High Versatility of IPP and DMAPP Methyltransferases Enables Synthesis of C₆, C₇ and C₈ Terpenoid Building Blocks

Laura Drummond†, Parab J. Haque†, Binbin Gu, Julia S. Jung, Hendrik Schewe, Jeroen S. Dickschat, and Markus Buchhaupt*
Table of Contents

S1  Chemicals, Media, and Microorganisms
S2  Vector construction
S3  Enzyme expression and purification
S4  \textit{In vitro} MTase reactions
S5  \textit{De novo} terpenoid production in \textit{E. coli} strains containing the mevalonate pathway and one of the MTases
S6  HS-SPME-GC/MS analysis

Supplementary Table 1 Primers used in this study

Supplementary Figure S1. SDS-PAGE analysis of purified enzymes

Supplementary Figure S2. Graphic representation of genes encoding putative SAM-dependent prenyl pyrophosphate MTases and flanking genes

Supplementary Figure S3. GC chromatograms (TIC) of the reference compounds which could be assigned to MTase products.

Supplementary Figure S4. Mass spectra of all compounds detected in \textit{in vivo} or \textit{in vitro} experiments and of respective reference compounds

Supplementary Figure S5. Total ion chromatograms of DMAPP conversion product and reference compounds

Supplementary Figure S6. Synthesis of A) (2S)-2-methyl-IPP and B) (2R)-2-methyl-IPP.

S7  Synthesis of (R)- and (S)-methyl 3-(\textit{tert}-butyldiphenylsilyloxy)-2-methylpropanoate (S2)
S8  Synthesis of (R)- and (S)-3-(\textit{tert}-butyldiphenylsilyloxy)-\textit{N}-methoxy-\textit{N},2-dimethylpropanamide (S3)
S9  Synthesis of (R)- and (S)-4-(\textit{tert}-butyldiphenylsilyloxy)-3-methylbutan-2-one (S4)
S10 Synthesis of (S)- and (R)-\textit{tert}-butyl((2,3-dimethylbut-3-en-1-yl)oxy)diphenylsilane (S5)
S11 Synthesis of (S)- and (R)-2,3-dimethylbut-3-en-1-ol (S6)
S12 Synthesis of (S)- and (R)-2,3-dimethylbut-3-en-1-yl 4-methylbenzenesulfonate (S7)
S13 Synthesis of (S)- and (R)-2,3-dimethylbut-3-en-1-yl diphosphate (S8)

Supplementary Figures S7 – S28 NMR analyses
S1: Chemicals, Media, and Microorganisms

_E. coli_ strain DH5α (New England Biolabs) was used for plasmid construction. _E. coli_ BL21 (DE3) (Novagen) was used for His-tag protein expression. _E. coli_ MG1655 (DE3) Δ_**recA**_Δ_**endA**_ ΔtnaA was used for _de novo_ terpenoid production experiments. It was constructed from _E. coli_ MG1655 (DE3) _ΔrecA_ Δ_**endA**_ (Addgene #37854) with the Quick and Easy _E. coli_ Gene Deletion Kit (GeneBridges, Heidelberg) and the oligonucleotides DtnaA-1 and DtnaA-2. _E. coli_ strains were grown in LB medium or TB medium containing the appropriate antibiotics (ampicillin at 50 μg/L, kanamycin at 50 μg/L, gentamycin 10 μg/L), at 37°C and 180 rpm. Solid medium was prepared by the addition of 1.5 % agar-agar (m/v).

Solid-Phase Micro-Extraction (SPME) fibers, DTT, acid phosphatase, IPP, S-Adenosylmethionine (SAM), RNAses, DNAses and protease inhibitor were purchased from Sigma-Aldrich. Reference compounds purchased from the company Enamine Ltd (Riga, Latvia) are named here with corresponding product numbers: (E)-4-methyl-prenol (Z3488308004), (Z)-4-methyl-prenol (Z3773228680), 2,4-dimethyl-isoprenol (Z1896357284), 2,5-dimethyl-isoprenol (Z1896382803), 4,4-dimethyl-prenol (FCH2520219), 4,5-dimethyl-prenol (EN300-2524336), 4,5-dimethyl-isoprenol (Z3681219426) and 5,5-dimethyl-isoprenol (EN300-123672) were synthesized by Enamine Ltd (Riga, Latvia). For all C7 compounds, mixtures of diastereomers or enantiomers were used. 3-ethyl-4-methylpent-4-en-1-ol was synthesized by Genosynth (Berlin, Germany). All standard compounds had purities of at least 95% and had their identities confirmed by NMR and GC-MS by the synthesizing companies.

S2: Vector construction

Construction of pET-28a(+) vectors was carried out by BioCat GmbH (Heidelberg, Germany) or via Gibson assembly. The following sequences were used in codon-optimized or natural forms to construct the respective vectors: pPJH-1 (WP_091072690 from _M. humi_, humMT), pPJH-2 (WP_032365004 from _R. fascians_, fasMT), pPJH-3 (WP_030281021 from _S. catenulae_, catMT), pPJH-4 (WP_076843458 from _Frankia_ sp., fraMT), pPJH-5 (WP_005164808 from _A. azurea_, azuMT), pPJH-6 (BAF98640 from _S. argenteolus_, argMT). Construction of pMK-24 containing the monMT-encoding gene has been described.[1] The methyltransferase-encoding genes from the extracted genomic DNA of _M. humi_ DSM 45647, _R. fascians_-1 DSM 20699 and _A. azurea_ DSM 43854 were amplified via PCR using genespecific primers (Supplementary Table 1) to form the vectors pPJH-1, pPJH-2 and pPJH-6. Successful construction of the vectors was validated by Sanger sequencing. Vectors pPJH-3, pPJH-4, pPJH-5, and pPJH-7 were synthesized by BioCat GmbH via standard restriction-cloning. Vector pLD-03 was used to provide high IPP and DMAPP levels and its construction has been described.[1]

S3: Enzyme expression and purification

_E. coli_ strain BL21 (DE3) was transformed with one of the pET-28a(+) derivatives containing one of the MTase-encoding genes. LB pre-cultures were used to inoculate main cultures with 1 L TB medium containing ampicillin (50 μg/ml) or kanamycin (50 μg/ml). After cultivation for 2 h at 37 °C (OD600 of 0.8), IPTG at 100 μM was added for induction. After cultivation at 18 °C, 110 rpm overnight, cells were harvested by centrifugation for 20 minutes at 4000 g and 4°C then re-suspended in 15 ml lysis buffer. Ultrasound sonication was performed on ice with 20 % amplitude (0.5 seconds pulse, 1 second pause, 4 minutes pulse time), followed by digestion with RNase (10 μg/ml) and DNase (5 μg/ml) on ice for 10 min. After centrifugation for 20 minutes at 4000 g and 4 °C, enzyme purification took place using His-Pur Ni-NTA Spin
Columns (Thermo Fischer Scientific) according to the manufacturer’s recommendations using the following buffers. Assay buffer (pH 6.7) consisted of 20 mM Tris-HCl, 100 mM NaCl, 15 mM MgCl₂, and 5 mM DTT in ultrapure water. Lysis, wash and elution buffers had pH of 8 and consisted of 50 mM Tris-HCl, 500 mM NaCl, 5 mM DTT with increasing concentrations of imidazole through each step: 10 mM (lysis), 30 mM (wash) and 250 mM (elution). Purified proteins were analyzed via SDS-PAGE using 15 µg of protein (Figure S1). Glycerol, Tris-HCl, NaCl, imidazole, ampicillin and kanamycin were purchased from Carl Roth GmbH.

**S4: In vitro MTase reactions**

Purified protein at 25 µM final concentration was added to 1 mL assay buffer (20 mM Tris-HCl, 100 mM NaCl, 15 mM MgCl₂, and 5 mM DTT, pH 6.7 in ultrapure water) containing 120 µM SAM and 60 µM of IPP, DMAPP, GPP or FPP. The enzymatic reaction was incubated at 30 °C overnight. Then 1 ml phosphatase solution (acid phosphatase 7.5 mg/ml in 0.1 M sodium acetate, pH 5.0) was added and the reaction was incubated at 30 °C for 2 h. Following the reaction, volatiles in the headspace were extracted with a SPME fibre for 20 minutes.

**S5: De novo terpenoid production in E. coli strains containing the mevalonate pathway and one of the MTases**

The production strain MG1655 harbored the plasmid pMK-16 for provision of high IPP and DMAPP levels and pET-28a derivatives containing the sequence of a MTase. Pre-cultures in reaction tubes containing 5 mL LB medium with appropriate antibiotics were incubated overnight at 37°C and 180 rpm. Main cultures with 15 mL TB medium and appropriate antibiotics in 100 ml baffled shake flasks were inoculated from pre-culture to an OD₆₀₀ of 0.1. After cultivation at 37°C to an OD₆₀₀ value of 1, gene expression was induced with isopropyl-β-D-1-thiogalactopyranoside (IPTG, 100 µM). Induced cultures had terpenoids extracted during the following 24 hours of cultivation at 30°C and 180 rpm with a magnetic adsorbent stir bar (Twister®, Gerstel). The twister was attached to the shake flask with the help of a magnet fixated to the external part of the flask, thus preventing the twister from being loose on the culture medium. If indicated, unlabeled L-methionine, ¹³C-methyl-L-methionine or (methyl-d₃)-L-methionine were supplemented (C_final = 3 g/L) to the pre- and main cultures.

**S6: HS-SPME-GC/MS analysis**

Volatile compounds in the headspace of in vitro assays and cultures were analyzed by extraction with an 85 µm stableflex SPME fiber composed of PDMS and Carboxen. The SPME fibre placed in a fibre holder (Gerstel) and attached to a stainless steel plunger sheathed by a protective needle (Supelco) was exposed in the headspace of each assay and culture and then inserted into the injection port of a GC-MS-QP2010 (Shimadzu) containing a DB-5 (5 % phenyl)-methylpolysiloxane column with 30 m length and 0.25 mm thickness. Measurements were conducted as follows: helium as carrier gas, splitless injections at 250 °C, 1 minute sampling time, and column flow of 1.1 mL/min. The column temperature was programmed to start with 40 °C for 1.5 minute then rising in steps of 10°C/min until 250 °C followed by rising in 20 °C/min steps up to 300°C. Compounds were identified via comparison of mass spectra and retention indices (RI) to the ones of reference substances or mass spectra of the NIST mass spectral library (v14).

For chiral analysis of the product of DMAPP methylation by humMT, an Astec CHIRALDEX B-DA cyclodextrin stationary phase column connected to an inactivated guard column was employed in a GC-MS QP2010 (Shimadzu) system. An SPME fibre was used for extraction of volatiles on the headspace of the in vitro assays with a final step of phosphatase reaction, as
described in the paragraph above. To elucidate product chirality, the two enantiomers (2R)-2-methyl-IPP and (2S)-2-methyl-IPP (synthesis described on Supplementary Information S3) were analyzed separately, by adding each synthetic prenyl pyrophosphate at a final concentration of 6 µM to a solution containing 7.5 mg/mL acid phosphatase. After incubation for 30 min at 40 °C, an SPME fibre was used to explore the headspace of the assay for 10 min at 40 °C. The retention times of both enantiomers were then compared to the one from the product generated by enzymatic assay. SPME-GC-MS measurements were conducted as follows: helium as carrier gas, splitless injections at 260 °C, 1 minute sampling time, and column flow of 0.84 mL/min. The column temperature was programmed to start with 40 °C for 2 minutes then rising in steps of 5 °C/min until 120 °C, hold for 40 minutes followed by rising in 10 °C/min steps up to 180 °C, hold for 5 minutes. Temperature was then decreased at a rate of -10 °C/min until 130 °C, hold for 1 minute and then decreased at a rate of -5 °C until 40 °C. Compounds were identified via comparison of mass spectra and retention indices (RI) to the ones of reference substances.

**Supplementary Table 1**

| Name    | Sequence                                                                 | Purpose                                   |
|---------|--------------------------------------------------------------------------|-------------------------------------------|
| pPJH-1A | GCTAGCATGACTGGTGGACAGCAAATGGGTATGTCGATC GATCTCGGC                        | Construction of pPJH-1                    |
| pPJH-1B | CGGATCTCAGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGTCGCGTGG                      | Construction of pPJH-1                    |
| pPJH-2A | GCTAGCATGACTGGTGGACAGCAAATGGGTATGCCGAAC CTCGACGTG                       | Construction of pPJH-2                    |
| pPJH-2B | CAGTGGTGGTGGTGGTGGTGGTCAGACGCCTCGACGC                                  | Construction of pPJH-2                    |
| pPJH-4A | CATGACTGGTGGACAGCAAATGGGTATGCTCCAACAA CAGCTCC                         | Construction of pPJH-4                    |
| pPJH-4B | GATCTCAGTGGTGGTGGTGGTGGTGGTGGCGATCGGTGTTCCG                           | Construction of pPJH-4                    |
| DtnaA-1 | TGTAATTTCACAGGGATCAGACTGTAATTTTTAATAATAGGA GATTATCTATATAGGGAAGACTGAAAA   | Deletion of tnaA                         |
| DtnaA-2 | TGTAATTTCACAGGGATCAGACTGTAATTTTTAATAATAGGA GATTATCTATATAGGGAAGACTGAAAA   | Deletion of tnaA                         |
**Supplementary Figure S1** SDS-PAGE analysis of purified enzymes. 15 µg of purified protein was used per lane. Gels were stained with Coomassie Brilliant Blue. Page ruler Prestained protein ladder, 10 – 180 kDa (Thermo Fischer Scientific) was used as protein size reference. Lanes were spliced to show the purified protein sample beside the respective protein ladder from the same gel, which is indicated by the black lines.
Supplementary Figure S2  Graphic representation of genes encoding putative SAM-dependent prenyl pyrophosphate MTases and flanking genes. The red arrows represent the putative prenyl pyrophosphate MTase-encoding genes selected for further analysis. Putative functions of the proteins encoded upstream and downstream of the MTase gene were identified by the search for specific protein domains and by the search for similar protein sequences with known function.
Supplementary Figure S3. GC chromatograms (TIC) of the reference compounds which could be assigned to MTase products. Red dots in the respective structures indicate the positions of the additional methyl groups. In case of 4,5-dimethyl-isoprenol it is possible to see two peaks corresponding to the two isomers present in the mixture, although it is unclear which peak represents which isomer.
Supplementary Figure S4. Mass spectra of all compounds detected in *in vivo* or *in vitro* experiments and of respective reference compounds. In all cases, in which an enzymatically or microbially synthesized compound could be clearly assigned to one of the reference compounds, both mass spectra are shown as comparison. In the case of unknown C7 compound 6 only the mass spectrum of detected compound is shown.

S4A. Comparison of MS of 1 detected (upper) and 5-methyl-isoprenol reference compound (lower)
S4B. Comparison of MS of 2 detected (upper) and (Z)-4-methyl-isoprenol (lower).
S4C. Comparison of MS of 3 detected (upper) and one of the stereoisomers of 4,5-dimethylisoprenol (lower).
S4D. Comparison of MS of 4 detected (upper) and one of the stereoisomers of 4,5-dimethyl-isoprenol (lower).
S4E. Comparison of MS of 5 detected (upper) and (E)-4-methyl-prenol (lower).

S4F. Mass spectrum of unknown C7 compound 6 detected.
Comparison of MS of 7 detected (upper) and (Z)-4-methyl-prenol (lower).
S4H. Comparison of MS of 8 detected (upper) and 4,4-dimethyl-prenol (lower).
S4I. Comparison of MS of 9 detected (upper) and (E)-4-methyl-isoprenol (lower).
S4J. Comparison of MS of 10 detected (upper) and 4,4-dimethyl-isoprenol (lower).
S4K. Comparison of MS of 11 detected (upper) and 5,5-dimethyl-isoprenol (lower).
S4L. Comparison of MS of 12 detected (upper) and prenol (lower).
S4M. Comparison of MS of 13 detected (upper) and a mixture of enantiomers of 2-methylisoprenol (lower).
S4N. Comparison of MS of 14 detected (upper) and 3-ethyl-4-methylpent-4-en-1-ol (lower).
Supplementary Figure S5. Total ion chromatograms of (A) the reaction product from DMAPP incubated with enzyme humMT and SAM followed by incubation with acid phosphatase, (B) incubation of synthetic prenyl pyrophosphate \((2R)\)-2-methyl-IPP with acid phosphatase and (C) incubation of synthetic prenyl pyrophosphate \((2S)\)-2-methyl-IPP with acid phosphatase.
Supplementary Figure S6. Synthesis of A) (2S)-2-methyl-IPP and B) (2R)-2-methyl-IPP.

S7: Synthesis of (R)- and (S)-methyl 3-(tert-butyldiphenylsilyloxy)-2-methylpropanoate (S2)

In a literature-known procedure, the (R)-Roche ester ((R)-S1, 2.00 g, 16.9 mmol, 1.0 eq.) and imidazole (1.62 g, 23.8 mmol, 1.4 eq.) were dissolved in CH₂Cl₂ (100 mL) and the mixture was stirred at room temperature. TBDPSCI (5.67 g, 20.4 mmol, 1.2 eq.) was added dropwise and the reaction mixture was stirred overnight at room temperature. The reaction was quenched by adding H₂O (200 mL). The mixture was extracted three times with diethyl ether (3 x 200 mL). The combined organic layers were dried with MgSO₄ and the solvent was removed under reduced pressure. Column chromatography on silica gel [cyclohexane/ethyl acetate (20:1)] resulted in the silylated (R)-Roche ester (R)-S2 (5.25 g, 14.7 mmol, 87%) as colorless oil. The same procedure was used to convert (S)-Roche ester ((S)-S1, 2.00 g, 16.9 mmol, 1.0 eq) into (S)-S2 (6.05 g, 16.9 mmol, 99%).

(R)-S2: 1H-NMR (500 MHz, C₆D₆): δ[ppm] = 7.80-7.74 (m, 4H, 4 x CH), 7.26-7.19 (m, 6H, 6 x CH), 3.86 (dd, J = 9.7, 7.0 Hz, 1H, 0.5 x CH₂), 3.72 (dd, J = 9.7, 5.5 Hz, 1H, 0.5 x CH₂), 3.39
(s, 3H, CH₃), 2.71-2.54 (m, 1H, CH), 1.15 (s, 9H, 3 x CH₃), 1.02 (d, J = 7.1 Hz, 3H, CH₃). ¹³C-NMR (126 MHz, C₆D₆) δ [ppm] = 174.7 (C₁₆), 136.0 (CH), 136.0 (CH), 134.0 (C₅), 133.9 (C₅), 130.1 (CH), 128.1 (CH), 128.1 (CH), 66.4(CH₃), 51.2 (CH₃), 42.7(CH), 27.0 (3 x CH₃), 19.5 (C₅), 13.6 (CH₃). TLC [cyclohexane/ethyl acetate (20:1)]: Rf = 0.36. GC (HP-5MS): I₆ = 2243. MS (EI, 70 eV): m/z (%) = 325 (3), 299 (82), 269 (4), 237 (9), 213 (100), 199 (16), 197 (16), 183 (56), 181 (26), 153 (20), 135 (13), 105 (24), 91 (10), 77 (8), 57 (9), 41 (9). [α]D²⁰ = −17.2° (c 1.8, CH₂Cl₂).

(S)-S2: [α]D²⁰ = +14.6° (c 1.5, CH₂Cl₂). Spectroscopic data as for the (R) enantiomer.

S8: Synthesis of (R)- and (S)-3-(tert-butyldiphenylsiloxy)-N-methoxy-N₂-dimethylpropanamide (S3)

Ester (R)-S2 (5.25 g, 14.7 mmol, 1.0 eq.) and N,O-dimethylhydroxylamine hydrochloride (4.28 g, 44.1 mmol, 3.0 eq.) were suspended in THF (65 mL) and cooled to −15 °C. A solution of tert-PrMgCl (2 M in Et₂O, 18.4 mL, 36.8 mmol, 2.5 eq.) was added dropwise and the reaction mixture was stirred at −15 °C for 1 h. After completion of the reaction (monitored by TLC), the reaction was quenched by addition of sat. aq. NH₄Cl (25 mL) at 0 °C. The mixture was extracted three times with EtOAc (3 x 60 mL). The combined organic layers were washed with sat. NaCl (40 mL), dried over MgSO₄, and concentrated in vacuo. Column chromatography on silica gel [cyclohexane/ethyl acetate (5:1)] gave the amide (R)-S3 (4.92 g, 12.8 mmol, 87%) as white solid. The same procedure was used to convert ester (S)-S2 (6.05 g, 16.9 mmol, 1.0 eq.) into (S)-S3 (6.05 g, 15.7 mmol, 93%).

(R)-S3: ¹H-NMR (500 MHz, C₆D₆): δ [ppm] = 7.89-7.85 (m, 2H, 2 x CH), 7.83-7.79 (m, 2H, 2 x CH), 7.29-7.19 (m, 6H, 6 x CH), 4.20 (dd, J = 9.5, 8.5 Hz, 1H, 0.5 x CH₂), 3.68 (dd, J = 9.4, 5.8 Hz, 1H, 0.5 x CH₂), 3.24 (m, 2H, 2 x CH₂), 2.93 (s, 3H, CH₃), 2.18 (s, 9H, 3 x CH₃), 1.04 (d, J = 6.9 Hz, 3H, CH₃). ¹³C-NMR (126 MHz, C₆D₆) δ [ppm] = 175.9 (C₁₆), 136.2 (2 x CH), 136.0 (2 x CH), 134.4 (C₆), 133.9 (C₅), 130.0 (CH), 130.0 (CH), 128.1 (2 x CH), 128.1 (2 x CH), 126.9 (CH₂), 61.1 (CH), 38.4 (CH₃), 32.1 (CH), 27.1 (3 x CH₃), 19.5 (C₅), 14.0 (CH₃). TLC [cyclohexane/ethyl acetate (5:1)]: Rf = 0.35. GC (HP-5MS): I₆ = 2497. MS (EI, 70 eV): m/z (%) = 328 (100), 296 (6), 213 (6), 199 (26), 197 (16), 183 (12), 181 (11), 135 (14), 105 (9), 91 (4), 82 (4), 77 (6), 57 (7), 41 (6). [α]D²⁰ = −13.9° (c 1.8, CH₂Cl₂).

(S)-S3: [α]D²⁰ = +10.2° (c 1.6, CH₂Cl₂). Spectroscopic data as for the (R) enantiomer.

S9 Synthesis of (R)- and (S)-4-(tert-butyldiphenylsiloxy)-3-methylbutan-2-one (S4)

Amide (R)-S3 (4.92 g, 12.8 mmol, 1.0 eq.) was dissolved in THF (50 mL) and the mixture was stirred at 0 °C until it dissolved. The MeMgBr (3 M in Et₂O, 12.8 mL, 38.4 mmol, 3.0 eq.) was added dropwise and the reaction mixture was stirred at 0 °C for 1 h. After completion of the reaction (monitored by TLC), the reaction was quenched by addition of sat. aq. NH₄Cl (25 mL) at 0 °C. The mixture was extracted three times with EtOAc (3 x 80 mL). The combined organic layers were washed with sat. NaCl (20 mL), dried over MgSO₄, and concentrated in vacuo. Column chromatography on silica gel [cyclohexane/ethyl acetate (20:1)] gave the methyl ketone (R)-S4 (3.94 g, 11.6 mmol, 91%) as colorless oil. The same procedure was used to convert amide (S)-S3 (6.05 g, 15.7 mmol, 1.0 eq.) into (S)-S4 (4.75 g, 14.0 mmol, 89%).

(R)-S4: ¹H-NMR (500 MHz, C₆D₆): δ [ppm] = 7.78-7.70 (m, 4H, 4 x CH), 7.26-7.19 (m, 6H, 6 x CH), 3.78 (dd, J = 10.0, 7.3 Hz, 1H, 0.5 x CH₂), 3.60 (dd, J = 10.0, 5.3 Hz, 1H, 0.5 x CH₂), 2.52-2.38 (m, 1H, CH), 1.85 (s, 3H, CH₃), 1.13 (s, 9H, 3 x CH₃), 0.82 (d, J = 7.0 Hz, 3H, CH₃). ¹³C-NMR (126 MHz, C₆D₆) δ [ppm] = 208.8 (C₁₆), 136.0 (2 x CH), 136.0 (2 x CH), 133.9
(C₆), 133.8 (C₆), 130.1 (2 × CH), 128.1 (4 × CH), 66.4 (CH₂), 49.2 (CH), 29.0 (CH₂), 27.0 (3 × CH₃), 19.5 (C₆), 12.9 (CH₃). TLC [cyclohexane/ethyl acetate (20:1)]; Rf = 0.26. GC (HP-5MS): l = 2239. MS (EI, 70 eV): m/z (%) = 283 (96), 253 (13), 239 (59), 205 (61), 199 (74), 197 (25), 187 (23), 183 (100), 181 (43), 175 (20), 135 (16), 123 (25), 105 (25), 77 (22), 43 (28). \([\alpha]\)D = −18.0° (c 1.5, CH₂Cl₂).

(S)-S₄: \([\alpha]\)_D = +20.9° (c 1.8, CH₂Cl₂). Spectroscopic data as for the (R) enantiomer.

S₁₀: Synthesis of (S)- and (R)-tert-butyl(2,3-dimethylbut-3-en-1-yl)oxy)diphenylsilane (S₅)

CH₃PPh₃ (8.44 g, 20.9 mmol, 1.8 eq.) was suspended in THF (100 mL) and cooled to 0 °C. The n-BuLi (1.6 M in hexane, 13.0 mL, 20.8 mmol, 1.8 eq.) was added dropwise and the mixture was stirred at 0 °C for 1 h. After cooling to the mixture to −78 °C, the ketone (R)-S₄ (3.94 g, 11.6 mmol, 1.0 eq.) was added dropwise and the reaction mixture was stirred at room temperature overnight. The reaction was quenched by pouring ice-water mixture (200 mL). The mixture was extracted three times with diethyl ether (3 × 200 mL). The combined organic layers were washed with sat. NaCl (20 mL), dried over MgSO₄, and concentrated under reduced pressure. Column chromatography on silica gel [cyclohexane/ethyl acetate (100:1)] gave the olefin (S)-S₅ (3.38 g, 10.0 mmol, 86%) as colorless oil. The same procedure was used to convert ketone (S)-S₅ (4.75 g, 14.0 mmol, 1.0 eq.) into (R)-S₅ (3.03 g, 8.95 mmol, 64%).

(S)-S₅: ¹H-NMR (500 MHz, CDCl₃): δ [ppm] = 7.84-7.73 (m, 4H, 4 × CH), 7.27-7.20 (m, 6H, 6 × CH), 4.83-4.79 (m, 2H, CH₂), 3.70 (dd, J = 9.9, 6.3 Hz, 1H, 0.5 × CH₂), 3.57 (dd, J = 9.9, 6.8 Hz, 1H, 0.5 × CH₂), 2.44-2.32 (m, 1H, CH), 1.57 (t, J = 1.2 Hz, 3H, CH₃), 1.19 (s, 9H, 3 × CH₃), 1.03 (d, J = 7.0 Hz, 3H, CH₃). ¹³C-NMR (126 MHz, CDCl₃) δ [ppm] = 147.7 (C₆), 136.1 (2 × CH), 136.1 (2 × CH), 134.4 (C₆), 134.4 (C₆), 130.0 (2 × CH), 128.1 (4 × CH), 111.0 (CH₂), 68.0 (CH₂), 43.8 (CH), 27.2 (3 × CH₃), 20.6 (CH₃), 19.6 (C₆), 16.3 (CH₃). TLC [cyclohexane/ethyl acetate (100:1)]; Rf = 0.40. GC (HP-5MS): l = 2122. MS (EI, 70 eV): m/z (%) = 281 (37), 239 (100), 211 (8), 203 (10), 199 (15), 197 (13), 135 (16), 121 (8), 105 (14), 77 (8), 57 (10), 41 (15). \([\alpha]\)D = −0.1° (c 1.8, CH₂Cl₂).

(R)-S₅: \([\alpha]\)_D = +0.2° (c 1.3, CH₂Cl₂). Spectroscopic data as for the (S) enantiomer.

S₁₁: Synthesis of (S)- and (R)-2,3-dimethylbut-3-en-1-ol (S₆)

Olefin (S)-S₅ (3.38 g, 10.0 mmol, 1.0 eq.) was suspended in THF (45 mL) and the solution was cooled to 0 °C. TBAF (1 M in THF, 12.0 mL, 12.0 mmol, 1.2 eq.) was added dropwise and the reaction mixture was stirred at room temperature for 2 h. The reaction was quenched by addition of water (50 mL). The mixture was extracted three times with diethyl ether (3 × 100 mL). The combined organic layers were washed with sat. NaCl (10 mL), dried over MgSO₄, and concentrated under reduced pressure (700 mbar, 40 °C, 20 min). Column chromatography on silica gel [n-pentane/diethyl ether (1:1)] gave the alcohol (S)-S₆ (0.83 g, 8.3 mmol, 83%) as colorless oil. The same procedure was used to convert olefin (R)-S₅ (3.03 g, 8.95 mmol, 1.0 eq.) into (R)-S₆ (0.57 g, 5.69 mmol, 64%).

(S)-S₆: ¹H-NMR (500 MHz, CDCl₃): δ [ppm] = 4.79-4.74 (m, 1H, CH), 4.73-4.70 (m, 1H, CH), 3.35-3.29 (m, 1H, 0.5 × CH₂), 3.29-3.24 (m, 1H, 0.5 × CH₂), 2.19-2.08 (m, 1H, CH), 1.51 (t, J =
The reaction mixture was stirred at room temperature overnight. The procedure was used to convert tosylate (S7) to an inseparable mixture (by NMR) of (S)-S7 and (R)-S7. GC (HP-5MS): $I/800$. MS (El, 70 eV): $m/z$ (%) = 100 (5), 82 (11), 70 (37), 69 (37), 67 (36), 55 (43), 53 (18), 41 (100), 39 (41). $\lbrack \alpha \rbrack^D_{D20} = -9.0^\circ$ (c = 0.67, CH2Cl2).

\((R)-S7\): $[\alpha]_{D20} = +10.5^\circ$ (c = 0.6, CH2Cl2). Lit: $[\alpha]_{D20} = +9.0^\circ$ (c 3.0, CHCl3).[3] Spectroscopic data as for the (S) enantiomer.

**S12: Synthesis of (S)- and (R)-2,3-dimethylbut-3-en-1-yl 4-methylbenzenesulfonate (S7)**

Alcohol (S)-S6 (0.83 g, 8.3 mmol, 1.0 eq.) was suspended in CH2Cl2 (80 mL) and the solution was cooled to 0 °C. After adding of DMAP (3.35 g, 27.4 mmol, 3.3 eq.), p-TsCl (partially dissolved in 10 mL CH2Cl2, 3.96 g, 20.8 mmol, 2.5 eq.) was added dropwise to the solution and the reaction mixture was stirred at room temperature overnight. The reaction was quenched by addition of sat. NH4Cl (100 mL). The mixture was extracted three times with diethyl ether (3 x 100 mL). The combined organic layers were washed with sat. NaCl (10 mL), dried over MgSO4, and concentrated under reduced pressure. Column chromatography on silica gel [cyclohexane/ethyl acetate (10:1)] gave the tosylate (S)-S7 (1.60 g, 6.29 mmol, 76%) as colorless oil. The same procedure was used to convert alcohol (R)-S6 (0.57 g, 5.69 mmol, 1.0 eq.) into (R)-S7 (1.00 g, 3.93 mmol, 69%).

\((S)-S7\): $^{1}H$-NMR (500 MHz, CdCl3): $\delta$ [ppm] = 7.76 (d, $J = 8.3$ Hz, 2H, 2 x CH), 6.69 (d, $J = 8.0$, 0.7 Hz, 2H, 2 x CH), 4.69-4.64 (m, 1H, 0.5 x CH2), 4.62-4.57 (m, 1H, 0.5 x CH2), 3.91 (dd, $J = 9.6$, 6.7 Hz, 1H, CH), 3.79 (dd, $J = 9.6$, 6.9 Hz, 1H, CH), 2.29-2.17 (m, 1H, CH), 1.82 (s, 3H, CH3), 1.37 (dd, $J = 1.5$, 0.8 Hz, 3H, CH3), 0.76 (d, $J = 7.0$ Hz, 3H, CH3). $^{13}$C-NMR (126 MHz, CdCl3): $\delta$ [ppm] = 145.3 (Cq), 144.2 (Cq), 134.5 (Cq), 129.8 (2 x CH), 128.2 (2 x CH), 112.1 (CH2), 72.9 (CH2), 40.4 (CH), 21.1 (CH3), 20.0 (CH3), 15.8 (CH3). TLC [cyclohexane/ethyl acetate (10:1)]; $R_f = 0.38$. GC (HP-5MS): $I = 1879$. MS (El, 70 eV): $m/z$ (%) = 173 (6), 155 (58), 91 (100), 82 (90), 69 (28), 67 (39), 65 (40), 41 (41), 39 (23). $[\alpha]_{D20} = +7.0^\circ$ (c = 1.0, CH2Cl2). Lit: $[\alpha]_{D20} = +7.4^\circ$ (c 0.91, CHCl3).[4]

\((R)-S7\): $[\alpha]_{D20} = -6.5^\circ$ (c 0.8, CH2Cl2). Lit: $[\alpha]_{D20} = -6.1^\circ$ (c 5.5, CHCl3).[6] Spectroscopic data as for the (S) enantiomer.

**S13: Synthesis of (S)- and (R)-2,3-dimethylbut-3-en-1-yl diphosphate (S8)**

Following a known pyrophosphorylation method,[6] (9NBu4)HPO3 (8.52 g, 9.44 mmol, 1.5 eq.) was added to acetonitrile (2 mL), followed by the dropwise addition of the tosylate (S)-S7 (1.60 g, 6.29 mmol, 1.0 eq., in 6 mL acetonitrile). The reaction mixture was stirred overnight, and the solvent was removed under reduced pressure. The residue was loaded onto an ion exchange resin column (DOWEX® 50W-X8, 100-200 mesh, NH4+ form), followed by elution with two column volumes of elution buffer (25 mm NH4HCO3 in 2% PrOH/H2O). The eluate was lyophilized, the residue was dissolved in NH4HCO3 solution (0.1 M, 8 mL) and mixed with 1:1 MeCN/PrOH (20 mL). The precipitate was separated by centrifugation (2000 x g, 5 min) and the liquid phase containing the target compound was collected. The procedure of dissolving the precipitate in NH4HCO3 solution (0.1 M, 8 mL) and mixing with 1:1 MeCN/PrOH (20 mL) followed by centrifugation was repeated three times, before the pooled liquid fractions were concentrated under reduced pressure. The residue was taken up in water (6 mL) and lyophilized again to yield the diphosphate (S)-S8 as an inseparable 1:1.7 mixture (by NMR peak integration) with ammonium tosylate as a white solid (0.90 g, 2.89 mmol, 46%). The same procedure was used to convert tosylate (R)-S7 (1.00 g, 3.93 mmol, 69%) into the (R)-S8 as an
inseparable 1:1.7 mixture (by NMR peak integration) with ammonium tosylate as a white solid (0.54 g, 1.73 mmol, 44%).

(S)-S8: $^1$H-NMR (500 MHz, D$_2$O): $\delta$ [ppm] = 4.86-4.83 (m, 2H, CH$_2$), 3.93-3.87 (m, 1H, 0.5 x CH$_2$), 3.87-3.80 (m, 1H, 0.5 x CH$_2$), 2.59-2.45 (m, 1H, CH), 1.74 (s, 3H, CH$_3$), 1.04 (dd, $J = 6.9$, 1.4 Hz, 3H, CH$_3$). $^{13}$C-NMR (126 MHz, D$_2$O) $\delta$ [ppm] = 148.9 (C$_q$), 110.6 (CH$_2$), 68.9 (d, $^2$J$_{C,P} = 6.0$ Hz, CH$_2$), 41.2 (d, $^3$J$_{C,P} = 7.6$ Hz, CH), 19.2 (CH$_3$), 15.5 (CH$_3$). $^{31}$P-NMR (202 MHz, D$_2$O): $\delta$ [ppm] = $-7.32$ (d, $^2$J$_{P,P} = 21.3$ Hz, 1P), $-10.47$ (d, $^2$J$_{P,P} = 21.3$ Hz, 1P). HRMS (TOF): $m/z$ = 259.0146 (calc. for [C$_6$H$_{13}$O$_7$P$_2$]$^-$ 259.0142).

(R)-S8: HRMS (TOF): $m/z$ = 259.0145 (calc. for [C$_6$H$_{13}$O$_7$P$_2$]$^-$ 259.0142). Spectroscopic data as for the (S) enantiomer.
Supplementary Figure S7. $^1$H-NMR (500 MHz, C$_6$D$_6$) of S2.

Supplementary Figure S8. $^{13}$C-NMR (126 MHz, C$_6$D$_6$) of S2.
**Supplementary Figure S9.** DEPT135 (126 MHz, C₆D₆) of S2.

**Supplementary Figure S10.** ¹H-NMR (500 MHz, C₆D₆) of S3.
Supplementary Figure S11. $^{13}$C-NMR (126 MHz, C$_6$D$_6$) of S3.

Supplementary Figure S12. DEPT135 (126 MHz, C$_6$D$_6$) of S3.
Supplementary Figure S13. $^1$H-NMR (500 MHz, C$_6$D$_6$) of S4.

Supplementary Figure S14. $^{13}$C-NMR (126 MHz, C$_6$D$_6$) of S4.
Supplementary Figure S15. DEPT135 (126 MHz, $\text{C}_6\text{D}_6$) of S4.

Supplementary Figure S16. $^1\text{H}$-NMR (500 MHz, $\text{C}_6\text{D}_6$) of S5.
Supplementary Figure S17. $^{13}$C-NMR (126 MHz, C$_6$D$_6$) of S5.

Supplementary Figure S18. DEPT135 (126 MHz, C$_6$D$_6$) of S5.
Supplementary Figure S19. $^1$H-NMR (500 MHz, C$_6$D$_6$) of S6.

Supplementary Figure S20. $^{13}$C-NMR (126 MHz, C$_6$D$_6$) of S6.
Supplementary Figure S21. DEPT135 (126 MHz, C_{6}D_{6}) of S6.

Supplementary Figure S22. 1H-NMR (500 MHz, C_{6}D_{6}) of S7.
Supplementary Figure S23. $^{13}$C-NMR (126 MHz, C$_6$D$_6$) of S7.

Supplementary Figure S24. DEPT135 (126 MHz, C$_6$D$_6$) of S7.
Supplementary Figure S25. $^1$H-NMR (500 MHz, D$_2$O) of S8.

Supplementary Figure S26. $^{13}$C-NMR (126 MHz, D$_2$O) of S8.
Supplementary Figure S27. DEPT135 (126 MHz, D$_2$O) of S8.

Supplementary Figure S28. $^{31}$P-NMR (202 MHz, D$_2$O) of S8.
References

[1] L. Drummond, M. J. Kschowak, J. Breitenbach, H. Wolff, Y. M. Shi, J. Schrader, H. B. Bode, G. Sandmann, M. Buchhaupt, ACS Synth Biol 2019, 8, 1303-1313.
[2] B. M. Trost, J. P. Papillon, J Am Chem Soc 2004, 126, 13618-13619.
[3] J. D. White, G. N. Reddy, G. O. Spessard, J. Am. Chem. Soc. 1988, 110, 1624.
[4] S. Sasaki, S. Samejima, T. Uruya, K. Anzai, N. Nishi, E. Kawakita, K. Takao, K. Tadano, J Antibiot (Tokyo) 2013, 66, 147-154.
[5] J. D. White, G. N. Reddy, G. O. Spessard, J Chem Soc, Perkin Trans 1 1993, 759-767.
[6] V. J. Davisson, A. B. Woodside, T. R. Neal, K. E. Stremler, M. Muehlbacher, C. D. Poulter, J Org Chem 1986, 51, 4768-4779.