Supporting Information

Biochemistry and Molecular Genetics of the Biosynthesis of the Earthy Odorant Methylisoborneol in *Streptomyces coelicolor*

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Material and methods

Materials. Reagents, solvents and substrates purchased from Sigma-Aldrich were of the highest quality available and were used without further purification. Restriction enzymes were from Promega. Isopropylthio-D-galactopyranoside (IPTG) was purchased from Invitrogen. Ni-NTA affinity resin was from Qiagen. PD-10 columns were from GE Healthcare and ultrafiltration devices (UltraFree-MC 5,000 MWCO and Amicon Ultra-15 10,000 and 50,000 MWCO) were purchased from Millipore.

Methods. All DNA manipulations were performed following standard procedures.¹ DNA sequencing was carried out at the U. C. Davis Sequencing Facility, Davis, CA. Protein concentrations were determined by Bradford assay using bovine serum albumin as standard.²

Methylisoborneol Synthase Sequence Alignment:

CLUSTAL 2.0.5 multiple sequence alignment

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S_scabies_4      MPTSGPLGPLTPEQVPTPASSAAVPDAAAPAAAPDHRPVAPTASGFLAALHPPVALLVPP 60
A3KI17|A3KI17_STRAM MPDSGPLGPHSPDHRPTPAT--TVPD--APASKPPDVAVTPTASEFLAALHPP----- 49
Q9F1Y6|Y7700_STRCO MPDSGTLGTTPPEQGPPTPT--TLPD--VPAPVIPSASVTSAAADFALAALHPP----- 49
Q9F1V8|YGLK_STRGR  MPVPELPPPRSSSLPEAVTRFGASVLG-AVAARAHDS EATVGGPSGGRRPLPSPPAG----- 54
A4FG19|A4FG19_SACEN -----

S_scabies_4      PTVPTPTSPPPVEPSAAVPGPPDTTAADDALRRILRAPTGPGTASLVVADRFAPLLSPSV 120
A3KI17|A3KI17_STRAM --VPIPSPPSPSGSASAAADTPDATTVGSALQRILRGPTGPGTAALALSVRHDDPS-LPG 106
Q9F1Y6|Y7700_STRCO --VTVPDPA PPPPPAPAAAGNPPDVTGDSVLQRILRGPTGPGTTS LAPAVRYGQP-GPE 106
Q9F1V8|YGLK_STRGR  --LSFGPPSPAAPPDVP--APEAPGWGADLERLLCGPHGLGTAGLR LTP-----GKE 103
A4FG19|A4FG19_SACEN -----MIELIGHETPVPSQQQHTGGVRG----- 23
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S_scabies_4      SRAPVEPAAGRAVPGLYHHVPVEPDVPRVEEVSRRIKRWAEEEVQLYPEEWEGQFDGFSV 180
A3KI17|A3KI17_STRAM SPAPAEPAAGRAVPGLYHHVPVEPDPA RVEEVSRRIKRWAEEDEVQLYPEDWEGFDGFSV 166
Q9F1Y6|Y7700_STRCO APASAPPAAGRAVPGLYHHVPVEPDVPRVEEVSRRIKRWAEEDEVQLYPEEWEGQFDGFSV 166
Q9F1V8|YGLK_STRGR  RPVPATAREGRPIPGLYHHVPVEPD EARVEEVSRRIKAWALDEVSLYPEEWEEQFDGFSV 163
A4FG19|A4FG19_SACEN TSACTPPGVGERTTVLYC P P P P P E R P E V A A E I N R R V V W M Q G - L G L G G E D N V A G V Y K H D P 82
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S_scabies_4      GRYMVACHPDAPTVDHMLLATRLMVAENAVDDCYCEDHG---GSPVGLGGRLLLAHTALD 237
A3KI17|A3KI17_STRAM GRYMVACHPDAPTVDHMLLATRLMVAENAVDDCYCEDHG---GSPVGLGGRLLLAHTAID 223
Q9F1Y6|Y7700_STRCO GRYMVACHPDAPTVDHMLLATRLMVAENAVDDCYCEDHG---GSPVGLGGRLLLAHTAID 223
Q9F1V8|YGLK_STRGR  GRYMVACHPDAPTVDHMLLATRLMVAENAVDDCYCEDHG---GSPVGLGERLLLAHTALD 220
A4FG19|A4FG19_SACEN RGITLCHPGSQDVERMTAAGKMIVAETAVDDYFCETNSRRDANDQTI GP NLSLAQSAID 142
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S_scabies_4      HFHTTA EYAPAWQESLASDAPRRAYRSAMDHFVGAATPSQADRYRHDMARLHLGYLAEEA 297
A3KI17|A3KI17_STRAM PFHTTA EYAPPWRSLTSDAPRRAYRSAMDYFVRAATPSQADRYRHDMARLHLGYLAEEA 283
Q9F1Y6|Y7700_STRCO HFHTTA EYTPTWQASLAADAPRRAYDSAMGYFVRAATPSQSDRYRHDMARLHLGYLAEEA 283
Q9F1V8|YGLK_STRGR  PLYTAREYQPGWAASLHADAPRRAYRSAMDYFVRAAGPSQADRLRHDMARLHLGYLAEEA 280
A4FG19|A4FG19_SACEN APRLT PDLQALWNKCRDDHPVLR AQHEAFGLERISSPAQAQRVRHDI AQLYLGYN AENG 202
                        : : . * . . . * . * : : : * * * : * * * : * * * * .

S_scabies_4      WAQTGHVPEVWEYLAMRQFNFRPCPTITDTVGGYELPADLHARPDMQRVIALAGNATTI 357
A3KI17|A3KI17_STRAM WAQTDHVPVWEYLAMRQFNFRPCPTITDTVGGYELPADLHARPDMQRVIALAGNATTI 343
Q9F1Y6|Y7700_STRCO WAQTGHVPEVWEYLAMRQFNFRPCPTITDTVGGYELPADLHARPDMQRVIALAGNATTI 343
Q9F1V8|YGLK_STRGR  WAQQDQVPEVWEYLAMRQFNFRPCPTITDTVGGYELPADLHAQAAMQKVIALASNATTI 340
A4FG19|A4FG19_SACEN WRLLNRLPPVWQYLANRQMNSFRPCNLTDALDGYELAPQLYAHPLVQDC TARA TLIATL 262
* . : : * * * : * * * * * . : * : : * * * * . : * * * : : *

S_scabies_4      VNDLYSYTKELDSPGHHLNLPVVAIERERLPVRDAYLKAVEVHNELQHAFEAASAE LAEA 417
A3KI17|A3KI17_STRAM VNDLYSYTKELDSPGRHLNLPVVAIERERLSE RDAYLKAVEVHNELQHAFEAASAE LAKA 403
Q9F1Y6|Y7700_STRCO VNDLYSYTKELNSPGRHLNLPVVAIEREQ LCE RDAYLKAVEVHNELQHSFEAAAAD LAEA 403
Q9F1V8|YGLK_STRGR  VNDLYSYTKELAAPGRHLNLPVVAIEREGLSDQDAY LK SVEIHNELMHAFES EAAALAAA 400
A4FG19|A4FG19_SACEN YNDLASCEREIREHGLPFNLPAVIAAEERIALDEAFVRACEIHNELIQALEEATGH AASA 322
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S_scabies_4      CPLPAVLRFLKGVAAWVDGNHDWHRTNTYRYSLP DFW 454
A3KI17|A3KI17_STRAM CPLPTVLRFLKGVAAWVDGNHDWHRTNTYRYSLP DFW 440
Q9F1Y6|Y7700_STRCO CPLPPVLRFLRGVAAWVDGNHDWHRTNTYRYSLP DFW 440
Q9F1V8|YGLK_STRGR  CPVPSVQRFLRGVAAWVDGNHDWHRSNTYRYSLP DFW 437
A4FG19|A4FG19_SACEN LADPALSRYL TGLWSWLAGSRHWHFTTARHRA----- 354
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Figure S1. ClustalW2 multiple sequence alignment of presumptive methylisoborneol synthases with *S. coelicolor* SCO7700 (UniProt ID Q9F1Y6). (EBI Clustal W Server, <http://www.ebi.ac.uk/Tools/clustalw2/index.html>. S_Scabies_4, transl. (rev) nt 572281-570620, http://www.sanger.ac.uk/Projects/S_scabies/, *S. scabies* strain 87.22; A3KI17, *S. ambofaciens* ATCC 23877; Q9F1Y6, *S. coelicolor* A3(2); Q9F1V8, *S. griseus* strain 13189; A4FG19, *Saccharopolyspora erythraea* NRRL 2338.

Expression and Purification of SCO7700. The *sco7700* gene was amplified by PCR from the genomic DNA of *S. coelicolor* A3(2). Primers (Fwd7700A: 5'- ATG GGC ATC AAC GAA CAG GC -3'; Rev7700A: 5'- GCG GTG GTG GTC TCA GTG GT -3') were designed to amplify a 1556-bp fragment flanking the targeted gene, which is then amplified by using the 1.56-kb amplified DNA fragment as a PCR template and the designed primers (Fwd7700: 5'- CAC ACA **CAT ATG** CCC GAC TCC GGG ACC CTC -3'; Rev7700: 5'- CAC ACA **CTC GAG** CTA CCA GAA GTC GGG CATG GCT -3') to introduce NdeI and XhoI restriction sites (**bold**), respectively. The amplificon was purified, treated with NdeI and XhoI restriction enzymes, and ligated into the expression vector pET-28a that had been digested with the same enzymes. The resulting plasmid pET28a-*sco7700* was transformed into *E. coli* XL-1 Blue competent cells by electroporation, and the insert sequence was verified by resequencing. The pET28a-*sco7700* construct was then transformed into *E. coli* BL21(DE3) competent cells by electroporation. The *E. coli* BL21(DE3) cells harboring the pET28a-*sco7700* construct were grown in Luria-Bertani broth containing 50 mg/L kanamycin. Cultures were incubated at 37 °C with shaking. At an OD₆₀₀ of 0.7, IPTG was added to a final concentration of 0.2 mM, and the culture was incubated overnight at 18 °C with shaking. The cells were harvested by centrifugation and suspended in 30 mL lysis buffer (50 mM Tris, 300 mM NaCl, 10 mM imidazole, 5 mM β-mercaptoethanol, pH 7.0). The suspension was lysed by sonication and centrifuged. The supernatant was bound to Ni-NTA resin (5 mL) that had been equilibrated with lysis buffer by shaking at 4 °C for 1 h. The resin slurry was applied to a column and prewashed with buffer containing 10 mM to 30 mM imidazole. The protein was eluted with 70 mM imidazole buffer (50 mM Tris, 300 mM NaCl, 70 mM imidazole, 5 mM β-mercaptoethanol, pH 7.0) at a flow rate of 2 ml · min⁻¹. The SCO7700-containing protein fractions (>95% pure) were pooled, concentrated by YM-10 centiprep, and

exchanged into storage buffer (50 mM PIPES, 20% (vol/vol) glycerol, 10 mM MgCl₂, 100 mM NaCl, 5 mM β-mercaptoethanol, pH 7.0) with a PD-10 desalting column. Typical yield is ~6 mg protein/L culture. The protein was flash-frozen in liquid nitrogen and stored at -80 °C.

Expression and Purification of SCO7701. The *sco7701* gene was amplified by PCR from the genomic DNA of *S. coelicolor* A3(2). Primers (Fwd7701A: 5'- CGC TAC AGC CTG CCC GAC TT -3'; Rev7701A: 5'- GGT GCG CTG ATG CGT TTC AT -3') were designed to amplify a 1056-bp fragment flanking the targeted gene, which is then amplified by using the 1.05-kb fragment as a PCR template and the designed primers (Fwd7701: 5'- GAC GTA **CAT ATG ACC** ACT GAG ACC ACC ACC -3'; Rev7701: 5'- GGT CCG **CTC GAG** TCA CAC GCG GTC GG -3') to introduce NdeI and XhoI restriction sites (**bold**), respectively. The amplificon was purified, treated with NdeI and XhoI restriction enzymes, and ligated into the expression vector pET-28a that had been digested with the same enzymes. The resulting plasmid pET28a-*sco7701* was directly electro-transformed into *E. coli* XL-1 Blue competent cells, and the insert sequence was verified by resequencing. The pET28a-*sco7701* construct was then transformed into *E. coli* BL21- CodonPlus competent cells by electroporation. The *E. coli* BL21(DE3) cells harboring the pET28a-*sco7701* construct were grown in Luria-Bertani broth containing 50 mg/L kanamycin. Cultures were incubated at 37 °C with shaking. At an OD₆₀₀ of 0.7, IPTG was added to a final concentration of 0.2 mM, and the culture was incubated overnight at 16 °C with shaking. The cells were harvested by centrifugation and suspended in 30 mL lysis buffer. The suspension was lysed by sonication and centrifuged. The supernatant was bound to Ni-NTA resin (5 mL) that had been equilibrated with lysis buffer by shaking at 4 °C for 1 h. The resin slurry was applied to a column and prewashed with buffer containing 10 mM to 30 mM imidazole. The protein was eluted with 70 mM imidazole buffer at a flow rate of 2 ml · min⁻¹. The SCO7701-

containing protein fractions (>95% pure) were pooled, concentrated by YM-10 centriprep, and exchanged into storage buffer with a PD-10 desalting column. Typical yield is ~5 mg protein/L culture. The protein was flash-frozen in liquid nitrogen and stored at -80 °C.

Incubation of SCO7700 with GPP and GC-MS Analysis. Purified recombinant SCO7700 (25 μ M) was incubated with 60 μ M GPP in 4 mL of assay buffer (50 mM PIPES, 10 mM MgCl_2 , 100 mM NaCl, pH 7.0), overlaid with 4 mL of HPLC-grade pentane, 18 h at 30 °C. The reaction mixture was initially extracted with 4 $\times$ 3 mL pentane, and after vigorous shaking (followed by centrifugation, if necessary, to facilitate separation of phases) the pentane extract was eluted through a mini silica gel column (0.5 $\times$ 3mL), which was further washed with another 0.5 mL of pentane. The original reaction mixture was then re-extracted with an equal amount of diethyl ether, and the ether extract was passed through the same silica gel column, which was further washed with an additional 1.5 mL of ether. Lastly, any traces of ether were removed from the extracted reaction mixture under a stream of nitrogen followed by the addition of 4 mL phosphatase solution (3 units of wheat germ acid phosphatase and 2 units of potato apyrase in 1.0 mL of 0.1 M sodium acetate, pH 5).³ The mixture was overlaid with HPLC-grade pentane, incubated for 2 h at 30 °C, and was then extracted with an equal amount of ether. The three combined pentane and ether eluates were dried over Na_2SO_4 and concentrated to 200 μ L under reduced pressure at 0 °C. A 1- μ L portion of the concentrated extract was analyzed by GC-MS (Hewlett-Packard Series 2 GC-MSD, 70 eV, EI, positive ion mode; 30 m $\times$ 0.25 mm HP5MS capillary column, using a temperature program of 50-280 °C, a temperature gradient of 20 °C/min and a solvent delay of 4.0 min). The incubation gave multiple monoterpene products, including cyclic hydrocarbons (parent peaks at m/z 136 and base peak at m/z 93): α -pinene (r.t. 6.01 min), β -pinene (r.t. 6.45 min), limonene (r.t. 6.91 min), γ -terpinene (r.t. 7.16 min), and δ -

terpinene (r.t. 7.43 min) (**Figure S2**) and alcohols (parent peaks at m/z 154 and base peak at m/z 93): β -terpineol (r.t. 7.26 min), 4-terpineol (r.t. 8.17 min), and α -terpineol (r.t. 8.26 min) (data not shown).

Figure S2. GC/MS TIC of pentane extract from the incubation of GPP (60 μ M) with SCO7700 (25 μ M) at 30 $^{\circ}$ C for 18 h.

Incubation of SCO7701 with SAM and GPP and GC-MS Analysis. Purified recombinant SCO7701 (25 μ M) was incubated with 60 μ M GPP and 120 μ M S-adenosyl-L-methionine (SAM) in 4 mL of assay buffer at 37 $^{\circ}$ C for 2 h. 4 mL of phosphatase solution was added to the reaction mixture, which was overlaid with HPLC-grade pentane, incubated for 2 h at 30 $^{\circ}$ C, and was then extracted with 4 $\times$ 3 mL ether. The combined organic layers were dried over Na_2SO_4 and concentrated to 200 μ L under reduced pressure at 0 $^{\circ}$ C. A 1- μ L portion of the concentrated extract was analyzed by GC-MS. The analysis of the ether-extractable products revealed the formation of 2-methylgeraniol with a characteristic m/z 168, corresponding to $\text{C}_{11}\text{H}_{20}\text{O}$ (r.t. 9.18 min) (**Figure S3A**), which shows an exact GC-MS result match with the spectrum of the synthetic trans-(*E*)-2-methylgeraniol (**Figure S3B**).

Figure S3. GC/MS of (A) (*E*)-2-methylgeraniol (**9**) from incubation of GPP (60 μ M) and SAM (120 μ M) with SCO7701 (25 μ M) at 37 $^{\circ}$ C for 18 h; (B) synthetic (*E*)-2-methylgeraniol (**9**).

Incubation of SCO7701 with SAM and GPP and NMR Analysis. 60 μ M GPP and 120 μ M SAM were incubated with purified recombinant SCO7701 (2 μ M) in 100 mL of assay buffer at 37 $^{\circ}$ C for 18 h. The reaction mixture was pre-extracted with ether to remove glycerol and any hydrolyzed GPP, then lyophilized down to a volume of 15 mL. The mixture was passed through a mini CHP-20P column (0.5 $\times$ 3mL) that had been equilibrated with H₂O. The resin slurry was prewashed with 10 mL H₂O followed by 10 mL of 5% aq. acetonitrile. The 5% acetonitrile eluate was collected and lyophilized. ³¹P NMR (400 MHz, D₂O) δ -4.60 (P _{β} , d, J = 21 Hz), -8.16 (P _{α} , d, J = 21 Hz) (**Figure S4**).

Figure S4. ^{31}P NMR (D_2O , 400 MHz) spectrum of enzymatically-generated 2-methylGPP.

Incubation of SCO7701 with SAM and GPP and NMR Analysis. GPP (60 μM) and 120 μM SAM were incubated with purified SCO7701 (2 μM) in 100 mL of assay buffer at 37 °C for 18 h. Phosphatase solution (100 mL) was added to the reaction mixture, which was overlaid with HPLC-grade pentane, and incubated for 2 h at 30 °C, then extracted with an equal volume of diethyl ether. The combined organic layers were dried over Na_2SO_4 and concentrated under reduced pressure at 0 °C and the ^1H NMR spectrum of the hydrolyzed product was recorded (**Figure S5**).

Figure S5. ¹H NMR (CDCl₃, 400 MHz) spectrum of A) enzymatically-generated **9**; B) synthetic (*E*)-2-methylgeraniol (**9**).

Coupled Incubation of SAM and GPP with SCO7700 plus SCO7701 and GC-MS Analysis.

Purified recombinant SCO7701 (25 μM) was incubated with 60 μM GPP and 120 μM SAM in 4 mL of assay buffer (18 h, 37 °C). Purified recombinant SCO7700 (25 μM) was then added to the reaction mixture, which was overlaid with 4 mL HPLC-grade pentane, and incubated for an additional 18 h at 30 °C. The reaction mixture was extracted with 4 × 3 mL diethyl ether, and the combined ether extracts were dried over Na₂SO₄ and concentrated to 200 μL under reduced

pressure at 0 °C. A 1- μ L portion of the concentrated extract was analyzed by GC–MS. The incubation gave a single product: 2-methylisoborneol (r.t. 8.25 min, m/z 168, base peak m/z 95) (**Figure S6**) with GC retention time and MS spectrum identical by direct comparison to authentic 2-methylisoborneol. 2-methylbornene (r.t. 6.83 min, m/z 150, base peak m/z 107) was also detected in trace amount if extracted only with pentane (data not shown).

Figure S6. GC/MS for the coupled incubation of SAM and GPP with SCO7701 plus SCO7700.

Incubation of SCO7700 with 2-MeGPP and GC-MS Analysis. Purified recombinant SCO7700 (25 μ M) was incubated with 60 μ M 2-methylGPP in 4 mL of assay buffer overlaid with 4 mL of HPLC-grade pentane (18 h, 30 °C). The reaction mixture was extracted with 4 $\times$ 3 mL diethyl ether, and after vigorous shaking (followed by centrifugation, if necessary, to

facilitate separation of phases) the ether extract was eluted through a mini silica gel column (0.5 × 3mL), which was further washed with additional 1.5 mL of ether. The combined ether eluate was dried over Na₂SO₄ and concentrated under reduced pressure at 0 °C to 200 µL. A 1-µL portion of the concentrated extract was analyzed by GC–MS. The incubation gave a single product: 2-methylisoborneol (r.t. 8.25 min, *m/z* 168, base peak *m/z* 95) (**Figure S7**), identical by GC retention time and MS spectrum to the incubation product of the coupled incubation of SAM and GPP with SCO7700 plus SCO7701 (**Figure S6**) as well direct comparison to an authentic sample (Sigma).

Figure S7. GC/MS for incubation of 2-methylGPP (60 µM) with SCO7700 (25 µM) at 30 °C overnight.

SCO7700 Kinetic Assays with GPP. Assay mixtures contained 50 mM PIPES, pH 7.0, 10 mM MgCl_2 , 100 mM NaCl, variable substrate $[1\text{-}^3\text{H}]\text{-GPP}$ (0.125 to 7.5 μM ; 170 Ci/mol), and 0.6 μM SCO7700 recombinant protein in a total volume of 1 mL. The assay mixtures, overlaid with 1.2 mL of hexanes, were incubated at 30 °C for 45 min. The reactions were terminated by the addition of 75 μL of 500 mM EDTA (pH 8.0) followed by immediate vortexing for 30 s. The organic layer was loaded onto a mini silica gel column (2 cm) in a Pasteur pipette and expelled with a stream of nitrogen into a scintillation vial containing 7 mL of Opti-Fluor. The samples were extracted further with two 1-mL portions of ether, and the extracts were applied to the same mini column, which was further washed with 750 μL of ether. The combined extracts were counted by liquid scintillation counter. Kinetic constants were determined by using the Kaleidagraph program and fitting the data to the Michaelis-Menten equation, which gave the values of $K_M = 2.8 \pm 0.5 \mu\text{M}$, $k_{\text{cat}} = 3.0 \pm 0.2 \times 10^{-5} \text{ s}^{-1}$ (**Figure S8**). Reported standard deviations in the steady-state kinetic parameters represent the calculated statistical errors in the non-linear, least squares regression analysis.

Figure S8. Michaelis-Menten plot of the reaction velocity of the formation of monoterpene products as a function of the concentration of GPP.

SCO7700 Kinetic Assays with 2-methylGPP. Assay mixtures contained 50 mM PIPES, pH 7.0, 10 mM MgCl₂, 100 mM NaCl, variable substrate [1-³H]-2-methylGPP (0.5 to 50 μM; 6.92 Ci/mol), and 0.12 μM SCO7700 recombinant protein in a total volume of 1 mL. The assay mixtures, overlaid with 1.2 mL of hexanes, were incubated at 30 °C for 15 min. The reactions were terminated by addition of EDTA, extracted, and analyzed as described above. Kinetic constants were determined by using the Kaleidagraph program and fitting the data to the Michaelis-Menten equation. $K_M = 26 \pm 12 \mu\text{M}$, $k_{\text{cat}} = 3.9 \pm 0.9 \times 10^{-2} \text{ s}^{-1}$ (**Figure S9**).

Figure S9. Michaelis-Menten plot of the reaction velocity of the formation of 2-methylisoborneol as a function of the concentration of 2-methylGPP.

SCO7701 Kinetic Assays. Kinetic parameters (V_{max} and K_M) were obtained for GPP and SAM (*S*-[methyl-³H]-SAM, PerkinElmer) under initial velocity conditions. Values for the variable substrate GPP (2 to 30 μM) were determined at one saturating SAM concentration (60 μM; 160 Ci/mol), and similarly the values for variable SAM (2 ~ 30 μM) were measured at a saturating concentration (60 μM) of GPP. The reactions were all carried out in 0.5 mL SCO7701 kinetics buffer (50 mM PIPES, 20% (vol/vol) glycerol, 10 mM MgCl₂, 100 mM NaCl, 5 mM β-

mercaptoethanol, pH 7.0) with 0.52 μM SCO7701 recombinant protein, and were incubated at 37 °C for 5 min. Reactions were quenched with 0.5 mL 2N HCl in 83% ethanol, and the mixtures, overlaid with 1.2 mL hexanes, incubated at 37 °C for another 10 min to hydrolyze the acid-labile pyrophosphate and then neutralized by adding 0.35 mL 10% NaOH.⁴ The reaction mixtures were extracted with Et₂O and the extracts were counted by liquid scintillation. Kinetic constants were determined by using the Kaleidagraph program and fitting the data to the Michaelis-Menten equation, which gave the values of $K_{\text{M_GPP}} = 13.1 \pm 1.4 \mu\text{M}$ and $K_{\text{M_SAM}} = 4.3 \pm 0.3 \mu\text{M}$ and the reaction $k_{\text{cat}} = 7.5 \pm 2.5 \times 10^{-3} \text{ s}^{-1}$ (Figure S10 and S11).

Figure S10. Michaelis-Menten plot of the reaction velocity of the formation of 2-methylgeranyl diphosphate (**3**) as a function of the concentration of GPP at a fixed saturating concentration of SAM. $K_{\text{M_GPP}} = 13.1 \pm 1.4 \mu\text{M}$; $k_{\text{cat}} = 9.2 \pm 0.5 \times 10^{-3} \text{ s}^{-1}$.

Figure S11. Michaelis-Menten plot of the reaction velocity of the formation of 2-methylgeranyl diphosphate (**3**) as a function of the concentration of SAM at a fixed saturating concentration of GPP. $K_{M_SAM} = 4.3 \pm 0.3 \mu\text{M}$; $k_{\text{cat}} = 5.7 \pm 0.2 \times 10^{-3} \text{ s}^{-1}$.

Synthesis of 2-methylgeraniol.

Scheme S1. Synthesis of (*E*)-2-Methylgeranyl Diphosphate (**S1a**).

Ethyl 2,3,7-Trimethylocta-2,6-Dienoate (3).⁵ Sodium hydride (344 mg, 8.60 mmol, 60% in mineral oil) was washed with hexanes and suspended in THF (4 ml) under nitrogen. Ethyl 2-(diethoxyphosphoryl)propanoate (**S6**) (2.05 g, 8.60 mmol) dissolved in THF (1 ml) was added drop-wise to the suspension at 0 °C over 5 min. The mixture was stirred at 30-40 °C for 1 h when hydrogen evolution ceased. After cooling to 0 °C, 6-methylhept-5-en-2-one (**S5**, 1.09 g, 8.60 mmol) in 1 mL THF was added and the mixture was stirred for 24 h at 60 °C (**Scheme S1**). Water (10 mL) was added, the layers were separated, and the aqueous layer was extracted with hexanes. The combined organic layers were washed with water, dried over Na₂SO₄, and concentrated to give 396 mg (21.9% crude yield) of yellow liquid. Column chromatography on silica gel using hexanes/diethyl ether (9:1) was performed to give a 321 mg (17.8%) of 1:1 trans(*E*):cis(*Z*)-**S3**.

(*E*)-2,3,7-trimethylocta-2,6-dien-1-ol (S2).⁶ LiAlH₄ (119 mg, 3.14 mmol, 2.0 equiv) in dry Et₂O (4 mL) under N₂ was cooled to 0 °C, followed by addition of absolute EtOH (190 μL, 3.24 mmol, 2.1 equiv) by syringe over 5 min. The mixture was stirred for another 10 min, and then a

solution of the (*E*)- and (*Z*)-**S3** mixture (321 mg, 1.53 mmol) in dry Et₂O (3 ml) was added over 5 min. Additional Et₂O (2 × 1 mL) was used to ensure complete transfer. The resulting gray suspension was stirred for 20 h at 0 °C, and then for another 12 h at room temperature. Reaction was quenched by recooling the mixture to 0 °C, and H₂O (120 μL), 15% NaOH (120 μL), and H₂O (360 μL) were added slowly at 10-min intervals. The white, flocculent precipitate was removed by suction filtration. The filtrate was dried over NaSO₄ and concentrated to give 257 mg (100% crude yield) of 1:1 trans(*E*)-**9**:cis(*Z*)-**10** (**Figure S12**). Initial purification was performed by flash chromatography on silica gel [hexanes/EtOAc (5:1)]. Fractions containing (*Z*)-**10** (38-46) were collected. The residue was further purified by flash chromatography on AgNO₃-impregnated silica gel [hexanes/EtOAc (10:1)]. Fractions #33-41 containing (*Z*)-**10** were combined with previously collected fractions and concentrated to give 90 mg (34.9%) of pure (*Z*)-**10** while fractions #92-140 were combined and concentrated to give 73 mg (28.2%) of pure (*E*)-**9** (**Figure S13**).

Figure S12. GC trace of purified 1:1 (*E*):(*Z*)-Ethyl 2,3,7-Trimethylocta-2,6-Dienoate (**S3**) isomer mixture.

Figure S13. GC trace of purified (*E*)-2,3,7-trimethylocta-2,6-dien-1-ol (**S2**).

The two isomers were also readily resolved by capillary GC-MS, with ret. times of 9.06 min ((*Z*)-**10**) and 9.17 min ((*E*)-**9**) (Figure S13). The (*E*)- and (*Z*)- isomers were unambiguously distinguished by a combination of HSQC, HMBC, and NOESY analysis (data not shown). (*E*)-2-methylgeraniol (**9**) showed a NOESY crosspeak between the allylic H-1 protons at δ 4.04 and the *cis*-C3-methyl at δ 1.69, while (*Z*)-**10** showed the corresponding crosspeak between the H-1 (δ 4.09) and the *cis*-H-4 methylene protons at δ 2.16. The chemical shifts of all protons were unambiguously assigned (**Scheme S2**). Arrows represent NOE correlations. ^1H NMR (C_6D_6) (**E**)-**9**: δ 1.66 (s, 3H), 1.69 (s, 3H), 1.77 (s, 3H), 1.84 (s, 3H), 2.17 (m, 4H), 4.04 (d, $J = 4$ Hz, 2H), 5.31 (tm, $J = 7$ Hz, 1H); (**Z**)-**10**: δ 1.62 (s, 3H), 1.68 (s, 3H), 1.76 (s, 3H), 1.81 (s, 3H), 2.17 (m, 4H), 4.09 (d, $J = 4$ Hz, 2H), 5.26 (tm, $J = 7$ Hz, 1H).

Scheme S2. ^1H NMR assignments for (*E*)-**9** and (*Z*)-**10**, showing key NOESY crosspeaks.

(E)-2-methylgeranial (S4). To a solution of (E)-S2 (9) (97.4 mg, 0.58 mmol) in CH₂Cl₂ (15 mL) was added the MnO₂ (988 mg, 11.60 mmol, 20 equiv.) The heterogeneous reaction mixture was stirred at room temperature for 48 h. The mixture was filtered and concentrated to give 85 mg (E)-2-methylgeranial (88 % crude yield). Product was checked by TLC (hexanes:EtOAc = 5:1; R_f = 0.61). (E)-S4 ¹H NMR (CD₂Cl₂): δ 1.56 (s, 3H), 1.66 (s, 3H), 1.72 (s, 3H), 1.76 (s, 3H), 2.21 (m, 4H), 5.18 (t, *J* = 7 Hz, 1H), 10.16 (s, 1H).

(E)-2-Methylgeranyl Pyrophosphate, [1-³H] (S1b). To NaBH₃T solution (aq. NaOH, 100 mCi, 1 mCi/mL, 1.18 μmol, American Radiolabeled Chemicals) under argon, cooled in an ice/H₂O bath, was added an ice-cold solution of (E)-S4 (84.7 mg, 0.51 mmol) in dry EtOH (1.5 mL). The resulting clear, colorless solution was stirred at 0 °C for 3 h, and the reaction was chased by slow addition of unlabeled NaBH₄ powder (15 mg, 0.4 mmol). The resulting solution was warmed to room temperature and stirred overnight. After a total of 22 h, the reaction was quenched by carefully transferring the mixture into ice-cold half-saturated aqueous NH₄Cl (5 mL). The mixture was gently swirled and then shaken to completely mix the layers. The aqueous layer was further extracted with Et₂O (5 × 5 mL). The combined extracts were dried, filtered, and concentrated to give 83 mg of yellowish liquid (97% crude yield). The residue was purified by flash chromatography on AgNO₃-impregnated silica gel [EtOAc/hexanes (1:5); 2 x 16 cm column]. Concentration of fractions #29-50 gave 64 mg of [1-³H]-(E)-S2 (9) (75% yield).

To [1-³H]-(E)-S2 (9) (64 mg, 0.38 mmol) CCl₄ (5 mL) was added triphenylphosphine (410 mg, 1.56 mmol), and the mixture was refluxed for 48 h. The mixture was dried, triturated with pentane (10 mL), and filtered by suction. Concentration of the pentane eluent provided 104 mg of crude [1-³H]-2-methylgeranyl chloride which was used in the subsequent reaction without further purification. To the chloride under nitrogen was added a solution of [(n-Bu₄)N]₃HP₂O₇ (1

g, 1.113 mmol, 2.0 equiv) in dry CH₃CN (3 mL) by syringe and the reaction was run at room temperature for 3 days. The solvent was removed under a gentle stream of N₂ to give a viscous, light brown liquid, followed by addition of 10 mL dH₂O, forming a white cloudy solution, and ether extraction (5 × 3 mL) to remove organic impurities.

The resulting aqueous phase was applied to a DEAE-Sephadex column that had been equilibrated with 0.05 M KHCO₃. The column was first washed with 100 mL 0.05 M KHCO₃ and then with a linear gradient of 0.05 M KHCO₃ (300 mL) to 1.0 M KHCO₃ (300 mL) at a flow rate of 2.0 mL/min. Fractions (8 mL) were collected automatically and analyzed by scintillation counting. Fractions #21-51 were combined and lyophilized. The resulting white powder was dissolved in 50 mL dH₂O and applied to a CHP-20P column equilibrated with dH₂O. The column was firstly washed with 100 mL dH₂O, followed by 100 mL 5% aq. CH₃CN and then 100 mL 10% aq. CH₃CN. Fractions were analyzed by scintillation counting, and fractions #4–15 of 5% acetonitrile eluent and #1-7 of 10% acetonitrile eluent were collected and lyophilized to yield 90 mg of purified [1-³H] trans-2-methylgeranyl pyrophosphate (72% yield, specific activity 6.92 mCi/mmol).

(*E*)-2-Methylgeranyl Pyrophosphate (S1a). To (*E*)-S2 (15.5 mg, 0.092 mmol) in CCl₄ (5 mL) was added triphenylphosphine (99 mg, 0.37 mmol), and the mixture was refluxed for 48 h. Reaction completeness was checked by TLC (hexanes:EtOAc = 5:1; product R_f = 0.57) The mixture was dried, triturated with pentane (10 mL), and filtered by suction. Concentration of the pentane eluate provided 28.7 mg crude 2-methylgeranyl chloride which was used in the subsequent reaction without further purification. To the chloride under nitrogen was added a solution of [(n-Bu₄)N]₃HP₂O₇ (165 mg, 1.11 mmol, 2.0 equiv) in dry CH₃CN (3 mL) by syringe and the reaction was run at room temperature for 3 days. The solvent was removed under a

gentle stream of N₂ to give a viscous, light brown liquid, followed by addition of 10 mL dH₂O, forming a white cloudy solution, and ether extraction (5 x 3 mL) to remove organic impurities. The resulting aqueous phase was purified by chromatography on DEAE-Sephadex and CHP-20P as was described for the purification of **S1b**. Fractions containing phosphates were checked by Malachite Green assay to give 10.3 mg of pure (*E*)-2-methylgeranyl pyrophosphate (34% yield). ¹H NMR (D₂O): δ 1.53 (s, 3H), 1.59 (s, 3H), 1.66 (s, 3H), 1.67 (s, 3H), 2.02 (m, 4H), 4.35 (d, *J* = 4.8 Hz, 2H), 5.14 (tm, *J* = 7 Hz, 1H).; ³¹P NMR (D₂O) δ -5.10 (P_β, d, *J* = 25 Hz), -9.04 (P_α, d, *J* = 25 Hz).

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