TPS homology model (blue). (*B*) Competitive inhibition of *Mh*TPS activity by DMAPP. *Mh*TPS was assayed with 50 µM (*E,E*)-FPP and various concentrations of DMAPP and IPP (n = 3, ± SD). One-way ANOVA, $P < 0.05$. (*C,D*) Homology models of *Mh*TPS wild type (blue) and protein variants featuring the tested site-specific mutations (orange) *Mh*TPS M2 - $S_{77}DAW_{80}$ to $K_{77}KVR_{80}$ (*C*) - and *Mh*TPS M3 - $K_{135}KG_{137}$ to $R_{135}PC_{137}$ (*D*). Equivalent residue positions in the wild type *Mh*FPPS structural model are superimposed in green.



(*E*,*E*)-FPP **2** Nerolidyl cation Bisabolyl cation Sesquipiperityl cation (1*S*,6*S*,7*R*)-Sesquipiperitol **3**

Fig. S10. Proposed cyclization mechanism for the formation of sesquipiperitol.

Table S1. NMR Data for Sesquipiperitols **3** and **6**

Position	**3**		**6**	
	δ ^{1}H (600 *MHz*, *J* in Hz)	δ ^{13}C (151 *MHz*)	δ ^{1}H (600 *MHz*, *J* in Hz)	δ ^{13}C (151 *MHz*)
1	4.01 m; OH ~1.20 m	69.2	4.12 m; OH 1.07 br d, 6.6	65.4
2	5.37 br s	125.7	5.60 br d, 5.4	123.7
3		137.4		139.7
4	4a, 1.87 m 4b, 1.93 m	30.5	4a,1.93 m 4b, 1.98 dd, 18.0, 5.4	30.9
5	5a, 1.27 m 5b, 1.60 m	20.8	5a, 1.34, dddd, 13.2, 12.6, 5.4 5b, 1.66 m	20.3
6	1.30 m	46.5	1.13 m	44.2
7	1.89 m	31.0	1.55 m	32.9
8	1.29 m	35.5	8a, 1.17 m 8b, 1.57 m	34.3
9	9a, 1.95 m 9b, 2.00 m	26.2	9a, 1.92 m 9b, 2.02 m	25.2
10	5.10 tm, 6.0	124.8	5.11 tm, 6.0	125.0
11		131.2		131.2
12	1.58 br s	17.7	1.58 br s	17.7
13	1.656 br s	25.7	1.66 br s	25.7
14	0.80 d, 6.6	14.4	0.94 d, 6.6	17.2
15	1.660 br s	23.1	1.67 br s	23.4

Assignments were aided by DEPT, COSY, and HSQC recordings

Table S2. BLAST query sequences

Gene	Query Sequences (tblastn)	*M. histrionica* Acc. Nos.
MhTPS	*Ips pini* FPPS (AAX55631.1) *Ips pini* GPPS/TPS (AY953508) *Bombyx mori* FPPS (NP_001036889) *Bombyx mori* FPPS2 (NP_001093301) *Drosophila melanogaster* FPPS (NP_477380)	GECQ01420512.1; MG662378.1
MhFPPS	*Ips pini* FPPS (AAX55631.1) *Ips pini* GPPS/TPS (AY953508) *Bombyx mori* FPPS (NP_001036889) *Bombyx mori* FPPS2 (NP_001093301) *Drosophila melanogaster* FPPS (NP_477380)	GECQ01414919.1; MG662379.1

Table S3. Primers

Gene	Primers (5'-3')	Amplicon size (bp)	Purpose
MhTPS	*Mh*TPS_1F ATGGTCTCCATTGCTGCTAAG *Mh*TPS_1158R TTACCCACTAATGTTCAAACATAAAGC	1158	TA cloning into pGEM-T Easy
	*Mh*TPS_FusF1 TCAGTCGACTGGATCCGG [ATGGTCTCCATTGCTGCTAAGT]* *Mh*TPS_FusR1 CTAGATATCTCGAGTGCGGCC [CTACCCACTAATGTTCAAACATAAAGCA]*	1197	Gibson assembly into F4/F5 amplified pENTR4^{NcoI-}
	*Mh*TPS_QF ACGATGTCAGCAGTTCTAGATG *Mh*TPS_QR AGCAGTACTTTCACCCCCTTG	99	qRT-PCR
	*Mh*TPS_dsRNA1F AAGGTTGGCATATGATCTGTC *Mh*TPS_dsRNA1R CGCAACAAATTGTCCTGC	260	Generate DNA template for synthesis of dsRNA
	*Mh*TPS_dsRNA1F_T7 TAATACGACTCACTATAGGGAGAAAGGTTGGCATATGATCTGTC *Mh*TPS_dsRNA1R_T7 TAATACGACTCACTATAGGGAGACGCAACAAATTGTCCTGC	306	Add T7 sites to DNA template for synthesis of dsRNA
	*Mh*TPS_M1F GTTATTTACGTTTTTCGCAGTTCTAGATGATATCAG *Mh*TPS_M1R AAATCCATTGTAGCTCTAATTAG *Mh*TPS_M2F GTTAGAAATTTTACGACTGAACTGATTTAC *Mh*TPS_M2R TTTCTTGAATGCTGGGTTGGTGTAG *Mh*TPS_M3F TTGTTGGCATATGATCTGTCAAG *Mh*TPS_M3R GGTCTACCTTTCCTAAACTCCGAC	3903	Site-directed mutagenesis
*Mh*FPPS	*Mh*FPPS_1F ATGCCGTTTACCAAAATGTGC *Mh*FPPS_1218R TTACTGCTTTCTACCATATAACTTATGGAGT	1218	TA cloning into pGEM-T Easy
	t*Mh*FPPS_1F GGAGTTGCAATACGTCCAC *Mh*FPPS_1218R TTACTGCTTTCTACCATATAACTTATGGAGT	1045	Truncation of *MhFPPS* to remove putative transit peptide (bp 175-1218)
	t*Mh*FPPS_M1F GCTTCAAGGGATGTCAGTTGTAATAGATGATC t*Mh*FPPS_M1R ATTTCAACACACCATCCTAAG t*Mh*FPPS_M2F GCTTGGGGTCTGACAGTTGTTTAC t*Mh*FPPS_M2R ATCACTGCCTCCAGATACATTATAC t*Mh*FPPS_M3F AGGTTGGTATAGATTACCTGGTG t*Mh*FPPS_M3R TTCTTCCCCCTTCTGGTTACAGA	3789	Site-directed mutagenesis
18S rRNA	*Mh*18S_448F TTAAGTCGAACAGCCCGAGC	99	qRT-PCR

	*Mh*18S_546R TCCGAAAAACCCCGCTTTTG		
pENTR4^{NcoI-}	pENTR4_F4 GGCCGCACTCGAGATATCTA pENTR4_R5 CCGGATCCAGTCGACTGAAT	2280	Linearize pENTR4^{NcoI-} for Gibson Assembly

* brackets denote gene specific sequence

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