Mikrobielle *de novo*-Produktion von neuartigen nicht-kanonischen Terpenen

Dissertation

zur Erlangung des Doktorgrades

der Naturwissenschaften

vorgelegt beim Fachbereich Biowissenschaften

der Johann Wolfgang Goethe-Universität

in Frankfurt am Main

von Max J. Lepple

geb. Kschowak

aus Hamburg

Frankfurt 2023

(D 30)

vom Fachbereich Biowissenschaften

der Johann Wolfgang Goethe-Universität als Dissertation angenommen.

Dekan: Prof. Dr. Sven Klimpel

Gutachter : Prof. Dr. Jens Schrader

Prof. Dr. Eckhard Boles

Datum der Disputation : 25.06.2024

Diese Dissertation beruht auf folgenden Publikationen:

1. Heterologous expression of 2-methylisoborneol / 2-methylenebornane biosynthesis genes in *Escherichia coli* yields novel C11-terpenes
   Kschowak et al. (2018) 10.1371/journal.pone.0196082
2. Expanding the Isoprenoid Building Block Repertoire with an IPP Methyltransferase from *Streptomyces monomycini*
   Drummond und Kschowak et al. (2019) 10.1021/acssynbio.8b00525
3. Analyzing and engineering the product selectivity of a 2-methylenebornane synthase
   Kschowak et al. (2020) 10.1021/acssynbio.9b00432

Die Arbeiten zur vorliegenden Dissertation wurden am DECHEMA-Forschungsinstitut in der Arbeitsgruppe Industrielle Biotechnologie angefertigt.

Danksagung

Mein herzlicher Dank gilt Prof. Dr. Jens Schrader für die Möglichkeit meine Promotion am DECHEMA Forschungsinstitut durchführen zu können und für seine hervorragende Betreuung.

Bei Prof. Dr. Eckhard Boles möchte ich mich für die Begutachtung dieser Dissertation als Zweitgutachter bedanken.

Mein besonderer Dank gilt Dr. Markus Buchhaupt für die exzellente Betreuung, die inspirierenden Diskussionen, die konstruktiven Ideen und Ratschläge und für die Zeit, die er sich für Fragen und Gespräche immer genommen hat.

Bei der Arbeitsgruppe Industrielle Biotechnologie bedanke ich mich für die großartige gegenseitige Unterstützung, die das Arbeiten sehr angenehm gemacht hat und für die vielen anregenden Diskussionen, insbesondere bei Laura Drummond, Ina Huth, Hendrik Schewe, Sebastian Bormann, Ingmar Strobel, Thomas Krieg und Anne Sydow.

Meinen Studierenden Hannah Wortmann und Felix Meier danke ich für ihre großartigen und engagierten Arbeiten.

Mein größter Dank gilt meiner Familie. Bei meinen Eltern und Brüdern möchte ich mich für die fortwährende und vielfältige Unterstützung in allen Lebenslagen bedanken. Meiner Frau und meinen Kindern danke ich besonders für ihre Geduld und die Kraft, die sie mir gegeben haben.

Inhaltsverzeichnis

Abkürzungsverzeichnis

2-MIB	2-Methylisoborneol
2-MB	2-Methylenbornan
IPP	Isopentenylpyrophosphat
DMAPP	Dimethylallylpyrophosphat
GPP	Geranylpyrophosphat
FPP	Farnesylpyrophosphat
GGPP	Geranylgeranylpyrophosphat
HPLC	*high performance liquid chromatography*
MS	Massenspektrometrie
SPME	*solid phase microextraction*
GC	Gaschromatographie
SBSE	*stir bar sorptive extraction*
TS	Terpensynthase
PTase	Prenyltransferase
MTase	Methyltransferase
MEP	Methylerythritolphosphat
DOXP	Desoxyxylulosephosphat
ATP	Adenosintriphosphat
VOC	*volatile organic compound*
GFPP	Geranylfarnesylpyrophophat
CoA	Coenzym A
HMG	3-Hydroxy-3-methylglutaryl
NADP	Nicotinamidadenindinukleotidphosphat
JH	Juvenilhormon
SAM	S-Adenosylmethionin

1 Zusammenfassung

Terpene bilden mit mehr als 81.000 Verbindungen die größte Klasse der Naturstoffe. Nichtsdestotrotz wird ihre strukturelle Vielfalt durch die Isoprenregel begrenzt. Diese besagt, dass alle primären Terpensynthaseprodukte aus Bausteinen mit fünf Kohlenstoffatomen hervorgehen. Ihre Produkte sind somit kanonisch, da sie durch ein Vielfaches von fünf Kohlenstoffatomen dargestellt sind. In dieser kumulativen Arbeit wird die mikrobielle Produktion einer Vielzahl neuartiger nicht-kanonischer Terpene beschrieben und somit der chemische Strukturraum von Terpenoiden über die Grenzen der Isoprenregel hinaus erweitert. Um dies zu erreichen, wurden in verschiedenen Ansätzen die Gene des Mevalonatwege, einschließlich einer IPP-Isomerase gemeinsam mit verschiedenen Prenylpyrosphosphat-Methyltransferasen und Terpensynthasen in *E. coli* exprimiert und die Produktspektren der Biosynthesewege detailliert untersucht.

Ein breites Spektrum neuer C_{11}-Terpene wurde als Nebenprodukt der bakteriellen 2-Methylisoborneol- oder 2-Methylenbornansynthasen entdeckt. Neben elf bekannten konnten 24 neuartige C_{11}-Terpene nachgewiesen werden, die bisher noch nicht als Terpensynthase-Produkte beschrieben wurden. Vier davon, 3,4-Dimethylcumol, 2-Methylborneol und die beiden Diastereomere von 2-Methylcitronellol, konnten identifiziert werden. Außerdem wurde das C_{16}-Terpen 6-Methylfarnesol als Produkt identifiziert.

Die Produktselektivität einer C_{11}-Terpensynthasen, die 2-Methylenbornansynthase aus *Pseudomonas fluorescens*, wurde durch einen semirationalen Protein-Engineering-Ansatz verändert. Aminosäuren des aktiven Zentrums mit Einfluss auf die Produktselektivität wurden identifiziert. Entsprechende Varianten des Enzyms führen zu gänzlich veränderten Produktspektren. So wurden neue Einblicke in die Struktur-Funktions-Beziehung für C_{11}-Terpensynthasen gewonnen und bisher unzugängliche nicht-kanonische Terpene produziert.

Eine IPP-Methyltranferase wurde identifiziert und charakterisiert, die den C_5-Baustein der Terpenbiosynthese in eine Vielzahl von C_6- und C_7-Prenylpyrophosphate umwandelt. Die heterologe Expression in *E. coli*

gemeinsam mit anderen Genen der Terpenbiosynthese erweitert das potenzielle Terpensynthase-Substratspektrum außerdem um C_{11}-, C_{12}-, C_{16}- und C_{17}-Prenylpyrosphopshate. Darüber hinaus konnten polymethylierte C_{41}-, C_{42}- und C_{43}-Carotinoide synthetisiert werden. So wurde die Terpenbiosynthese durch die Modifikation ihrer Bausteine erweitert und neue ungewöhnliche Terpene produziert.

2 Einleitung

Mit mehr als 81.000 bekannten Substanzen sind Isoprenoide die größte Gruppe der Naturstoffe (Buckingham 2020). Sie weisen eine enorme strukturelle Diversität auf, obwohl ihre Grundgerüste alle aus einer oder mehreren Isopreneinheiten (s. Abb. 1) bestehen. Isoprenoide werden meist Terpenoide genannt, zu diesen Gruppen zählen ebenfalls Steroide und Carotinoide. Steroide werden auch als eigene Naturstoffgruppe neben den Terpenoiden geführt, obwohl der Vorläufer aller Steroide das Terpenoid Squalen ist.

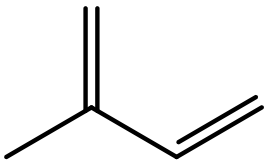


Abb. 1: Strukturformel von Isopren, dem namensgebenden Molekül der Isoprenoide

Terpene sind Terpenoide, die keine funktionelle Gruppe besitzen und somit reine Kohlenwasserstoffe sind. Mittlerweile werden die Begriffe Terpen, Terpenoid und Isoprenoid häufig analog verwendet, so auch in dieser Arbeit.
Gregory und Himly isolierten 1835 unabhängig voneinander Isopren, als sie Kautschuk destillierten und die leichteste Fraktion mit einem Siedepunkt von 36 °C bzw. 33 -44 °C beschrieben (Liebig 1835; Bouchardat 1838). Der Hauptbestandteil von Kautschuk ist cis-1,4-Polyisopren. Greville Williams destillierte einige Jahre später ebenfalls Kautschuk, fasste die Ergebnisse seiner Kollegen zusammen und kam zu dem Schluss, dass diese leichteste Fraktion eine reine Substanz mit fünf Kohlenstoffatomen sein muss. Er gab dieser Substanz den Namen Isopren (Williams 1860):

“I have given the substance thus examined the name isoprene. It would have been more grateful to me to have retained one of the names given by the previous observer, if that course had been possible…”

Inzwischen weiß man, dass das einfachste Terpen Isopren auf der Erde allgegenwertig ist. Schätzungen, die auf experimentell bestimmten Daten beruhen, gehen von einer Emission von Isopren durch Pflanzen in Mengen von 600 Milliarden kg Kohlenstoff pro Jahr aus. Dabei haben Gras- und Strauchlandschaften sowie der natürliche Regenwald mit 88 bzw. 84 Milliarden kg Kohlenstoff pro Jahr den größten Anteil (Guenther et al. 1995). Neben Pflanzen emittieren auch Bakterien und Menschen Isopren (Kuzma et al. 1995; Gelmont et al. 1981). So erhöhte sich die Isoprenkonzentration in der Luft eines Raumes mit Zuschauern eines Fußballspiels der Europameisterschaft 2016, wenn diese jubelten und verringerte sich, wenn ihnen der Atem stockte (Stönner and Williams 2016).

Erhöhte Konzentrationen von Isopren in der Atmosphäre führen zu einem blauen Dunst oder einer blauen Trübung. Dieser Schleier entsteht durch Brechung des Sonnenlichts an Isopren, anderen Terpenoiden und flüchtigen organischen Verbindungen (*volatile organic compounds* – VOCs) (Went 1960). Diesen blauen Schleier kann man besonders gut an warmen Tagen über vegetationsreichen Landschaften wahrnehmen, wenn sie zum Beispiel weitentfernte Hügel oder Berge verdecken.

Abb. 2: Das Ölgemälde (links) Mona Lisa (da Vinci 1503) gemalt vor einer toskanischen Landschaft zeigt trotz Vergilbung den durch Isopren hervorgerufenen blauen Dunstschleier, ebenso wie eine Fotografie (rechts) aus den Blue Ridge Mountains (van der Crabben 2003).

Nach diesem Phänomen sind aller Wahrscheinlichkeit nach Gebirge wie die Blue Mountains im australischen Bundestaat New South Wales oder die Blue Ridge

Mountains der Appalachen in den USA benannt (s. Abb. 2). Auch Leonardo da Vinci beschrieb diesen Dunst in der Toskana in seinen Notizen und arbeitete ihn in seine Kunstwerke wie Mona Lisa ein (s. Abb. 2) (Went 1960).

2.1 Biosynthese kanonischer Terpenoide

„Für die Erlangung einer allgemeinen Vorstellung von der Constitution der Terpene ist von größtem Belang zunächst jedenfalls die Thatsache, daß dieselben zum Theil wenigstens durch Polymerisation des Pentens, C_5H_8, entstehen und daß sich aus dem Penten nicht nur die gewöhnlichen Terpene, $C_{10}H_{16}$, sondern auch Polyterpene, $C_{15}H_{24}$,$C_{20}H_{32}$ u. s. f., aufbauen können. Das bekannteste terpenbildende Penten ist das Isopren.“

(Wallach 1887)

Diese Beschreibung des Aufbaus der Terpene von 1887 durch den Chemie-Nobelpreisträger Otto Wallach ist die erste Version der bis heute bestehenden Isoprenregel. Sie besagt, dass alle Terpene aus C_5-Körpern entstehen und tatsächlich sind die Bausteine aller Terpene die durch Pyrophosphatgruppen aktivierten C_5-Moleküle Isopentenylpyrophosphat (IPP) und Dimethylallylpyrophosphat (DMAPP). Nach der Anzahl an C_5-Einheiten aus denen Terpene aufgebaut sind, werden sie in Hemiterpene (C_5), Monoterpene (C_{10}), Sesquiterpene (C_{15}), Diterpene (C_{20}), Sesterterpene (C_{25}), Triterpene (C_{30}), Tetraterpene (C_{40}) und Polyterpene (C_{n*5}, n>8) klassifiziert. Bei der Biosynthese von Terpenen wird zumeist ein DMAPP-Molekül mit einem oder mehreren IPP-Molekülen durch Prenyltransferasen kondensiert. So entstehen die Substrate der Terpensynthasen Geranylpyrophosphat (GPP, C_{10}), Farnesylpyrophosphat (FPP, C_{15}) Geranylgeranylpyrophosphat (GGPP, C_{20}) und Geranylfarnesylpyrophophat (GFPP, C_{25}) (s. Kap 2.1.2). Terpensynthasen dephosphorylieren diese Prenylpyrophosphate, so dass hochreaktive Carbokationen entstehen (s. Kap.2.1.3).

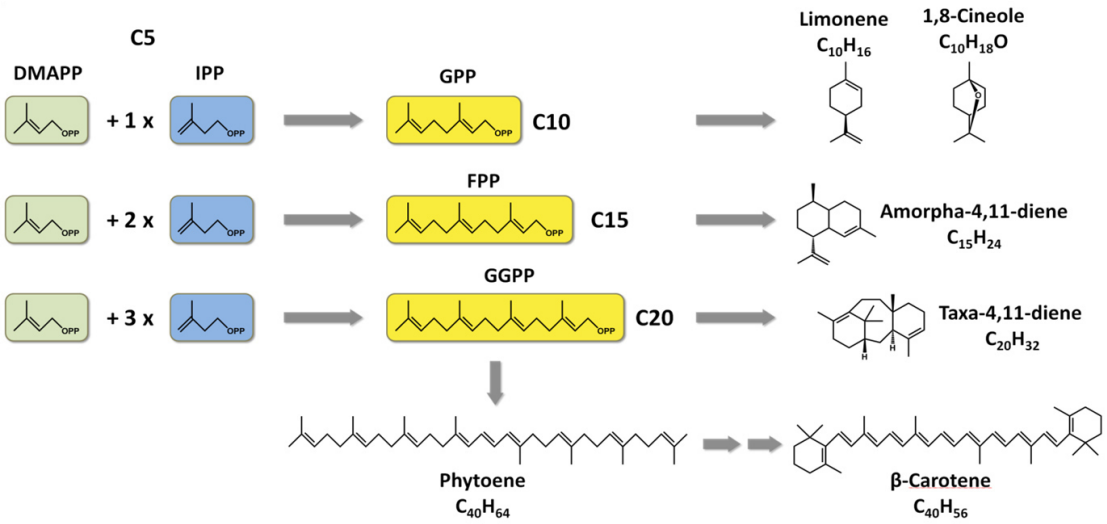


Abb. 3: Biosynthese von Terpenen inklusive der Bausteine IPP und DMAPP, sowie der Grundgerüste GPP, FPP und GGPP (Drummond und Kschowak et al. 2020)

Im aktiven Zentrum der Terpensynthasen kommt es zu Wasserstoffumlagerungen, Zyklisierungen und anderen Umlagerungen der Carbokationen, bis die Reaktionsfolge durch Deprotonierung oder Addition von Wasser gestoppt wird (s. Kap. 2.4). Diese unterschiedlichen und vielfältigen Reaktionskaskaden erklären die enorme strukturelle Vielseitigkeit der Terpene.

2.1.1 Biosynthesewege der allgemeinen Bausteine von Terpenoiden

Die Bausteine aller kanonischer Terpene sind die C_5-Moleküle Isopentenylpyrophosphat (IPP) und Dimethylallylpyrophosphat (DMAPP). Sie werden durch zwei verschiedene Biosynthesewege produziert: den Mevalonatweg und den Methylerythritolphosphatweg (MEP-Weg), der auch 1-Desoxyxylulosephosphatweg (DOXP-Weg) genannt wird. Der Mevalonatweg findet hauptsächlich in höheren Eukaryoten, in Archaeen und im Cytosol von Pflanzen statt, der MEP-Weg in Bakterien und in den Plastiden von Pflanzen (Lange et al. 2000).

Mevalonatweg

Der Mevalonatweg wurde in den 1950er Jahren aufgeklärt (Chaykin et al. 1958; Lynen et al. 1958; Popják and Cornforth 1960; Goodwin 1964) und beginnt mit

einem der wichtigsten Zwischenprodukte im Stoffwechsel vieler Lebewesen: Acetyl-CoA. Im ersten Schritt wird ein Acetylrest von Acetyl-CoA von der Acetyl-CoA-Acetyltransferase auf ein anderes Molekül Acetyl-CoA übertragen, so dass Acetoacetyl-CoA entsteht (s. Abb. 3). Dieses kondensiert mit einem weiteren Molekül Acetyl-CoA zu 3-Hydroxy-3-methylglutaryl-CoA (HMG-CoA), katalysiert durch die HMG-CoA-Synthase. HMG-CoA wird unter Umsetzung zweier Moleküle NADPH/H+ von der HMG-CoA-Reduktase zu Mevalonat reduziert. Mevalonat wird in zwei Reaktionen von der 5-Mevalonat- bzw. 5-Phosphomevalonat-Kinase über 5-Phospho- zu 5-Pyrophosphamevalonat phosphoryliert, jeweils unter Umsatz eines ATPs. In dem letzten Schritt wird 5-Pyrophosphomevalonat von der 5-Pyrophosphomevalonat-Decarboylase durch Abspaltung eines Kohlendioxids und unter Umsetzung eines ATPs zu IPP umgesetzt. IPP kann von der Isopentenylpyrophosphat-Isomerase zu DMAPP und zurück isomerisiert werden (Boronat and Rodríguez-Concepción 2015).

In manchen Archaeen gibt es alternative Mevalonatwege. 5-Phosphomevalonat kann von einer 5-Phosphomevalonat-Decarboxylase zu Isopentenylphosphat decarboxyliert werden (Dellas et al. 2013). Isopentenylphosphat wird von einer Isopentenylphosphat-Kinase eine weitere Phosphat-Gruppe hinzugefügt, so dass ebenfalls IPP entsteht (Grochowski et al. 2006).

In einer anderen propagierten Route wird Mevalonat zu 3-Phosphomevalonat phosphoryliert und in weiteren Schritten vermutlich über 3,5-Biphosphamevalonat zu Isopentenylphosphat umgesetzt (Azami et al. 2014; Vinokur et al. 2014; Rossoni et al. 2015). Letzteres kann, wie in der erstgenannten alternativen Route, von einer Isopentenylphosphat-Kinase zu IPP phosphoryliert werden.

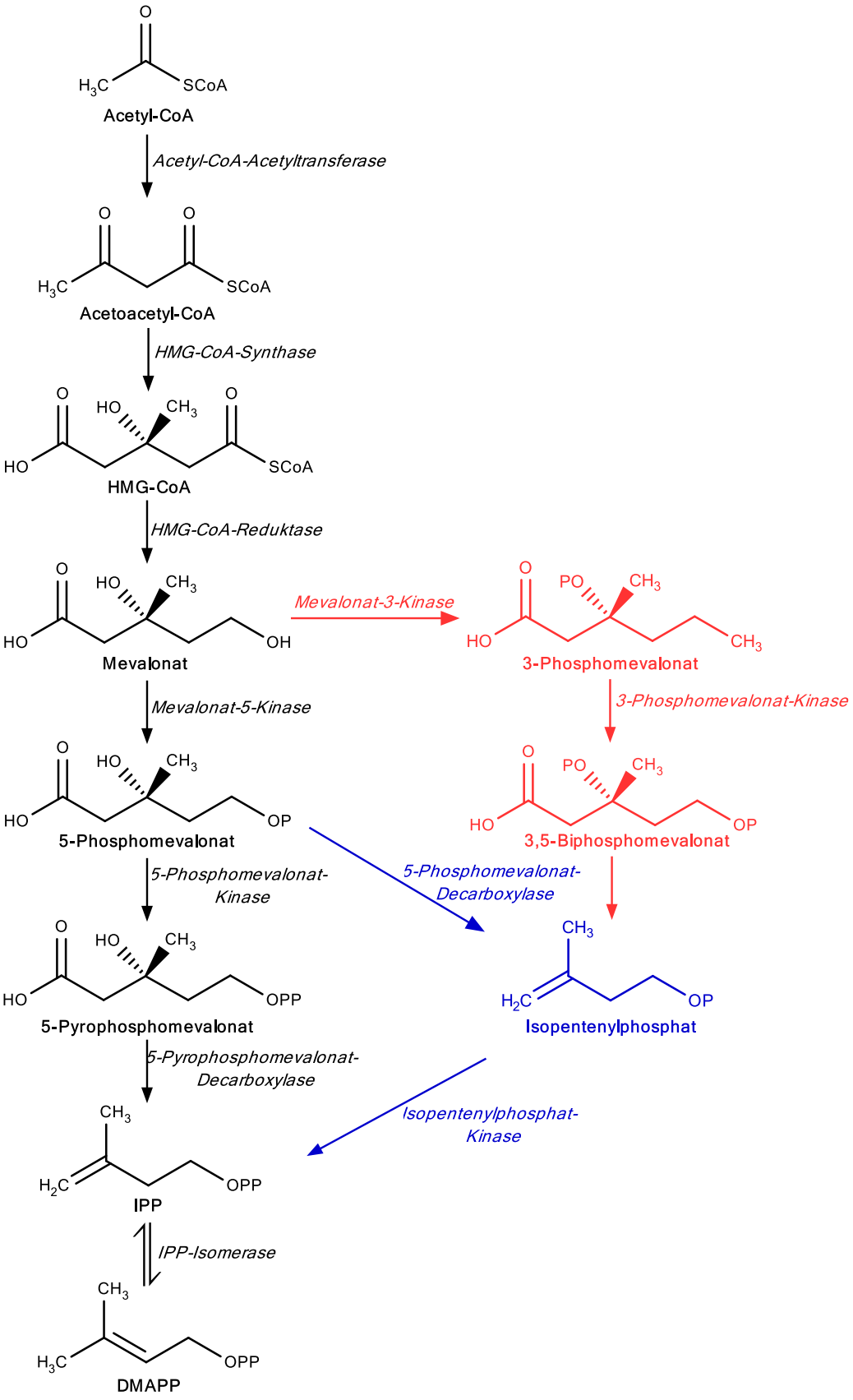


Abb. 4: Der Mevalonatweg inklusiver zweier alternativer Routen (rot und blau) aus Archaeen

Methylerythritolphosphatweg

Bis in die 1990er Jahre war der Mevalonatweg der einzig bekannte Biosyntheseweg der beiden Isoprenoidbausteine IPP und DMAPP. Jedoch gab es schon in den 1950er Jahren Widersprüche und Anzeichen dafür, dass nicht alle Terpenoide über den Mevalonatweg synthetisiert werden (Rohmer 1999). 1993 wurde dann die Existenz des alternativen Wegs, des Methylerythritolphosphatwegs (MEP-Weg), vollständig nachgewiesen (Rohmer et al. 1993).

Dieser beginnt mit der Verknüpfung der Glycolyse-Produkte bzw. Zwischenprodukte Pyruvat und Glycerinaldehyd-3-phosphat. Bei der von der Desoxyxylulose-Phosphat-Synthase katalysierten Reaktion entsteht durch Abspaltung von Kohlendioxid 1-Desoxy-D-xylulose-5-phosphat. Dieses wird unter Umsatz von $NAPH/H^+$ zu 2C-Methyl-D-erythritol-4-phosphat von der Desoxyxylulosephosphat-Reduktoisomerase reduziert und isomerisiert. Durch die Cytidindiphosphat-Methylerythritol-Synthase wird 2C-Methyl-D-erythritol-4-phosphat anschließend mit Cytidintriphosphat unter Abspaltung einer Pyrophosphatgruppe verknüpft. Eine Cytidyl-Methyl-Kinase phosphoryliert 4-Diphosphocytidyl-2C-methyl-D-erythritol unter ATP-Umsatz zu 4-Diphosphocytidyl-2C-methyl-D-erythritol-2-phosphat. Die Methyl-Erythritol-Cyclo-Diphosphat-Synthase spaltet Cytidinmonophosphat ab und cyclisiert das Produkt zu 2C-Methyl-D-erythritol-2,4-cyclodiphosphat. Dieses wird von der Hydroxy-Methyl-Butenyl-Diphosphat-Synthase zu (*E*)-4-Hydroxy-3-methylbut-2-enyldiphosphat dehydratisiert, wobei zwei Ferrodoxine oxidiert werden. Im letzten Schritt werden von der Hydroxy-Methyl-Butenyl-Diphosphat-Reduktase unter Umsetzung von NADPH/H+ und Abspaltung von Wasser IPP und DMAPP in einem Verhältnis von 5:1 synthetisiert (Boronat and Rodríguez-Concepción 2015).

2.1.2 Kondensation der Bausteine zu Terpengrundgerüsten

Zur Synthese von Terpenen mit mehr als fünf Kohlenstoffatomen wird zumeist ein DMAPP mit einem oder mehreren IPPs kondensiert. Isoprenylpyrophosphat-Synthasen (Prenyltransferasen, PTasen) katalysieren diese Kondensation

(nukleophile Substitution). Die Produkte sind ebenfalls lineare Prenylpyrophosphate mit eine Kohlenstoff-Kettenlänge von $C_{n*5.}$ Sie bilden die Grundgerüste der Terpene und sind die Substrate der Terpensynthasen (TS) (Tholl 2015). Für Produkte ab einer Kettenlänge von C_{30} werden nicht mehr einzelne C_5-Bausteine zu Prenylpyrophosphaten addiert, sondern auch längere Prenylpyrophosphate, wie zum Beispiel FPP (C_{15}) als Substrate genutzt (K. C. Wang and Ohnuma 2000).

Man unterscheidet zwischen *cis*- und *trans*-PTasen, je nachdem welche Stereochemie die Doppelbindung(en) des Produktes aufweisen (s. Abb. 5). Die meisten Terpensynthasen nutzen *trans*-Prenylpyrophosphate als Substrate, so dass auch deren PTasen häufiger vorkommen und besser untersucht sind (Tholl 2015).

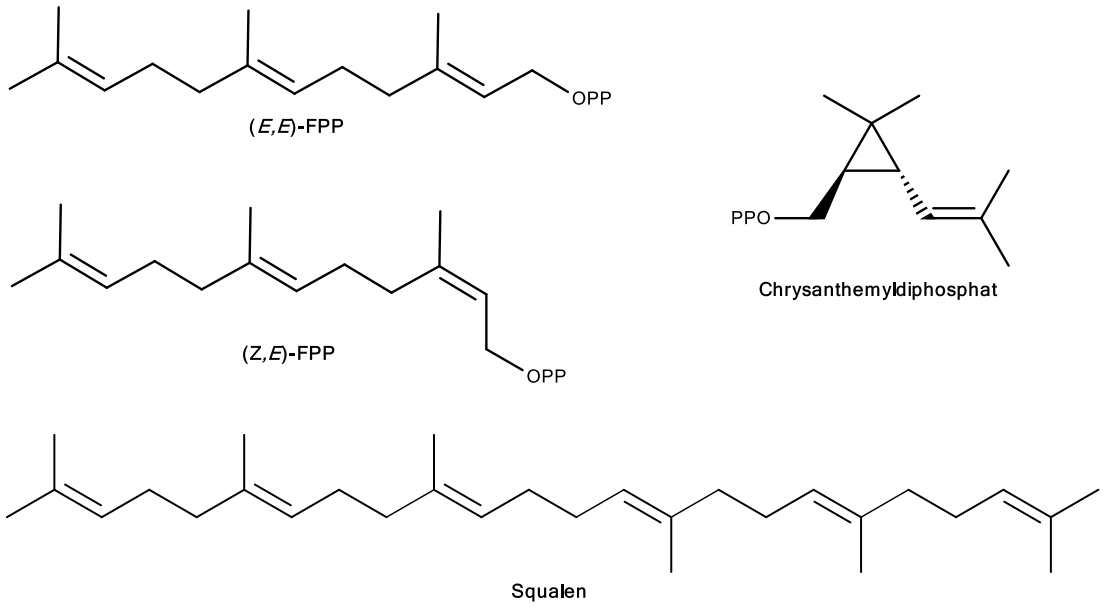


Abb. 5: Produkte verschiedener Prenyltransferasen: *cis*- und *trans*-PTasen synthetisieren verschiedene Isomere wie (*E,E*)-FPP und (*Z,E*)-FPP. Das Produkt einer Kopf-zu-Kopf-Kondensation ist Squalen und das einer ungewöhnlichen nicht-Kopf-zu-Schwanz-Kondensation ist Chrysanthemyldiphosphat

In der Regel erfolgt eine Kopf-zu-Schwanz-Kondensation (1'→4) der Prenylpyrophosphate (Poulter et al. 1978). Es gibt jedoch auch verschiedenste andere Anordnungen, so findet beispielsweise während der Squalensynthese eine Kopf-zu-Kopf Kondensation zweier FPPs statt (Tansey and Shechter 2000). Die Chrysanthemyldiphosphat-Synthase kondensiert zwei FPPs zu einem

cyclischen Prenylpyrophosphat (s. Abb. 6) durch eine der sogenannten nicht-Kopf-zu-Schwanz-Kondensationen (c1‘-2-3) (Thulasiram et al. 2007).

2.1.3 Umsetzung der Grundgerüste zu Terpenen

Terpensynthasen (TS) setzen die Prenylpyrophosphat-Grundgerüste DMAPP (C_5), GPP (C_{10}), FPP (C_{15}), GGPP (C_{20}), GFPP (C_{25}) oder deren Kondensationsprodukte zu Terpenen um. Aufgrund der eingeschränkten Anzahl an Substraten, die sich meistens lediglich in ihrer Kettenlänge unterscheiden, könnte man meinen, dass die Produktvielfalt dieser Enzymklasse ebenfalls eingeschränkt ist. Allerdings ist das Gegenteil der Fall: Terpene sind mit mehr als 81 000 verschiedenen Strukturen die größte Klasse der Naturstoffe (Buckingham 2020). Diese enorme strukturelle Vielfalt beruht auf dem Reaktionsmechanismus der TS. Bei diesem wird die Pyrophosphatgruppe abgespalten und es kommt im Durchschnitt bei mehr als der Hälfte der Kohlenstoffatome zu Änderungen in der Bindung, Hybridisierung oder Stereochemie (Christianson 2017), bevor die Reaktionskaskade durch Deprotonierung des Carbokations oder Addition von Wasser zu diesem gestoppt wird (vgl. Kap. 2.4). So entstehen lineare, monocyclische und polycyclische reine Kohlenwasserstoffe und Alkohole (s. Abb. 6).

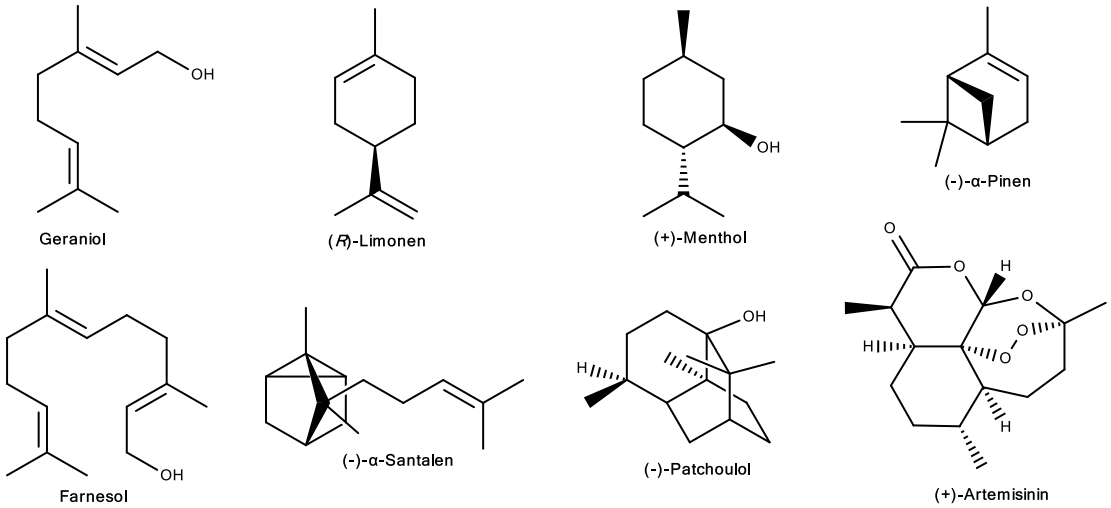


Abb. 6: Strukturelle Vielfalt der Terpenoide: Die Strukturformeln der Monoterpene Geraniol, Limonen, Menthol und Pinen, sowie der Sesquiterpene Farnesol, Santalen, Patchoulol und Artemisinin

2.2 Biosynthese nicht-kanonischer Terpenoide

> *From the "biogenetic isoprene rule", however, it would follow that the carbon skeleton of the biological end product is not necessarily identical to the carbon skeleton of the precursor. In other words the validity of the "empirical isoprene rule" depends on the mechanisms of formation of the natural compounds, and the failure of a terpene to obey this rule does not necessarily disprove its origin from isoprene units.*
>
> (Ružička 1953)

1953 führte ein weiterer Nobelpreisträger - Leopold Ružička - die „empirische Isoprenregel“ Otto Wallachs fort und definierte die „biogenetische Isoprenregel“. In dieser formulierte er, dass die Isoprenregel auch für Terpene gültig ist, dessen Grundgerüste nicht aus einem Vielfachen des C_5-Körpers Isopren bestehen. Denn Studien von Reaktionsmechanismen nicht-kanonischer Isoprenoide zeigten, dass diese ihren Ursprung ebenfalls in Isopreneinheiten haben. (Ružička 1953; 1963). Diese nicht-kanonischen Terpene entstehen also nach der biogenetischen Isoprenregel ebenfalls aus den gleichen Terpen-Grundgerüsten IPP (C_5), GPP (C_{10}), FPP (C_{15}), GGPP (C_{20}), etc.. Ein Beispiel hierfür ist das C_{12}-Terpen Geosmin. Es ist das Produkt einer Terpensynthase-katalysierten Reaktion mit FPP als Substrat, während der Aceton frei wird (Jiang et al. 2006). Andere nicht-kanonische Terpene sind Produkte sogenannter dekorierender Enzyme, wie Oxidoreduktasen, Acetyltransferasen oder Methyltransferasen (Dudareva et al. 2004). Man differenziert nicht-kanonische Terpene teilweise weiter in Homoterpene, die eine oder mehrere zusätzliche Methylgruppen besitzen, und Meroterpene, das sind Biomoleküle mit nur partieller Terpenstruktur.

2.2.1 Natürliche Biosynthese

Es gibt wenige nicht-kanonische Terpene, die auch der biogenetischen Isoprenregel widersprechen, da bereits die Substrate der entsprechenden

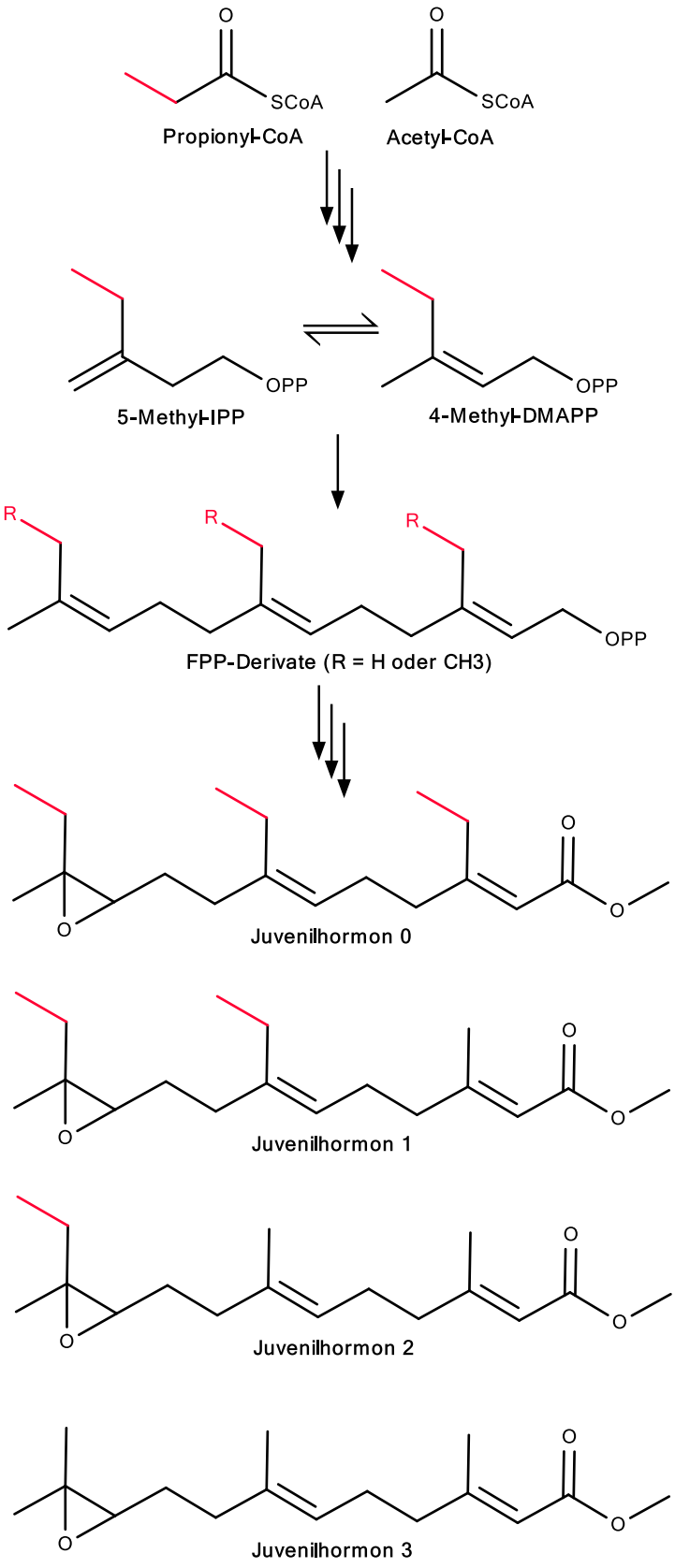


Abb. 7: Überblick über die Substrate und Produkte des nicht-kanonischen Mevalonatweges aus Insekten, ihre FPP-Derivate und Juvenilhormone. Zusätzliche Methylgruppen sind rot dargestellt.

Terpensynthasen, nicht-kanonisch sind. Diese Ausnahmen beruhen auf zwei unterschiedlichen Mechanismen und wurden jeweils bei der Aufklärung der Biosynthese des Juvenilhormons in Insekten (Schooley et al. 1973) und der Aufklärung der Biosynthese des 2-Methyisoborneols in Bakterien (Dickschat et al. 2007; Komatsu et al. 2008; C.-M. Wang and Cane 2008) aufgedeckt.

In Insekten

Die Juvenilhormone (JH) kommen in Insekten vor und repräsentieren eine Gruppe von linearen, teilweise methylierten Sesquiterpenen, wobei die methylierten Formen bisher nur aus Schmetterlingen (Lepidoptera) isoliert wurden. Neben der Strukturaufklärung des dreifach methylierten Juvenilhormons 0 (Bergot et al. 1980), des zweifach methylierten Juvenilhormons 1 (Röller et al. 1967; Heinz Dahm et al. 1968), des einfach methylierten Juvenilhormons 2 (Meyer et al. 1968) und des unmethylierten Juvenilhormons 3 (Bowers et al. 1965; Judy et al. 1973) erfolgte die Aufklärung der Biosynthese. Diese zeigte, dass der Mevalonatweg neben dem gewöhnlichen Eintrittsintermediat Acetyl-CoA (vgl. Abb. 4) auch Propionyl-CoA akzeptiert (Schooley et al. 1973). Die zusätzliche Methylgruppe des Proprionyl-CoA findet sich somit auch in den Produkten des Mevalonatweges IPP und DMAPP wieder, die somit auch als methylierte Derivate vorliegen. Diese methylierten Derivate werden gemeinsam mit den unmethylierten Derivaten von den Prenyltransferasen unterschiedlich kombiniert, so dass ein- und mehrfach-methylierte FPP-Derivate vorliegen, die in weiteren Schritten zu den Juvenilhormonen umgesetzt werden (s. Abb. 7).

Neben den Juvenilhormonen JH0 bis JH3 und ihrer Derivate, wurden auch weitere methylierte Sesquiterpene und Monoterpene aus Insekten isoliert (s. Abb. 8). Hierzu gehören die Homo-Monoterpene 3-methyl-7-methylene-1,3,8-Nonatrien aus dem Borkenkäfer *Ips typographus* (Francke et al. 1995), Homoocimen aus der Ameise *Lapidus praedator* (Keegans et al. 1993), Fettsäureester von 4-Methylgeraniol und Bishomogeraniol aus der Ameise *Gnamptogenys striatula* (Blatrix et al. 2002), sowie die Homosesquiterpene

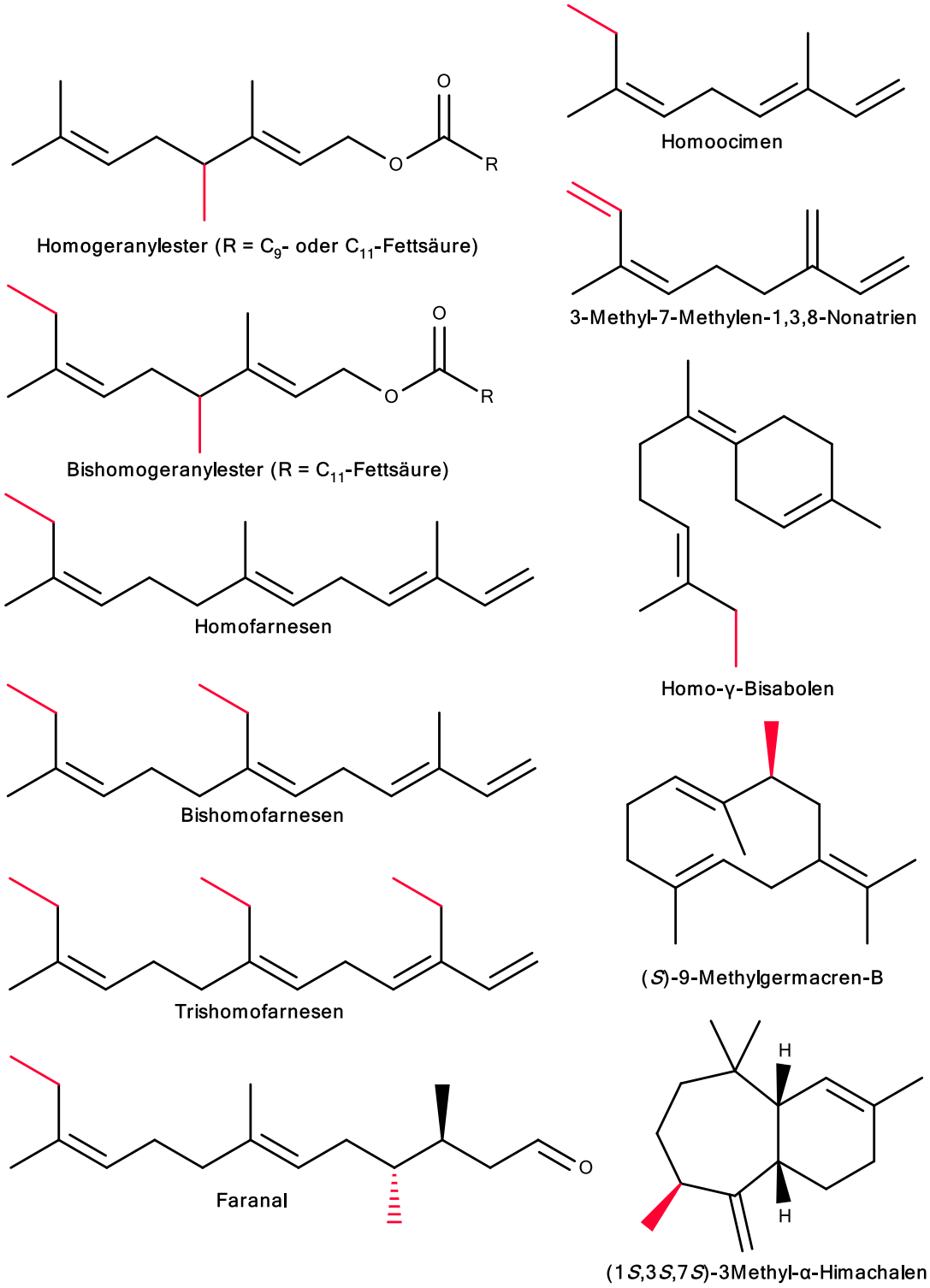


Abb. 8: Nicht-kanonische Terpene von verschiedenen Insekten: Die Strukturformeln der Homomonoterpene Homoocimen, 3-Methyl-7Methylen-1,3,8-Nonatrien, Ester von Homogeraniol und Bishomogeraniol, sowie der Homosesquiterpene Faranal, Homo-γ-Bisabolen, 9-Methylgermacren-B, 3-Methyl-α-Himachalen, Homo-, Bishomo-, und Trishomofarnesen mit ein bis drei zusätzlichen Methylgruppen (rot).

Homo-, Bishomo-, und Trishomofarnesen aus der Ameise *Myrmica scabrinodis* (Morgan et al. 1979), Faranal aus der Ameise *Monomorium pharaonis* (Ritter et al. 1977), Homo-γ-Bisabolen aus der Schildlaus *Eurygaster integriceps* (Staddon et al. 1994), 9-Methyl-Germacren und 3-Methyl-α-Himachalen aus verschiedenen Unterarten der Sandfliege *Lutzomyia longipalpis* (Hamilton et al. 1996a; 1996b).

In Bakterien

Der andere Weg zur Produktion von nicht-kanonischen Terpenen findet in Bakterien statt. Bei diesem werden die kanonischen Terpengrundgerüste IPP, GPP oder FPP von C-Methyltransferasen in einer S-Adenosylmethionin (SAM) abhängigen Reaktion methyliert. Die entstandenen methylierten Prenylpyrophosphate dienen als Substrate für weitere Enzyme, meist Terpensynthasen, die die Reaktion zu den nicht-kanonischen Isoprenoiden katalysieren (s. Abb. 9).

Der bakterielle Mechanismus zur Produktion nicht-kanonischer Terpene wurde bei der Aufschlüsselung des Biosyntheseweges des bizyklischen C_{11}-Terpens 2-Methylisoborneol (2-MIB) von drei verschiedenen Arbeitsgruppen basierend auf Fütterungsexperimenten mit isotopisch markierten Vorstufen (Dickschat et al. 2007) oder auf *in-vitro*-Experimenten mit rekombinanten Enzymen (Komatsu et al. 2008; C.-M. Wang and Cane 2008) entdeckt. In einem ersten Schritt katalysiert die Geranylpyrophosphat-C-Methyltransferase (GPP-MTase) die elektrophile Methylierung an der C-2-Position von GPP. Das 2-Methyl-GPP dient als Substrat für die 2-MIB-Synthase, die unter Abspaltung der Pyrophosphatgruppe die Zyklisierung zu dem Terpenalkohol 2-MIB katalysiert (s. Abb. 9 Mitte). Diese Reaktion verläuft über eine Reihe von carbokationischen Zwischenstufen. Wie bei anderen Terpensynthasen können diese Carbokationen durch Addition von Wasser oder durch Deprotonierung neutralisiert werden (Dickschat 2016). Dies kann zu den Nebenprodukten 2-Methylgeraniol, 2-Methylnerol, 2-Methyllinalool, 2-Methylmyrcen, 2-Methyl-α-Terpineol, 2-Methyl-β-Fenchol, 2-Methyllimonen, 2-Methyl-2-Bornen, 1-Methylcamphen und 2-Methylenbornan (2-MB) führen (Brock et al. 2013; Chou et al. 2011; 2017). 2-Methylbornyl-Kation ist das letzte Carbokation im Reaktionsmechanismus, der

durch die 2-MIB-Synthase katalysiert wird, und ist der Vorläufer für die beiden Produkte 2-MIB und 2-MB. Neben 2-MIB-Synthasen gibt es auch zwei C_{11}-TS, die die Reaktion zu 2-MB als Hauptprodukt katalysieren (Chou et al. 2011; Komatsu et al. 2008)

2-MIB kann modrig oder nach feuchten Waldboden riechen und weist eine niedrigen Geruchs- und Geschmacksschwellenwert von unter 5 ng/L auf (Z. Wang et al. 2006; Piriou et al. 2009). Es zeigt bakterielles Wachstum an und kann gleichzeitig aufgrund des penetranten Geruchs zur Ungenießbarkeit von Fisch (Martin 1992), Trinkwasser und Wein führen (Callejón et al. 2016). Es wird aber auch mit dem erdigen Aroma von verschiedenen Weißschimmelkäsesorten wie beispielsweise Camembert in Verbindung gebracht (Karahadian et al. 1985a). 1969 wurde 2-MIB erstmals isoliert und seine Struktur aufgeklärt (Gerber 1969; Medsker et al. 1969). Seitdem wurde seine Freisetzung hauptsächlich von Bakterien wie Actinomyceten und Cyanobakterien (Tabachek and Yurkowski 1976), aber auch von Eukaryoten wie Pilzen (Karahadian et al. 1985b; Fravel et al. 2002) und Moosen (Toyota et al. 1990) beschrieben.

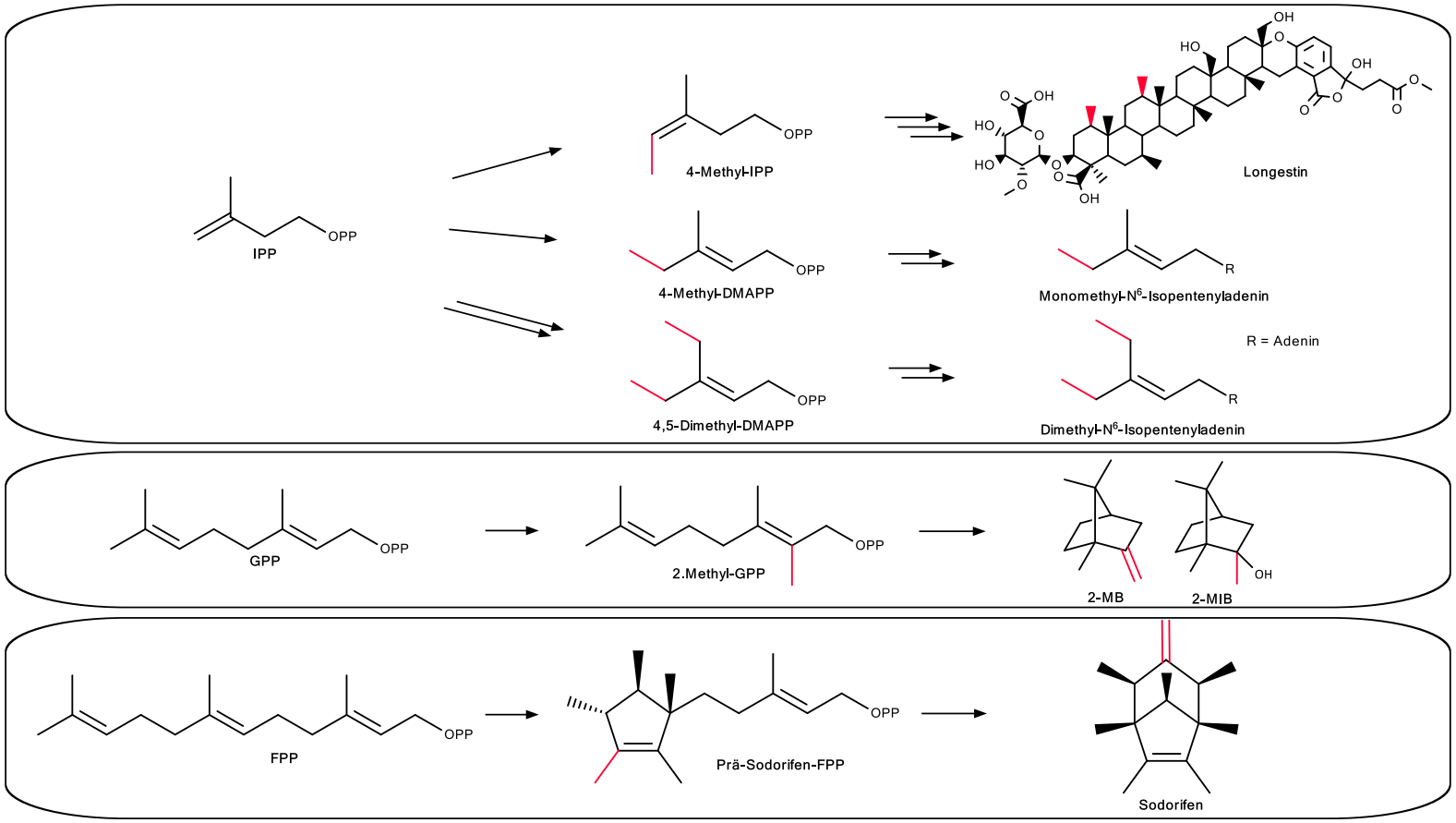


Abb. 9: Übersicht der Biosynthese von nicht-kanonischen Terpenen in Bakterien: In einem ersten Schritt werden die kanonischen Vorläufermoleküle IPP (oben), GPP (Mitte) bzw. FPP (unten) von verschiedenen Isoprenyl-Pyrophosphat-Methyltransferasen methyliert (die entsprechenden Methylgruppen sind rot dargestellt). Im weiteren Verlauf werden die methylierten Prenylpyrophosphate teilweise verlängert und auf andere Moleküle übertragen (oben) oder von Terpensynthasen zu den Endprodukten umgewandelt (Mitte und unten)

Zwei IPP-MTasen wurden bei der Untersuchung der Biosynthese von Cytokininen in *Rhodococcus fascians* entdeckt. Die als MT2 bezeichnete SAM-abhängige MTase methyliert IPP am C4-Atom, so dass 4-Methyl-DMAPP entsteht. Dies ist auch eines der Produkte des nicht-kanonischen Mevalonatweges in Insekten. Ein zweifach methyliertes Prenylpyrophosphat - 4,5-Dimethyl-DMAPP - entsteht wahrscheinlich durch Umsetzung von IPP durch die gleiche MTase MT2 gemeinsam mit einer weiteren, als MT1 bezeichneten, MTase (s. Abb. 9). Unter Abspaltung der Phosphatgruppen werden die methylierten Zwischenprodukte auf Adenin übertragen, sodass die methylierten Cytokinine synthetisiert werden (Radhika et al. 2015).

Eine weitere IPP-MTase wurde in *Streptomyces argenteolus* entdeckt. Das Enzym Lon23 katalysiert die Synthese von 4-Methyl-IPP. Dieses C6-Prenylpyrophosphat wird mit hoher Positionsgenauigkeit mit C5-Prenylpyrophosphaten zu einem GGPP-Derivat kondensiert, sodass sich die zusätzlichen Methylgruppen je an seinen C4- und C12-Atomen wiederfinden. In einem weiteren Kondensationsschritt wird das lineare C42-Molekül 20,32-Dimethyl-Octaprenylpyrophospat synthetisiert, bevor dieses auf nichtisoprenoide Moleküle übertragen und durch weiter enzymatische Schritte Longestin hergestellt wird (Ozaki et al. 2018).

Eine FPP-MTase, die den C_{15}-Terpenvorläufer methyliert, fungiert gleichzeitig als Cyclase und synthetisiert so das ungewöhnliche Intermediat Prä-Sodiferen-FPP (s. Abb. 9). Dieses besitzt einen C5-Ring und wird ebenfalls SAM-abhängig produziert. Das entsprechende Gen aus Serratia *plymuthica* 4Rx13 liegt in einem Gencluster gemeinsam mit zwei Genen für Enzyme des MEP-Weges und einem Gen für eine Terpensynthase, die die nachfolgende Reaktion zu dem bizyklischen Sodiferen katalysiert (von Reuss et al. 2018).

2.2.2 Artifizielle Biosynthese

Neben der beschriebenen natürlichen Biosynthese wurden bereits viele *in vitro* und auch *in vivo* Versuche beschrieben, bei der nicht-kanonische oder andere nicht-natürliche Isoprenoide durch Einsatz von Substratanaloga synthetisiert wurden (Harms, Kirschning, et al. 2020). Häufig dienten diese zur Aufklärung von detaillierten Reaktionsmechanismen von Prenyltransferasen oder

Terpensynthasen. So wurde beispielsweise der Reaktionsmechanismus der 2-MIB-Synthase durch Fütterungsexperimente mit [*Methyl*-^{13}C]Methionin und anderen markierten Vorläufermolekülen aufgeklärt (Dickschat et al. 2007). Auch werden Substratanaloga als Inhibitoren für Terpensynthasen eingesetzt, um 3D-Kristallstrukturen der Enzyme mit der Konformation des Substratanalogs im aktiven Zentrum zu erlangen. Für die Aufklärung der Kristallstruktur der 2-MIB-Synthase aus *S. coelicolor* wurde 2-Fluoro-GPP als Analog für das natürliche Substrat 2-Methyl-GPP verwendet (Köksal et al. 2012).

Mit Prenyltransferasen

Vor allem in den 1970er, 1980er und 1990er Jahren wurden viele Arbeiten von Kyozo Oguras Arbeitsgruppe um Tanetoshi Koyama zur Untersuchung der Substratspezifitäten von Prenyltransferasen und IPP-Isomerasen durchgeführt, bei der auch viele nicht-kanonische Prenylpyrophosphat-Derivate synthetisiert wurden (Popják et al. 1969; Ogura et al. 1970; Polito et al. 1972; Tanetsohi Koyama et al. 1973; Nishino et al. 1973; Shinka et al. 1975; Tanetoshi Koyama et al. 1983; 1988; Shinichi Ohnuma et al. 1989; Nagaki et al. 2000; 2002; Shin-ichi Ohnuma et al. 1998).

So wurden beispielsweise 5-Methyl-IPP, (*E*)- und (*Z*)-4-Methyl-IPP, 4,4,5-Trimethyl-IPP, 5-Ethyl-4(*E*)-Methyl-IPP, 5-Ethyl-4(*Z*)-Methyl-IPP und auch cyclische IPP-Derivate zusammen mit DMAPP und/oder GPP als Substrate (Tanetoshi Koyama et al. 1980) von einer FPP-Synthase aus einer Schweineleber zu den entsprechenden GPP- bzw. FPP-Derivaten und ihren Alkoholen umgesetzt.

Mit Terpensynthasen

Die Promiskuität von verschiedenen Terpensynthasen wurde vielfach nachgewiesen. Studien aus den späten 1990er Jahre zeigten erstmals, dass einige Sesquiterpensynthasen neben ihrem natürlichen Substrat FPP auch GPP akzeptieren und die Reaktionen zu Limonen und anderen Monoterpenen katalysieren (Crock et al. 1997; Bohlmann et al. 1998; Colby et al. 1998). Seitdem wurde auch für andere Terpensynthasen, wie Mono- und Diterpensynthasen, gezeigt, dass sie kurzkettigere kanonische Prenylpyrophosphate als Substrat

akzeptieren (Pazouki and Niinemets 2016). Es gibt auch Terpensynthasen, die eine noch größere Bandbreite an kanonischen Substraten (C_{10}- C_{25}) akzeptieren (Bian et al. 2017).

Mit nicht-natürlichen Prenylpyrophosphat-Derivaten, die andere Atome (Heteroatome) enthalten oder verschobene Methylgruppen wurden nicht-natürliche Terpensynthase-Produkte synthetisiert. So katalysieren Aristolochen-Synthasen beispielsweise die Reaktion von 6-Fluoro-FPP und 2-Fluoro-FPP zu 1- bzw. 2-Fluorogermacren A (Faraldos et al. 2007; Miller et al. 2007). Vier verschiedene FPP-Derivate mit verschobenen Methylgruppen wurden von einer Presilphiperfolanol-Synthase umgesetzt (Harms, Schröder, et al. 2020).

Auch chemisch hergestellte, nicht-kanonische Prenylpyrophosphatderivate wurden bereits mit kanonischen Terpensynthasen getestet. Eine Trichodien-Synthase synthetisiert aus 4-Methyl-FPP das entsprechenden Trichodien-Derivat (Vedula et al. 2007). Eine Germacren-A-Synthase setzten neben verschiedenen Fluoro-FPP-Derivaten ebenfalls 14-Methyl-FPP zu 14-Methyl-Germacren und sechs weiteren Kohlenwasserstoffen um (Cascón et al. 2012).

2.3 Funktionen und Anwendungen von Terpenen

Terpene werden von allen Domänen des Lebens produziert und sind in der Natur allgegenwärtig. Die meisten wurden aus Pflanzen und Pilzen isoliert. Es wird jedoch eine steigende Anzahl von bakteriellen Terpenen bekannt (Rudolf et al. 2021). Die strukturelle Diversität spiegelt sich auch in ihren Funktionen wieder.
Man unterscheidet dabei zwischen primären und sekundären Metaboliten. Es gibt Terpene die eine grundlegende, primäre Funktion in den entsprechenden biologischen Zellen haben, wie photosynthetische Pigmente (Carotinoide, Phytolester in Chlorophyll), Elektronenüberträger in der Atmungskette (Prenylseitenketten von Plastochinon und Ubichinon), Elemente der Membranstruktur (Phytosterole), prenylierte Proteine (z. B. GTPase), Vitamine (z.B. Retinol und Calciol) und Wachstums- und Entwicklungshormone (z.B.: Gibberelline, Abscisinsäure und Cytokinine) (Tholl 2015; Tetali 2019).
Als sekundäre Metabolite werden Terpenoide häufig als Antwort auf biotischen oder abiotischen Stress von Organismen produziert und ausgestoßen. Sie spielen eine wichtige Rolle bei intra- und interspezifischer Kommunikation. So wurde für verschiedene Pflanzen gezeigt, dass sie bei thermischem Stress vermehrt Isopren und Monoterpene produzieren, vermutlich werden diese in photosynthetische Membranen eingelagert, um deren Funktionalität zu verbessern (Behnke et al. 2007; Velikova et al. 2014). Als Bestandteil des Blütenduftes können verschiedene Terpene potenzielle Bestäuber anlocken (Byers et al. 2014), oder die Blüte vor mikrobiellen Krankheitserregern schützen (Junker et al. 2011; M. Huang et al. 2012). Pflanzen stoßen Terpene auch zur Insektenabwehr aus, so schützt sich die Tomatenpflanze gegen weiße Fliegen mit den Sesquiterpenen Zingiberen und Kurkumen (Bleeker et al. 2011). Nadelbäume produzieren verschiedene flüchtige Terpene und Terpenderivate, um Krankheitserreger und andere potenzielle Schädlinge abzuwehren (Zulak and Bohlmann 2010). Eine andere Strategie von Pflanzen ist die Anlockung von natürlichen Feinden von Herbivoren (Gols 2014).
Die Biosynthese und biologische Funktion von bakteriellen Terpenen wurde erst in den letzten Jahren so detailliert untersucht, wie die der Pflanzen, jedoch konnte auch hier schon gezeigt werden, dass sie ebenfalls wichtige Funktionen bei der Signalübertragung und Kommunikation, der Stressantwort auf Temperatur-, pH-

und osmotische Schwankungen, Interaktionen mit Wirten sowie bei der Abwehr von Feinden einnehmen (zusammengefasst von (Boronat and Rodríguez-Concepción 2015) und (Avalos et al. 2022)).

Viele Terpenoide finden Anwendung in den unterschiedlichsten wirtschaftlichen Bereichen, dazu gehören unter anderem die pharmazeutische, kosmetische, die Kunststoff-, sowie die Aroma- und Duftstoffindustrie. In letzterer ist bisher die am weitesten verbreitete Anwendung. Für Isoprenoide wurden in verschiedenen *in vivo* und *in vitro* Studien entzündungshemmende, antioxidative, gerinnungshemmende, tumorhemmende, sedierende und schmerzstillende Wirkungen nachgewiesen (Tetali 2019). Dies macht sie vor allem für die kosmetische und pharmazeutische Industrie relevant. Prominente Beispiele sind Artemisinin, das als Antimalariawirkstoff und Taxol, das als Antitumorwirkstoff eingesetzt wird. Aber auch in weniger wertvollen Produkten wie Haushaltsreinigern (Limonen) oder als Biotreibstoff (Farnesen) finden und fanden Terpenoide Anwendung (George et al. 2015). Carotinoide werden als Lebensmittelfarbstoffe, Tierfutter-Zusatzstoff und auf Grund ihrer antioxidativen Eigenschaften als therapeutische Wirkstoffe eingesetzt (Sandmann 2015).
Einige Terpene werden chemisch synthetisiert oder aus natürlichen Materialien extrahiert. Da ersteres für viele komplexe Strukturen nicht wirtschaftlich ist und letzteres auf Grund geringer Konzentrationen oder geringen Vorkommens in der Natur nicht möglich ist, werden viele Terpenoide biotechnologisch hergestellt (Schempp et al. 2018), zum Beispiel von den Firmen Amyris, Isobionics oder Evolova.

2.4 Strukturchemie der Terpensynthasen

Trotz der Limitierung durch die Isoprenregel, der relativ einfachen nicht zyklischen Prenylpyrophosphate als Substrate und der wenigen Ausnahmen, gibt es eine enorme Anzahl an verschiedenen Terpenoiden von über 80 000 (Buckingham 2020) mit sehr diversen Strukturen (s. Abb. 3 und Abb. 6). Diese enorme Diversität entsteht im ersten Schritt durch die von Terpensynthasen katalysierte Reaktion. Hierbei wird das Prenylpyrophosphat-Substrat zunächst dephosphoryliert und das entstandene Carbokation unterläuft mehrstufigen Veränderungen bezüglich der Bindung, Hybridisierung und Stereochemie. Es wird dabei häufig ein- oder mehrfach cyclisiert, man spricht dann auch von Terpencyclasen. Die hochreaktiven Carbokation-Intermediate werden dabei von unpolaren und aromatischen Aminosäureresten im aktiven Zentrum der Enzyme stabilisiert. Zum Schluss der komplexen und vielfältigen Reaktionskaskade wird das Kation entweder zu einem reinen Kohlenwasserstoff deprotoniert oder seltener durch Addition von Wasser zu einem Alkohol umgesetzt (Christianson 2017).

Je nachdem auf welche Weise das Substrat zu dem Carbokation ionisiert wird, unterscheidet man zwei verschiedenen Klassen von Terpensynthasen: Klasse I Terpensynthasen ionisieren durch Abstraktion der Pyrophosphatgruppe mittels eines dreikernigen Metallclusters, so dass ein reaktives Allylkation entsteht. Klasse II Terpensynthasen protonieren die endständige Doppelbindung des Substrates mit einer Asparaginsäure-Seitenkette des aktiven Zentrums zu einem tertiären Carbokation (Wendt and Schulz 1998). Diese Unterteilung spiegelt sich auch in unterschiedlichen Proteinstrukturen wieder, wie schon die ersten verfügbaren Röntgenkristallstrukturen von Terpensynthasen aufzeigten (Lesburg et al. 1997; Starks et al. 1997; Wendt et al. 1997).

Man unterscheidet in Terpensynthasen generell die drei unterschiedlichen Strukturdomänen α,β und γ, die sich durch unterschiedlich angeordnete α-Helices auszeichnen. Terpensynthasen können aus einer oder aus einer Kombination mehrerer dieser Domänen bestehen. Terpensynthasen der Klasse I haben ihr aktives Zentrum dabei innerhalb der α-Domäne und solche der Klasse II zwischen der β- und der γ-Domäne (Cao et al. 2010; Köksal et al. 2011).

Zwischen der β- und der γ-Domäne befindet sich das für diese Klasse charakteristische Sequenzmotiv DXDD. Die mittlere Asparaginsäure ist die katalytisch aktive Aminosäure, die die Doppelbindung des Substrats zu dem Carbokation protoniert (Cao et al. 2010; Wendt et al. 1997)
Im Folgenden werden die Struktur und die Sequenzmotive der Terpensynthaseklasse I genauer beschrieben, da die für diese Arbeit wichtigen C_{11}-Terpensynthasen ebenfalls dieser Klasse angehören.

2.4.1 Terpensynthasen der Klasse I

Terpensynthasen der Klasse I weisen zwar häufig keine ähnlichen Sequenzen auf, sie besitzen jedoch die ähnliche α-helikale Struktur und ihr aktives Zentrum weist eine Reihe von konservierten Sequenzmotiven auf.

Aspartatreiches Motiv und NSE-Triade

Zunächst wurden die konservierten Sequenzen und die Funktionen des aspartatreichen Motivs DDXX(XX)D und der NSE-Triade (N,D)D(L,I,V)X(S,T)XXXE aufgeschlüsselt. Sie sind beide für die Koordinierung der Mg2+-Cofaktoren im aktiven Zentrum maßgeblich. Das aspartatreiche Motiv und seine Funktion wurden aber zunächst nicht für Terpensynthasen, sondern für Prenyltransferasen bestimmt (Ashby and Edwards 1990; Tarshis et al. 1994). Die konservierte Sequenz der NSE-Triade wurde bei einer Analyse einer Aristolochen-Synthase dargestellt (Cane and Kang 2000) und die Funktion bei der Strukturaufklärung einer Trichodien-Synthase bestätigt (Rynkiewicz et al. 2001), auch wenn sie schon früher beobachtet wurde (Starks et al. 1997). Das aspartatreiche Motiv ist für die Bindung bzw. Koordinierung zweier Mg^{2+}-Kationen verantwortlich (Mg^{2+}_A und MG^{2+}_C), während die NSE-Triade ein Drittes bindet (Mg^{2+}_B). Die Ionen koordinieren zum einen das Substrat über die Pyrophosphat-Gruppe, zum anderen sind sie für die Abstraktion dieser verantwortlich, woraufhin das reaktive Carbokation entsteht.

Effektortriade

2014 wurde ein weiteres konserviertes Sequenzmotiv genauer beschrieben. Für die Effektortriade bestehend aus Pyrophosphatsensor, Linker und Effektor wurde

ein Mechanismus mit großer Bedeutung für die Abstraktion der Pyrophosphatgruppe und der damit einhergehenden Ionisierung des Substrats, vorgeschlagen (Baer, Rabe, Fischer, et al. 2014). Bei Substratbindung vollzieht das Enzym einen *Induced-Fit*-Mechanismus, bei dem das Enzym von seiner offenen (apo) Konformation, in die geschlossene (substratgebundene) Konformation übergeht. Im Zuge des Übergangs dreht sich der sogenannte Pyrophosphat-Sensor - eine Argininseitenkette - zur Pyrophosphatgruppe des Substrats und es können zwei Wasserstoffbrückenbindungen zwischen Sensor und Pyrophosphatgruppe gebildet werden. Gleichzeitig orientiert sich der sogenannte Linker - eine polare Seitenkette (z.B. Asp, Asn, Thr oder Ser) - ebenfalls neu und bildet eine Wasserstoffbrückenbindung zum Pyrophosphat-Sensor aus, so dass die Konformationsänderung stabilisiert wird und der benachbarte Effektor - eine kleine Seitenkette (z.B. Gly) - in das katalytisch aktive Zentrum ragt und auf das C3-Atom des Substrats zeigt (Baer, Rabe, Fischer, et al. 2014). Das Carbonylsauerstoffatom des Effektors nimmt hierbei eine Schlüsselrolle ein: Gemeinsam mit den Wasserstoffbrückenbindungen des positiv geladenen Pyrophosphatsensors zu der negativ geladenen Pyrophosphatgruppe des Substrats und seiner hohen Elektronendichte, die auf das Π-Orbital der C2-C3-Doppelbindung wirkt, löst es die Ionisierung des Substrates aus (Starks et al. 1997; Baer, Rabe, Citron, et al. 2014).

WXXXXXRY-Motiv

Die WXXXXXRY-Sequenz ist ebenfalls ein konserviertes Motiv am C-Terminus von bakteriellen Terpensynthasen. Die C-terminale Region scheint sich bei dem *Induced-Fit*-Mechanismus über die aktive Tasche des Enzyms zu legen und so eine Art Deckel in der substratgebundenen Konformation zu bilden (Driller et al. 2018). Die Arginin- und Tyrosinseitenketten bilden ebenfalls Wasserstoffbrückenbindungen zur Pyrophosphatgruppe des Substrates und zu Seitenketten der NSE-Triade aus (Rabe et al. 2016). Die aromatische Tryptophanseitenkette mit der hohen Elektronendichte des aromatischen Ringes hat stabilisierende Effekte auf die hochreaktiven Carbokation-Intermediate, wie Mutationsstudien mit Phenylalanin und nicht-aromatischen Aminosäuren zeigen (Starks et al. 1997; Seemann et al. 2002; Driller et al. 2018).

Aromatische und aliphatische Seitenketten

Dieser oben beschriebene stabilisierende Effekt von den aromatischen Seitenketten ist für fast alle Terpensynthasen bekannt und wird durch Kation-Π-Interaktionen erklärt. Neben den polaren Seitenketten der metallbindenden Motive ragen hauptsächlich hydrophobe und aromatische Aminosäuren in das aktive Zentrum, die so eine konturgebende Tasche bilden und mit dieser den komplexen Reaktionsmechanismus des Carbokations leiten und zur korrekten Faltung der Intermediate und des Produktes führen (Lesburg et al. 1997; Christianson 2017). Einflussreiche aromatische und aliphatische Seitenketten von bakteriellen und anderen Terpensynthasen liegen hierbei jeweils drei und vier Positionen vor dem aspartatreichen Motiv und der NSE-Triade, sechs und sieben Positionen vor dem WXXXXXRY-Motiv, sowie eine, drei und vier Positionen vor und fünf, sechs, acht und neun Positionen nach dem Pyrophosphatsensor (Xu and Dickschat 2022).

2.5 Zielsetzung der Arbeit

Ziel dieser Arbeit ist die mikrobielle *de novo* Produktion neuartiger nicht-kanonischer Terpene und somit die Erweiterung des chemischen Strukturraumes von Terpenoiden über die Grenzen der Isoprenregel hinaus. Hierfür wurden verschiedene Ansätze gewählt. In allen werden die Gene verschiedener Prenylpyrophosphat-Methyltransferasen und C_{11}-Terpensynthasen gemeinsam mit den Genen des Mevalonatweges einschließlich einer IPP-Isomerase in *E. coli* heterolog exprimiert, um die nicht-kanonischen Terpenprodukte zu bilden und detailliert zu untersuchen.

Dabei sollen die Produktspektren von zwei 2-MIBS-Terpensynthasen, zwei 2-MB-Terpensynthasen und einer neuidentifizierten IPP-Methyltransferase mit hoch sensitiven Methoden analysiert werden. Für eine der 2-MB-Synthasen wird ein halbrationaler *Protein-Engineering* Ansatz gewählt, um den Einfluss einzelner Aminosäuren und die Möglichkeit der Modifizierung des Produktspektrums hin zu neuen Terpensynthase-Hauptprodukten aufzuzeigen.

Die Produktion neuartiger C_6-und C_7-Prenylpyrophosphate mittels der IPP-Methyltransferase und die Promiskuität von Biosynthesewegen soll genutzt werden, um eine Vielzahl von methylierten Carotinoiden zu synthetisieren.

2.6 Überblick über die Publikationen

2.6.1 Heterologous expression of 2-methylisoborneol / 2-methylenebornane biosynthesis genes in *Escherichia coli* yields novel C11-terpenes

Kschowak MJ, Wortmann H, Dickschat JS, Schrader J, Buchhaupt M (2018) PLoS ONE 13(4): e0196082

In dieser Publikation wird das diverse Produktspektrum von vier C_{11}-Terpensynthasen aus verschiedenen Mikroorganismen beschrieben. Dafür wurden die entsprechenden Gene gemeinsam mit denen des Mevalonatweges, einer GPP-Synthase und einer GPP-Methyltransferase heterolog in *E. coli* exprimiert, so dass die C_{11}-Terpene *de novo* produziert wurden. Die volatilen Produkte wurden mittels SPME-Fasern aus dem Kopfraum der Kulturen extrahiert und anschließend mittels GC-MS analysiert. Bei den C_{11}-Terpensynthasen handelte es sich um zwei 2-MIB-Synthasen aus *Streptomyces coelicolor* und *Streptomyces griseus* sowie zwei 2-MB-Synthasen aus *Pseudomonas fluorescens* und *Micromonospora olivasterospora*.
Bei den qualitativen Analysen wurden neben den Hauptprodukten 2-MIB bzw. 2-MB 33 weitere C_{11}-Terpene detektiert. Die neun C_{11}-Terpene 2-Methyl-2-bornene, 1-Methylcamphene, 2-Methylmyrcene, 2-Methyllimonene, 2-Methyl-β-fenchol, 2-Methyllinalool, 2-Methyl-α-terpineol, 2-Methylgeraniol und 2-Methylnerol wurden bereits zuvor als Nebenprodukte von C_{11}-Terpensynthasen beschrieben. 24 neue Nebenprodukte konnten identifiziert werden und bei vier von Ihnen die Struktur aufgeklärt werden: 2-Methylborneol, 3,4-Dimethylcumene und die beiden Diastereomere von 2-Methylcitronellol.
Die Bildung von weniger volatilen Terpenoiden durch die Produktionsstämme wurde ebenfalls untersucht. Dabei wurde ein mit Polydimethylsiloxan beschichteter Rührfisch in der Flüssigphase der Kultur inkubiert (Stir Bar Sorptive Extraction, SBSE), die Analyten mit Hexan extrahiert und mit der GC-MS analysiert. Neben den fünf Sesquiterpenoiden Nerolidol, 2,3-Dihydrofarnesol, (*Z,E*)-Farnesol, (*E,E*)-Farnesol und Farnesal wurde das C_{16}-Terpen 6-Methylfarnesol produziert. Letzteres wurde auch von einem Kontrollstamm

produziert, der kein Gen einer C_{11}-Terpensynthase exprimierte, aber nicht von einem Kontrollstamm ohne GPP-Methyltransferase. Vermutlich katalysiert die endogene FPP-Synthase die Kondensation von 2-Methyl-GPP mit IPP zu 6-Methyl-FPP, das anschließend entweder spontan oder durch ein anderes endogenes Enzym zu 6-Methylfarnesol hydrolysiert wird.

2.6.2 Analyzing and engineering the product selectivity of a 2-methylenebornane synthase

Kschowak MJ, Maier F, Wortmann H, Buchhaupt M (2020) ACS Synthetic Biology 9, 5, 981–986

Diese Publikation beschreibt ein semirationales Protein-Engineering der 2-MB-Synthase aus *Pseudomonas fluorescens,* die bereits in der zuvor beschriebenen Arbeit untersucht wurde. Dafür wurde zunächst ein Sequenzvergleich und eine Homologiemodellierung der 2-MB-Synthase durchgeführt. Auf Grundlage derer wurden 13 Aminosäuren in Umgebung des aktiven Zentrums ausgewählt, die durch Alanin substituiert wurden. Diese 13 Gene und das des Wildtyps wurden wie in der vorrangegangenen Studie jeweils gemeinsam mit denen des Mevalonatweges, einer GPP-Synthase und einer GPP-Methyltransferase heterolog in *E. coli* exprimiert. Die Produktselektivität und die 2-MB-Konzentration wurden mittels SBSE-GC-MS bestimmt.

Alle Stämme, die eines der mutierten 2-MB-Synthase-Gene exprimieren, zeigten im Vergleich zu dem Stamm, der das Wildtyp-Gen exprimiert, deutlich reduzierte 2-MB-Konzentrationen. Das zeigt die Bedeutung der ausgewählten Aminosäurereste in der Nähe des aktiven Zentrums für die Substratbindung oder -umsetzung. Das Produktspektrum der F205A-Mutante zeigte eine starke Änderung auf. So wurde deutlich weniger des Hauptproduktes 2-MB produziert und stattdessen das Nebenprodukt 2-Methyllimonen und drei C_{11}-Terpene mit unbekannter Struktur freigesetzt.

Um die Schlüsselrolle der Aminosäure F205 in der Produktbildung genauer zu untersuchen, wurde an dieser Stelle eine Sättigungsmutagenese durchgeführt. Der 2-MB-Anteil an der gesamten C_{11}-Terpen-Produkt-Peakfläche war bei allen Stämmen mit Enzymvarianten im Vergleich zu dem Stamm mit dem Wildtyp-

Enzym reduziert. Nur geringe Mengen an C_{11}-Terpensynthase-Produkten wurden in Kulturen von Stämmen gefunden, bei denen F205 durch eine der basischen oder sauren Aminosäuren ausgetauscht wurde. Die anderen Mutanten zeigten verschiedene Produktselektivtäten und insgesamt konnten vier neue, zuvor nicht beschriebene, C_{11}-Terpene produziert werden.

2.6.3 Expanding the Isoprenoid Building Block Repertoire with an IPP Methyltransferase from *Streptomyces monomycini*

Drummond L, Kschowak MJ*, Breitenbach J, Wolff H, Shi YM, Schrader J, Bode HB, Sandmann G, Buchhaupt M (2019) ACS Synthetic Biology 8, 6, 1303-1313*
**zu gleichen Teilen beigetragen*

In diesem Artikel wird eine weitere Möglichkeit beschrieben, nicht-kanonische Terpene herzustellen; mit einer IPP-Methyltransferase aus *Streptomyces monomycini,* die IPP einfach zu (*E*)-4-Methyl-IPP, (*Z*)-4-Methyl-IPP, 4-Methyl-DMAPP und zweifach zu 4,4-Dimethyl-IPP und 4,4-Dimethyl-DMAPP methylieren kann. Dies zeigten *in vitro*-Studien. Das enzymkodierende Gen befindet sich im Genom von *S. monomycini* vor den Genen einer putativen Prenyltransferase und einer Terpensynthase, deren genaue Funktionen noch nicht aufgeklärt wurden. Das Struktur-Homologiemodell zeigt im Vergleich zu der GPP-Methyltransferase aus *S. coelicolor* einen Tryptophanrest, der in die Substratbindetasche ragt und diese somit deutlich verkleinert. Das Gen wurde gemeinsam mit denen des Mevalonatweges in *E. coli* exprimiert und die volatilen Stoffe aus dem Kopfraum mit SPME-GC-MS analysiert. Neben den Produkten aus den *in vitro*-Studien wurde mit diesem *in vivo*-Ansatz außerdem 5-Methyl-IPP produziert. Zusätzlich konnten Kondensationsprodukte aus 4-Methyl-IPP mit DMAPP oder IPP, sowie 4-Methyl-DMAPP mit IPP zu 4-Methyl-GPP und 4-Methyl-FPP bzw. 8-Methyl-GPP produziert werden.
Zur Detektion weiterer Produkte der Methyltransferase und Prenyltransferase im *in vivo*-Ansatz wurden Fütterungsexperimente mit (methyl-^{13}C)-Methionin umgesetzt. So konnten durch Vergleich der Massenspektren aus GC-Analysen von Kulturen mit unmarkiertem Methionin alle durch die Methyltransferase katalysierten Übertragungen von Methylgruppen detektiert werden. Dieser

Vergleich zeigte nicht nur die Umsetzung zu C_{11}- und C_{16}-Terpenoide, sondern auch zu C_{12}- und C_{17}-Terpenoiden.

Um zu überprüfen, ob die methylierten, nicht-kanonischen Prenylpyrophosphatbausteine auch von nachfolgenden Enzymen eines längeren Terpenoid-Biosyntheseweges umgesetzt werden und in die Produkte eingebaut werden, wurde das Gen der IPP-Methyltransferase in *E. coli* Stämmen zusätzlich zur heterologen *de novo*-Produktion von Carotinoiden exprimiert. Entlang des Biosyntheseweges zu Zeaxanthin – über GGPP, Phytoen, Lycopin, und β-Carotin – stellten Stämme zur Produktion von Phytoen und Lycopin einfach (C_{41}), zweifach (C_{42}) und dreifach methylierte Carotinoide (C_{43}) her und Stämme zur Produktion von β-Carotin und Zeaxanthin einfach und zweifach methylierte Carotinoide. Aufgrund der Möglichkeit des Einbaus von methylierten oder nicht methylierten Prenylpyrophosphatbausteinen an verschiedenen Positionen der Produkte gibt es eine Reihe von Möglichkeiten, an welchen Positionen die Carotinoide methyliert sind. Dass die nicht-kanonischen Carotinoide an verschiedenen Positionen methyliert sind, wurde exemplarisch an Monomethyl-β-Carotin mittels HPLC-MS-MS Analyse gezeigt.

3 Zusammenfassende Diskussion

Zwei Wege, C_{11}-Terpene zu produzieren, wurden im Rahmen dieser Arbeit aufgezeigt (vgl. Publikationszusammenfassungen Kap. 2.6.1 und Kap. 2.6.2). Der in der ersten Arbeit propagierte Reaktionsmechanismus für die 2-MIB- und 2-MB-Synthasen zeigt, dass die Nebenprodukte aus Abbruchreaktionen der Carbokationreaktionskaskaden zum 2-Methybornylkation entstanden sind. Wie bereits eingeführt (s. Kap. 2.4.1) spielen insbesondere aromatische Seitenketten eine wichtige Rolle bei der Stabilisierung der Carbokationen im aktiven Zentrum der Terpensynthasen. Die Schlüsselrolle, die F205 in der 2-MB-Synthase aus *Pseudomonas fluorescens* einnimmt, wurde durch eine Sättigungsmutagenese untersucht. Ein Einfluss beim Übergang von der *trans*- zur *cis*-Form der Carbokationintermediate wird vermutet (Hyatt et al. 2007). Auch die anderen in der vorliegenden Arbeit untersuchten C_{11}-Terpensynthasen weisen an dieser Position ein Phenylalaninrest auf (F305 in MIBSc, F302 in MIBSg, F268 in MBSm). Eine besondere Rolle scheinen die Aminosäuren in dieser Position bei C_{11}-Terpensynthasen einzunehmen. Eine andere Studie untersuchte die Umsetzung von 2-Methyl-GPP zu C_{11}-Terpenen durch Monoterpensynthasen. Hierbei zeigte sich, dass ein Austausch von Phenylalanin bzw. Histidin an dieser Position mit den Aminosäuren Histidin, Tryptophan, Isoleucin, Valin oder Leucin zu einer erhöhten Spezifität gegenüber 2-Methyl-GPP im Vergleich zu GPP führte (Ignea et al. 2018). Eine Vergleich der Aminosäuresequenzen von nicht-kanonischen Terpensynthasen mit denen von kanonischen, insbesondere der metallbindenden Motive zeigte eine hohe Ähnlichkeit und keine Besonderheiten (Piechulla et al. 2021).

Ebenso ist es sehr wahrscheinlich, dass der Reaktionsmechanismus der Prenylpyrophosphat-Methyltransferasen, die insbesondere in der dritten Publikation dieser Arbeit im Fokus stehen (s. Kap. 2.6.3), über Carbokationen führt. Dies wurde bereits für die GPP-MTase propagiert (Ariyawutthiphan et al. 2012) und auch die in der vorliegenden Arbeit identifizierten Produkte der IPP-MTase aus *S. monomycini* lassen darauf schließen. Hierbei findet ein elektrophiler Angriff von SAM auf die Doppelbindung des Substrates statt, sodass dieses ionisiert wird und ein Carbokation entsteht. Je nachdem an welcher

Position dieses deprotoniert, entsteht 5-Methyl-IPP, 4-Methyl-IPP oder 4-Methyl DMAPP als Produkt. Kürzlich wurden aufbauend auf dieser Publikation weitere IPP-MTasen untersucht und es konnten weitere IPP-MTase-Produkte identifiziert werden, insgesamt fünf C_6- und sechs C_7-Moleküle, sowie ein C_8-Molekül bei einer *in vivo* Inkubation (Drummond et al. 2022). Die genannten Produkte der IPP-MTasen und der Reaktionsmechanismus über Carbokationen zeigen, dass analog zu den TS-katalysierten Reaktionen nicht nur Deprotonierungen als finaler Schritt in der MTase-Reaktion möglich sind, sondern auch Hydridverschiebungen stattfinden. Ebenso wurde eine Zyklisierung während der Methylierung von FPP (s.Abb. 9) durch eine MTase beschrieben (von Reuss et al. 2018). Insbesondere die Arbeit an den IPP- und DMAPP-MTasen zeigt die Möglichkeiten auf, die durch Einführung der Methylgruppen hinsichtlich der neu entstandenen Stereoisomere besteht. Die meisten untersuchten MTasen weisen eine hohe Substratselektivität, Selektivität der Deprotonierungsstellen und Stereoselektivität bzw. Enantioselektivität auf (Drummond et al. 2022). Die selektiv entstandenen Isomere führen auch in den Folgereaktionen spezifisch zu Stereoisomeren. So kondensiert beispielsweise eine Prenyltransferase (*E*)-4-Methyl-IPP mit GPP zu (*R*)-4-Methyl-FPP kondensiert und (*Z*)-4-Methyl-IPP mit GPP zu (*S*)-4-Methyl-FPP (Tanetoshi Koyama et al. 1980).

Jeweils zehn Aminosäurereste des aktiven Zentrums der Prenylpyrophosphat-Methyltransferasen IPP-MTase aus *S. monomycini*, der GPP-MTase aus *S. coelicolor* und der FPP-MTase aus *Serratia plymuthica* sind hydrophob und fördern die Bindung der hydrophoben Enden der Substrate. Auch ein Tripel-Histidin-Motiv zur Bindung der Pyrophoshatgruppe ist in allen drei Methyltransferasen konserviert (Lemfack et al. 2021). Die in der dritten Publikation dieser Arbeit aufgestellte Hypothese, dass der Tryptophanrest im aktiven Zentrum der IPP-MTase, dieses im Vergleich zum Glycinrest der GPP-MTase verkleinert, wurde in der Zwischenzeit untermauert. Der Wildtyp der GPP-MTase setzt kein DMAPP oder IPP um. Ein Austausch der kurzen Seitenkette des Glycins mit größeren führte zu einem Umsatz von DMAPP (Xia et al. 2023). Andersherum wurde für eine C6-GPP-MTase, deren Wildtyp ausschließlich GPP umsetzt, gezeigt, dass der Austausch eines Tryptophanrests gegen einen Alaninrest zur Akzeptanz von FPP als Substrat und C6-Metyhlierung führt (Tsutsumi et al. 2022).

Die Relevanz, der hier zusammengefassten und gemeinsam betrachteten Publikationen, zeigt sich auch in weiteren Publikationen, die sich mit Prenylpyrophosphat-Methyltransferasen und der Produktion nicht-kanonischer Terpene beschäftigt und zeitgleich oder kurze Zeit später veröffentlicht wurde. Ein Teil der hier mit *E. coli* produzierten C_{11}-Terpene wurde *de novo* mit *Saccharomyces cerevisiae* mit einer GPP-MTase und verschiedenen Monoterpensynthasen hergestellt (Ignea et al. 2018). Die gleiche Gruppe nutzte die FPP-MTase der Sodorifen-Biosynthese und sechs Mutanten der FPP-MTase gemeinsam mit zwei Diterpensynthasen und einer Cytochrom P450, um 28 verschiedene C_{16}-Terpene *de novo* in *S. cerevisiae* zu synthetisieren (Ignea et al. 2022). Die Gene des Biosyntheseweges nicht-kanonischer Terpene aus Insekten wurde in *E. coli* heterolog exprimiert, um C_{16} -Terpene zu produzieren (Eiben et al. 2019).

In 2019 veröffentlichten vier verschiedene Arbeitsgruppen einen neuen Weg über zwei Enzyme, um ausgehend von Isopentenol und Dimethylallylalkohol IPP und DMAPP zu synthetisieren (Lund et al. 2019; Chatzivasileiou et al. 2019; Couillaud et al. 2019; Clomburg et al. 2019). Für die erste Phosphorylierung nutzten die verschiedenen Arbeitsgruppen unterschiedliche Enzyme aus *S. cerevisiae*, *Shigella flexneri*, *E. coli* oder *Xanthomonas translucens.* Für die zweite Phosphorylierung nutzten alle eine Isopentenyl-Phosphat-Kinase aus einem Archaeon (Grochowski et al. 2006). Die Substrat-Promiskuität dieses Weges wurde kurze Zeit später beschrieben und bereits verschiedene Sesquiterpenderivate, unter anderem auch einfach und zweifach methylierte, synthetisiert.

Einige Strukturen der methylierten C_{41}-Carotenoide, dessen Produktion in der dritten Publikation der vorliegenden Arbeit gezeigt wurde, sind aufgeklärt, es handelt sich hierbei um 16-Methyl-Derivate von 15-*cis*-Phytoen, Lycopin, β-Carotin und Zeaxanthin (Takagi et al. 2020).

Über die natürliche Funktion nicht-kanonischer Isoprenoide ist relativ wenig bekannt. Das weitverbreitetste C_{11}-Terpen 2-Methylisoborneol, dessen Synthase auch in dieser Arbeit genauer untersuch wurde, wird von vielen *Streptomyceten* zusammen mit Geosmin produziert. Beide Substanzen locken Springschwänze

an, die unter anderem Mikroorganismen als Nahrungsquelle nutzen. Sie werden von Streptomyceten bei der Sporulierung gebildet und die Springschwänze verbreiten die Sporen über Anhaftung oder ihre Ausscheidungen (Becher et al. 2020). Die rote Feuerameise bevorzugt 2-MIB- und Geosmin-enthaltende Böden als Nistplätze. Es wird vermutet, dass die terpenausstoßenden Bakterien entomopathogene Pilze hemmen (H. Huang et al. 2020). Von einem anderen bakteriellen nicht-kanonischen Terpen ist die Funktion bekannt: Das phytopathogene Bakterium *Rhodococcus fascians* produziert Cytokinine, Phytohormone, die Pflanzenwachstum und -entwicklung regulieren. Die methylierten Cytokinine verbleiben im Gegensatz zu kanonischen Cytokininen länger in Pflanzen und sind schlechtere Substrate für eine abbauende Dehydrogenase. Hier erfüllen die Methylgruppen durch eine Erhöhung der biologische Stabilität eine spezifische Funktion, was ihren Produzenten einen Vorteil verschaffen könnte (Radhika et al. 2015). *Serratia plymuthica* setzt unter Einfluss von bestimmten volatilen Stoffen von Pilzen verstärkt Sodorifen frei und scheint eine wichtige Rolle in der Kommunikation mit Pilzen zu spielen (Schmidt et al. 2017).

Über die Funktion von Insekten produzierten Homoterpenen ist etwas mehr bekannt. Homo-γ-Bisabolen, 9-Methylgermacren-B und 3 Methyl-α-Himachalen sind männliche Sex-Pheromone (Hamilton et al. 1996b; 1996a; Staddon et al. 1994). 3-Methyl-7-Methylen-1,3,8-Nonatrien wird unter bestimmten Stressbedingungen vom Borkenkäfer *I. typographus* freigesetzt (Francke et al. 1995). Faranal wird als *true trail pheromone* der Ameise *Monomorium pharaonis* bezeichnet und hat eine Lockwirkung auf Ameisen der gleichen Art (Ritter et al. 1977).

Die dargelegten Funktionen zeigen, dass die produzierenden Organismen einen Vorteil durch die Produktion und Interaktionen der nicht-kanonischen Terpene erlangen, der sich von den Funktionen der kanonischen Terpene teilweise abgrenzt. In Umgebung mit hohen Konzentrationen von durch Pflanzen freigesetzten, kanonischen Terpenen, könnte dieser „Dialekt“ der neuartigen Terpene eine spezifischere Wechselwirkung zulassen und einen evolutionären Vorteil bringen. Eine oder mehrere zusätzliche Methylgruppe könnten im Fall von Kommunikationssignalen die Bindung an für kanonische Terpene angepasste Rezeptoren deutlich verringern oder unmöglich machen. Andererseits wird in der

pharmazeutischen Forschung der *magic methyl effect* beschrieben, der zu einer vielfach erhöhte Affinität durch Einführung einer Methylgruppe im Wirkstoff führt (Schönherr and Cernak 2013). So wäre es denkbar für ein natürlicherweise kanonisches Terpen mit mehreren Funktionen, durch Einführung einer Methylgruppe eine Funktion abzuschalten. Auch andere Vorteile von Derivaten, wie ein verlangsamter Abbau oder eine veränderte Membrangängigkeit sind für methylierte Terpene vorstellbar und wurden wie oben beschrieben für einzelne Terpene bereits aufgezeigt.

2-Methylisoborneol hat einen unangenehmen Geruch, mit dem Probleme in der Trinkwasseraufbereitung und der Fischzucht einhergehen (Jüttner and Watson 2007). Für die meisten der während dieser Arbeit produzierten methylierten Hemi-, Mono- und Sesquiterpene wurden mittlerweile die Geruchseigenschaften genauer untersucht (Sommer et al. 2022). Besonders methylierte Monoterpene wiesen dabei intensive und angenehme Gerüche auf und hatten einen ähnlichen Geruchsschwellenwert, im Vergleich zu ihren nicht-methylierten Äquivalenten. Sie unterschieden sich jedoch teilweise hinsichtlich ihres Geruches. So wurde der Geruch von Methylcaren als fruchtig, süßlich und nadelwaldartig beschrieben, während (S)-Caren einen harzigen Geruch hat. Der Einfluss der Stereoisomerie und der Position der Methylierung wurde anhand der *cis-trans*-Isomere Nerol und Geraniol untersucht. Geraniol hat einen blumigen, zitrusartigen Geruch, während sein Isomer Nerol zusätzlich einen harzigen Geruch besitzt. Die Nerolderivate zeigten jeweils Unterschiede auf. Der Geruch 2-Methylnerols wurde als süßlich, blumig, frisch, zitrus- und orangenähnlich beschrieben. Im Gegensatz dazu wurde 4-Methylnerol als grün, fruchtig, blumig und zitrusartig beschrieben. 2-Methylgeraniol wies keinen Unterschied zu Geraniol im Geruch auf, während 8-Methylgeraniol einen harzigeren Geruchseindruck und 4-Methylgeraniol vor allem einen zitronenartigen Geruchseindruck vermittelte. Das Racemat Linalools hat einen frischen, blumigen, holzigen und herben Geruch. Der Geruch von *(R/S)*-2-Methyllinalool wurde ebenfalls als angenehm und insgesamt ähnlich, jedoch zusätzlich als zitronen- und bergamotteartig beschrieben (Sommer et al. 2022).

Dies zeigt das Potenzial, dass die stereospezifische Einführung von Methylgruppen bei Terpenen für neue Aromastoffe hat.

4 Literaturverzeichnis

Ariyawutthiphan, Orapin, Toyoyuki Ose, Atsushi Minami, Sandip Sinde, Muneya Tsuda, Y-G Gao, Min Yao, Hideaki Oikawa, and Isao Tanaka. 2012. “Structure Analysis of Geranyl Pyrophosphate Methyltransferase and the Proposed Reaction Mechanism of SAM-Dependent C-Methylation.” *Acta Crystallographica Section D: Biological Crystallography* 68 (11): 1558–69.

Ashby, M N, and P A Edwards. 1990. “Elucidation of the Deficiency in Two Yeast Coenzyme Q Mutants. Characterization of the Structural Gene Encoding Hexaprenyl Pyrophosphate Synthetase.” *Journal of Biological Chemistry* 265 (22): 13157–64. https://doi.org/10.1016/S0021-9258(19)38280-8.

Avalos, Mariana, Paolina Garbeva, Lisa Vader, Gilles P. van Wezel, Jeroen S. Dickschat, and Dana Ulanova. 2022. “Biosynthesis, Evolution and Ecology of Microbial Terpenoids.” *Natural Product Reports* 39 (2): 249–72. https://doi.org/10.1039/D1NP00047K.

Azami, Yasuhiro, Ai Hattori, Hiroto Nishimura, Hiroshi Kawaide, Tohru Yoshimura, and Hisashi Hemmi. 2014. “*(R)*-Mevalonate 3-Phosphate Is an Intermediate of the Mevalonate Pathway in Thermoplasma Acidophilum.” *Journal of Biological Chemistry* 289 (23): 15957–67. https://doi.org/10.1074/jbc.M114.562686.

Baer, Philipp, Patrick Rabe, Christian A. Citron, Carina C. de Oliveira Mann, Norman Kaufmann, Michael Groll, and Jeroen S. Dickschat. 2014. “Hedycaryol Synthase in Complex with Nerolidol Reveals Terpene Cyclase Mechanism.” *ChemBioChem* 15 (2): 213–16. https://doi.org/10.1002/cbic.201300708.

Baer, Philipp, Patrick Rabe, Katrin Fischer, Christian A. Citron, Tim A. Klapschinski, Michael Groll, and Jeroen S. Dickschat. 2014. “Substratinduzierte Konformationsänderungen in Klasse-I-Terpencyclasen.” *Angewandte Chemie* 126 (29): 7783–87. https://doi.org/10.1002/ange.201403648.

Becher, Paul G., Vasiliki Verschut, Maureen J. Bibb, Matthew J. Bush, Béla P. Molnár, Elisabeth Barane, Mahmoud M. Al-Bassam, et al. 2020. “Developmentally Regulated Volatiles Geosmin and 2-Methylisoborneol Attract a Soil Arthropod to Streptomyces Bacteria Promoting Spore Dispersal.” *Nature Microbiology* 5 (6): 821–29. https://doi.org/10.1038/s41564-020-0697-x.

Behnke, Katja, Barbara Ehlting, Markus Teuber, Martina Bauerfeind, Sandrine Louis, Robert Hänsch, Andrea Polle, Jörg Bohlmann, and Jörg-Peter Schnitzler. 2007. “Transgenic, Non-Isoprene Emitting Poplars Don’t like It Hot: Thermotolerance in Isoprene Emission Knock-down Mutants of Poplar.” *The Plant Journal* 51 (3): 485–99. https://doi.org/10.1111/j.1365-313X.2007.03157.x.

Bergot, B. John, Gene C. Jamieson, Milton A. Ratcliff, and David A. Schooley. 1980. “JH Zero: New Naturally Occurring Insect Juvenile Hormone from Developing Embryos of the Tobacco Hornworm.” *Science* 210 (4467): 336–38. https://doi.org/10.1126/science.210.4467.336.

Bian, Guangkai, Yichao Han, Anwei Hou, Yujie Yuan, Xinhua Liu, Zixin Deng, and Tiangang Liu. 2017. “Releasing the Potential Power of Terpene

Synthases by a Robust Precursor Supply Platform." *Metabolic Engineering* 42 (July): 1–8. https://doi.org/10.1016/j.ymben.2017.04.006.

Blatrix, Rumsaïs, Claudia Schulz, Pierre Jaisson, Wittko Francke, and Abraham Hefetz. 2002. "Trail Pheromone of Ponerine Ant *Gnamptogenys Striatula*: 4-Methylgeranyl Esters from Dufour's Gland." *Journal of Chemical Ecology* 28 (12): 2557–67. https://doi.org/10.1023/A:1021444321238.

Bleeker, Petra M., Paul J. Diergaarde, Kai Ament, Stefan Schütz, Bettina Johne, Jan Dijkink, Henk Hiemstra, et al. 2011. "Tomato-Produced 7-Epizingiberene and R-Curcumene Act as Repellents to Whiteflies." *Phytochemistry* 72 (1): 68–73. https://doi.org/10.1016/j.phytochem.2010.10.014.

Bohlmann, Jörg, John Crock, Reinhard Jetter, and Rodney Croteau. 1998. "Terpenoid-Based Defenses in Conifers: CDNA Cloning, Characterization, and Functional Expression of Wound-Inducible (*E*)-α-Bisabolene Synthase from Grand Fir (*Abies Grandis*)." *Proceedings of the National Academy of Sciences* 95 (12): 6756–61. https://doi.org/10.1073/pnas.95.12.6756.

Boronat, Albert, and Manuel Rodríguez-Concepción. 2015. "Terpenoid Biosynthesis in Prokaryotes." In *Biotechnology of Isoprenoids*, edited by Jens Schrader and Jörg Bohlmann, 3–18. Advances in Biochemical Engineering/Biotechnology. Cham: Springer International Publishing. https://doi.org/10.1007/10_2014_285.

Bouchardat, A. 1838. "Ueber Die Destillationsproducte Des Kautschucks." *Annalen Der Pharmacie* 27 (1): 30–42. https://doi.org/10.1002/jlac.18380270110.

Bowers, William S., Malcolm J. Thompson, and Edward C. Uebel. 1965. "Juvenile and Gonadotropic Hormone Activity of 10,11-Epoxyfarnesenic Acid Methyl Ester." *Life Sciences* 4 (23): 2323–31. https://doi.org/10.1016/0024-3205(65)90256-0.

Brock, Nelson L., Srinivasa R. Ravella, Stefan Schulz, and Jeroen S. Dickschat. 2013. "A Detailed View of 2-Methylisoborneol Biosynthesis." *Angewandte Chemie International Edition* 52 (7): 2100–2104. https://doi.org/10.1002/anie.201209173.

Buckingham, John. 2020. "Dictionary of Natural Products 28.2." In . CHEMnetBASE. London: Chapman and Hall / CRC Press. https://dnp.chemnetbase.com.

Byers, Kelsey J. R. P., H. D. Bradshaw Jr, and Jeffrey A. Riffell. 2014. "Three Floral Volatiles Contribute to Differential Pollinator Attraction in Monkeyflowers (Mimulus)." *Journal of Experimental Biology* 217 (4): 614–23. https://doi.org/10.1242/jeb.092213.

Callejón, R. M., C. Ubeda, R. Ríos-Reina, M. L. Morales, and A. M. Troncoso. 2016. "Recent Developments in the Analysis of Musty Odour Compounds in Water and Wine: A Review." *Journal of Chromatography A* 1428: 72–85. https://doi.org/10.1016/j.chroma.2015.09.008.

Cane, David E, and Ilgu Kang. 2000. "Aristolochene Synthase: Purification, Molecular Cloning, High-Level Expression in *Escherichia Coli*, and Characterization of the *Aspergillus Terreus* Cyclase." *Archives of Biochemistry and Biophysics* 376 (2): 354–64. https://doi.org/10.1006/abbi.2000.1734.

Cao, Rong, Yonghui Zhang, Francis M. Mann, Cancan Huang, Dushyant Mukkamala, Michael P. Hudock, Matthew E. Mead, et al. 2010. “Diterpene Cyclases and the Nature of the Isoprene Fold.” *Proteins: Structure, Function, and Bioinformatics* 78 (11): 2417–32. https://doi.org/10.1002/prot.22751.

Cascón, Oscar, Sabrina Touchet, David J. Miller, Veronica Gonzalez, Juan A. Faraldos, and Rudolf K. Allemann. 2012. “Chemoenzymatic Preparation of Germacrene Analogues.” *Chemical Communications* 48 (78): 9702–4. https://doi.org/10.1039/C2CC35542F.

Chatzivasileiou, Alkiviadis Orfefs, Valerie Ward, Steven McBride Edgar, and Gregory Stephanopoulos. 2019. “Two-Step Pathway for Isoprenoid Synthesis.” *Proceedings of the National Academy of Sciences* 116 (2): 506–11. https://doi.org/10.1073/pnas.1812935116.

Chaykin, S., J. Law, A. H. Phillips, T. T. Tchen, and K. Bloch. 1958. “Phosphorylated Intermediates in the Synthesis of Squalene.” *Proceedings of the National Academy of Sciences of the United States of America* 44 (10): 998–1004. https://doi.org/10.1073/pnas.44.10.998.

Chou, Wayne KW, Colin A. Gould, and David E. Cane. 2017. “Incubation of 2-Methylisoborneol Synthase with the Intermediate Analog 2-Methylneryl Diphosphate.” *The Journal of Antibiotics* 70 (5): 625–31. https://doi.org/10.1038/ja.2017.24.

Chou, Wayne KW, Haruo Ikeda, and David E Cane. 2011. “Cloning and Characterization of Pfl_1841, a 2-Methylenebornane Synthase in *Pseudomonas Fluorescens* PfO-1.” *Tetrahedron* 67 (35): 6627–32. https://doi.org/10.1016/j.tet.2011.05.084.

Christianson, David W. 2017. “Structural and Chemical Biology of Terpenoid Cyclases.” *Chemical Reviews* 117 (17): 11570–648. https://doi.org/10.1021/acs.chemrev.7b00287.

Clomburg, James M., Shuai Qian, Zaigao Tan, Seokjung Cheong, and Ramon Gonzalez. 2019. “The Isoprenoid Alcohol Pathway, a Synthetic Route for Isoprenoid Biosynthesis.” *Proceedings of the National Academy of Sciences* 116 (26): 12810–15. https://doi.org/10.1073/pnas.1821004116.

Colby, Sheila M., John Crock, Barbara Dowdle-Rizzo, Peggy G. Lemaux, and Rodney Croteau. 1998. “Germacrene C Synthase from *Lycopersicon Esculentum* Cv. VFNT Cherry Tomato: CDNA Isolation, Characterization, and Bacterial Expression of the Multiple Product Sesquiterpene Cyclase.” *Proceedings of the National Academy of Sciences* 95 (5): 2216–21. https://doi.org/10.1073/pnas.95.5.2216.

Couillaud, Julie, Juan Rico, Alyssa Rubini, Tarek Hamrouni, Elise Courvoisier-Dezord, Jean-Louis Petit, Aline Mariage, et al. 2019. “Simplified in Vitro and in Vivo Bioaccess to Prenylated Compounds.” *ACS Omega* 4 (4): 7838–49. https://doi.org/10.1021/acsomega.9b00561.

Crabben, Jan van der. 2003. *Shining Rock Wilderness Area, Blue Ridge Mountains, North Carolina*. Fotografie. https://de.wikipedia.org/wiki/Blue_Ridge_Mountains#/media/Datei:Blue_Ridge_NC.jpg.

Crock, John, Mark Wildung, and Rodney Croteau. 1997. “Isolation and Bacterial Expression of a Sesquiterpene Synthase CDNA Clone from Peppermint (*Mentha* x *Piperita*, L.) That Produces the Aphid Alarm Pheromone (*E*)-β-

Farnesene." *Proceedings of the National Academy of Sciences* 94 (24): 12833–38. https://doi.org/10.1073/pnas.94.24.12833.

Dellas, Nikki, Suzanne T. Thomas, Gerard Manning, and Joseph P. Noel. 2013. "Discovery of a Metabolic Alternative to the Classical Mevalonate Pathway." *ELife* 2 (December): e00672. https://doi.org/10.7554/eLife.00672.

Dickschat, Jeroen S. 2016. "Bacterial Terpene Cyclases." *Natural Product Reports* 33 (1): 87–110. https://doi.org/10.1039/C5NP00102A.

Dickschat, Jeroen S., Thorben Nawrath, Verena Thiel, Brigitte Kunze, Rolf Mueller, and Stefan. Schulz. 2007. "Biosynthesis of the Off-Flavor 2-Methylisoborneol by the Myxobacterium *Nannocystis Exedens*." *Angewandte Chemie, International Edition* 46 (Copyright (C) 2016 American Chemical Society (ACS). All Rights Reserved.): 8287–90. https://doi.org/10.1002/anie.200702496.

Driller, Ronja, Sophie Janke, Monika Fuchs, Evelyn Warner, Anil R. Mhashal, Dan Thomas Major, Mathias Christmann, Thomas Brück, and Bernhard Loll. 2018. "Towards a Comprehensive Understanding of the Structural Dynamics of a Bacterial Diterpene Synthase during Catalysis." *Nature Communications* 9 (1): 1–8. https://doi.org/10.1038/s41467-018-06325-8.

Drummond, Laura, Parab J. Haque, Binbin Gu, Julia S. Jung, Hendrik Schewe, Jeroen S. Dickschat, and Markus Buchhaupt. 2022. "High Versatility of IPP and DMAPP Methyltransferases Enables Synthesis of C6, C7 and C8 Terpenoid Building Blocks." *ChemBioChem* 23 (14): e202200091. https://doi.org/10.1002/cbic.202200091.

Dudareva, Natalia, Eran Pichersky, and Jonathan Gershenzon. 2004. "Biochemistry of Plant Volatiles." *Plant Physiology* 135 (4): 1893–1902. https://doi.org/10.1104/pp.104.049981.

Eiben, Christopher B., Tristan de Rond, Clayton Bloszies, Jennifer Gin, Jennifer Chiniquy, Edward E. K. Baidoo, Christopher J. Petzold, Nathan J. Hillson, Oliver Fiehn, and Jay D. Keasling. 2019. "Mevalonate Pathway Promiscuity Enables Noncanonical Terpene Production." *ACS Synthetic Biology* 8 (10): 2238–47. https://doi.org/10.1021/acssynbio.9b00230.

Faraldos, Juan A., Yuxin Zhao, Paul E. O'Maille, Joseph P. Noel, and Robert M. Coates. 2007. "Interception of the Enzymatic Conversion of Farnesyl Diphosphate to 5-Epi-Aristolochene by Using a Fluoro Substrate Analogue: 1-Fluorogermacrene A from (2*E*,6*Z*)-6-Fluorofarnesyl Diphosphate." *ChemBioChem* 8 (15): 1826–33. https://doi.org/10.1002/cbic.200700398.

Francke, Wittko, Jochen Bartels, Holger Meyer, Frank Schröder, Ulrich Kohnle, Ernst Baader, and Jean Pierre Vité. 1995. "Semiochemicals from Bark Beetles: New Results, Remarks, and Reflections." *Journal of Chemical Ecology* 21 (7): 1043–63. https://doi.org/10.1007/BF02033807.

Fravel, Deborah R., Connick William J., Casey C. Grimm, and Steven W. Lloyd. 2002. "Volatile Compounds Emitted by *Sclerotia* of *Sclerotinia Minor*, *Sclerotinia Sclerotiorum* and *Sclerotium Rolfsii*." *Journal of Agricultural and Food Chemistry* 50 (13): 3761–64. https://doi.org/10.1021/jf0117098.

Gelmont, David, Robert A. Stein, and James F. Mead. 1981. "Isoprene — The Main Hydrocarbon in Human Breath." *Biochemical and Biophysical Research Communications* 99 (4): 1456–60. https://doi.org/10.1016/0006-291X(81)90782-8.

George, Kevin W., Jorge Alonso-Gutierrez, Jay D. Keasling, and Taek Soon Lee. 2015. “Isoprenoid Drugs, Biofuels, and Chemicals—Artemisinin, Farnesene, and Beyond.” In *Biotechnology of Isoprenoids*, edited by Jens Schrader and Jörg Bohlmann, 355–89. Advances in Biochemical Engineering/Biotechnology. Cham: Springer International Publishing. https://doi.org/10.1007/10_2014_288.

Gerber, Nancy N. 1969. “A Volatile Metabolite of Actinomycetes, 2-Methylisoborneol.” *The Journal of Antibiotics* 22 (10): 508–9. https://doi.org/10.7164/antibiotics.22.508.

Gols, Rieta. 2014. “Direct and Indirect Chemical Defences against Insects in a Multitrophic Framework.” *Plant, Cell & Environment* 37 (8): 1741–52. https://doi.org/10.1111/pce.12318.

Goodwin, T. W. 1964. “Chapter 19 - The Biogenesis of Terpenes and Steroids.” In *Rodd’s Chemistry of Carbon Compounds (Second Edition)*, edited by S. Coffey, 54–137. Amsterdam: Elsevier. https://doi.org/10.1016/B978-044453345-6.50248-0.

Grochowski, Laura L., Huimin Xu, and Robert H. White. 2006. “*Methanocaldococcus Jannaschii* Uses a Modified Mevalonate Pathway for Biosynthesis of Isopentenyl Diphosphate.” *Journal of Bacteriology* 188 (9): 3192–98. https://doi.org/10.1128/JB.188.9.3192-3198.2006.

Guenther, Alex, C. Nicholas Hewitt, David Erickson, Ray Fall, Chris Geron, Tom Graedel, Peter Harley, et al. 1995. “A Global Model of Natural Volatile Organic Compound Emissions.” *Journal of Geophysical Research: Atmospheres* 100 (D5): 8873–92. https://doi.org/10.1029/94JD02950.

Hamilton, J. G. C., G. W. Dawson, and J. A. Pickett. 1996a. “9-Methylgermacrene-B; Proposed Structure for Novel Homosesquiterpene from the Sex Pheromone Glands of *Lutzomyia Longipalpis* (Diptera: Psychodidae) from Lapinha, Brazil.” *Journal of Chemical Ecology* 22 (8): 1477–91. https://doi.org/10.1007/BF02027726.

———. 1996b. “3-Methyl-α-Himachalene: Proposed Structure for Novel Homosesquiterpene Sex Pheromone of *Lutzomyia Longipalpis* (Diptera: Psychodidae) from Jacobina, Brazil.” *Journal of Chemical Ecology* 22 (12): 2331–40. https://doi.org/10.1007/BF02029550.

Harms, Vanessa, Andreas Kirschning, and Jeroen S. Dickschat. 2020. “Nature-Driven Approaches to Non-Natural Terpene Analogues.” *Natural Product Reports* 37 (8): 1080–97. https://doi.org/10.1039/C9NP00055K.

Harms, Vanessa, Benjamin Schröder, Clara Oberhauser, Cong Duc Tran, Sven Winkler, Gerald Dräger, and Andreas Kirschning. 2020. “Methyl-Shifted Farnesyldiphosphate Derivatives Are Substrates for Sesquiterpene Cyclases.” *Organic Letters* 22 (11): 4360–65. https://doi.org/10.1021/acs.orglett.0c01345.

Heinz Dahm, Karl, Herbert Röller, and Barry M. Trost. 1968. “The Juvenile Hormone - Iv. Stereochemistry of Juvenile Hormone and Biological Activity of Some of Its Isomers and Related Compounds.” *Life Sciences* 7 (4): 129–37. https://doi.org/10.1016/0024-3205(68)90296-8.

Huang, Hongmei, Lu Ren, Huijing Li, Axel Schmidt, Jonathan Gershenzon, Yongyue Lu, and Daifeng Cheng. 2020. “The Nesting Preference of an Invasive Ant Is Associated with the Cues Produced by Actinobacteria in Soil.” *PLOS Pathogens* 16 (9): e1008800. https://doi.org/10.1371/journal.ppat.1008800.

Huang, Mengsu, Adela M. Sanchez-Moreiras, Christian Abel, Reza Sohrabi, Sungbeom Lee, Jonathan Gershenzon, and Dorothea Tholl. 2012. “The Major Volatile Organic Compound Emitted from *Arabidopsis Thaliana* Flowers, the Sesquiterpene (*E*)-β-Caryophyllene, Is a Defense against a Bacterial Pathogen.” *New Phytologist* 193 (4): 997–1008. https://doi.org/10.1111/j.1469-8137.2011.04001.x.

Hyatt, David C., Buhyun Youn, Yuxin Zhao, Bindu Santhamma, Robert M. Coates, Rodney B. Croteau, and ChulHee Kang. 2007. “Structure of Limonene Synthase, a Simple Model for Terpenoid Cyclase Catalysis.” *Proceedings of the National Academy of Sciences* 104 (13): 5360–65. https://doi.org/10.1073/pnas.0700915104.

Ignea, Codruta, Marianna Pontini, Mohammed S. Motawia, Massimo E. Maffei, Antonios M. Makris, and Sotirios C. Kampranis. 2018. “Synthesis of 11-Carbon Terpenoids in Yeast Using Protein and Metabolic Engineering.” *Nature Chemical Biology* 14 (12): 1090. https://doi.org/10.1038/s41589-018-0166-5.

Ignea, Codruta, Morten H. Raadam, Aikaterini Koutsaviti, Yong Zhao, Yao-Tao Duan, Maria Harizani, Karel Miettinen, et al. 2022. “Expanding the Terpene Biosynthetic Code with Non-Canonical 16 Carbon Atom Building Blocks.” *Nature Communications* 13 (1): 5188. https://doi.org/10.1038/s41467-022-32921-w.

Jiang, Jiaoyang, Xiaofei He, and David E. Cane. 2006. “Geosmin Biosynthesis. Streptomyces Coelicolor Germacradienol/Germacrene D Synthase Converts Farnesyl Diphosphate to Geosmin.” *Journal of the American Chemical Society* 128 (25): 8128–29. https://doi.org/10.1021/ja062669x.

Judy, Kenneth J., David A. Schooley, Loren L. Dunham, M. S. Hall, B. John Bergot, and John B. Siddall. 1973. “Isolation, Structure, and Absolute Configuration of a New Natural Insect Juvenile Hormone from *Manduca Sexta*.” *Proceedings of the National Academy of Sciences* 70 (5): 1509–13.

Junker, R. R., C. Loewel, R. Gross, S. Dötterl, A. Keller, and N. Blüthgen. 2011. “Composition of Epiphytic Bacterial Communities Differs on Petals and Leaves.” *Plant Biology* 13 (6): 918–24. https://doi.org/10.1111/j.1438-8677.2011.00454.x.

Jüttner, Friedrich, and Susan B. Watson. 2007. “Biochemical and Ecological Control of Geosmin and 2-Methylisoborneol in Source Waters.” *Applied and Environmental Microbiology* 73 (14): 4395–4406. https://doi.org/10.1128/AEM.02250-06.

Karahadian, Carol, D. B. Josephson, and R. C. Lindsay. 1985a. “Contribution of *Penicillium Sp.* to the Flavors of Brie and Camembert Cheese1.” *Journal of Dairy Science* 68 (8): 1865–77. https://doi.org/10.3168/jds.S0022-0302(85)81043-2.

Karahadian, Carol, David B. Josephson, and Robert C. Lindsay. 1985b. “Volatile Compounds from *Penicillium Sp.* Contributing Musty-Earthy Notes to Brie and Camembert Cheese Flavors.” *Journal of Agricultural and Food Chemistry* 33 (3): 339–43. https://doi.org/10.1021/jf00063a005.

Keegans, Sarah J., Johan Billen, E. David Morgan, and Oya A. Gökcen. 1993. “Volatile Glandular Secretions of Three Species of New World Army Ants, *Eciton Burchelli*, *Labidus Coecus*, and *Labidus Praedator*.” *Journal of Chemical Ecology* 19 (11): 2705–19. https://doi.org/10.1007/BF00980702.

Köksal, Mustafa, Wayne KW Chou, David E Cane, and David W Christianson. 2012. “Structure of 2-Methylisoborneol Synthase from *Streptomyces Coelicolor* and Implications for the Cyclization of a Noncanonical C-Methylated Monoterpenoid Substrate.” *Biochemistry* 51 (14): 3011–20. https://doi.org/10.1021/bi201827a.

Köksal, Mustafa, Yinghua Jin, Robert M. Coates, Rodney Croteau, and David W. Christianson. 2011. “Taxadiene Synthase Structure and Evolution of Modular Architecture in Terpene Biosynthesis.” *Nature* 469 (7328): 116–20. https://doi.org/10.1038/nature09628.

Komatsu, Mamoru, Muneya Tsuda, Satoshi Ōmura, Hideaki Oikawa, and Haruo Ikeda. 2008. “Identification and Functional Analysis of Genes Controlling Biosynthesis of 2-Methylisoborneol.” *Proceedings of the National Academy of Sciences* 105 (21): 7422–27. https://doi.org/10.1073/pnas.0802312105.

Koyama, Tanetoshi, Michio Ito, Shinichi Ohnuma, and Kyozo. Ogura. 1988. “Novel Behavior of Undecaprenyl Diphosphate Synthase toward an Artificial Substrate. Formation of the (*S*)-4-Methyl Derivative of *Z,E,E*-Geranylgeranyl Diphosphate.” *Tetrahedron Letters* 29 (Copyright (C) 2016 American Chemical Society (ACS). All Rights Reserved.): 3807–10. https://doi.org/10.1016/S0040-4039(00)82120-6.

Koyama, Tanetoshi, Yukio Katsuki, and Kyozo. Ogura. 1983. “Studies on Isopentenyl Pyrophosphate Isomerase with Artificial Substrates: *Z-E* Isomerization of *Z*-3-Methyl-3-Pentenyl Pyrophosphate.” *Bioorganic Chemistry* 12 (Copyright (C) 2016 American Chemical Society (ACS). All Rights Reserved.): 58–70. https://doi.org/10.1016/0045-2068(83)90007-X.

Koyama, Tanetoshi, Akio Saito, Kyozo Ogura, and Shuichi. Seto. 1980. “Substrate Specificity of Farnesylpyrophosphate Synthetase. Application to Asymmetric Synthesis.” *Journal of the American Chemical Society* 102 (Copyright (C) 2016 American Chemical Society (ACS). All Rights Reserved.): 3614–18. https://doi.org/10.1021/ja00530a050.

Koyama, Tanetsohi, Kyozo Ogura, and Shuichi. Seto. 1973. “Isopentenyl Pyrophosphate Isomerase with Artificial Substrates.” *Journal of Biological Chemistry* 248 (Copyright (C) 2016 American Chemical Society (ACS). All Rights Reserved.): 8043–51. https://doi.org/10.1016/S0021-9258(19)43191-8.

Kuzma, Jennifer, Michele Nemecek-Marshall, Walter H. Pollock, and Ray Fall. 1995. “Bacteria Produce the Volatile Hydrocarbon Isoprene.” *Current Microbiology* 30 (2): 97–103. https://doi.org/10.1007/BF00294190.

Lange, B. Markus, Tamas Rujan, William Martin, and Rodney Croteau. 2000. “Isoprenoid Biosynthesis: The Evolution of Two Ancient and Distinct Pathways across Genomes.” *Proceedings of the National Academy of Sciences* 97 (24): 13172–77. https://doi.org/10.1073/pnas.240454797.

Lemfack, Marie Chantal, Wolfgang Brandt, Katja Krüger, Alexandra Gurowietz, Jacky Djifack, Jan-Philip Jung, Marius Hopf, et al. 2021. “Reaction Mechanism of the Farnesyl Pyrophosphate C-Methyltransferase towards the Biosynthesis of Pre-Sodorifen Pyrophosphate by *Serratia Plymuthica* 4Rx13.” *Scientific Reports* 11 (1): 3182. https://doi.org/10.1038/s41598-021-82521-9.

Lesburg, Charles A., Guangzhi Zhai, David E. Cane, and David W. Christianson. 1997. “Crystal Structure of Pentalenene Synthase: Mechanistic Insights on Terpenoid Cyclization Reactions in Biology.” *Science* 277 (5333): 1820–24. https://doi.org/10.1126/science.277.5333.1820.

Liebig, Justus. 1835. “Bemerkung Über Die Methoden Der Darstellung Und Reinigung Flüchtiger, Durch Trockene Destillation Organischer Materien Erhaltene Producte.” *Annalen Der Pharmacie* 16 (1): 61–62. https://doi.org/10.1002/jlac.18350160107.

Lund, Sean, Taylor Courtney, and Gavin J. Williams. 2019. “Probing the Substrate Promiscuity of Isopentenyl Phosphate Kinase as a Platform for Hemiterpene Analogue Production.” *Chembiochem : A European Journal of Chemical Biology* 20 (17): 2217–21. https://doi.org/10.1002/cbic.201900135.

Lynen, F., H. Eggerer, U. Henning, and Ingrid Kessel. 1958. “Farnesyl-pyrophosphat und 3-Methyl-Δ3-butenyl-1-pyrophosphat, die biologischen Vorstufen des Squalens. Zur Biosynthese der Terpene, III.” *Angewandte Chemie* 70 (24): 738–42. https://doi.org/10.1002/ange.19580702403.

Martin, J. F. 1992. “The Use of Sodium Carbonate Peroxyhydrate to Treat Off-Flavor in Commercial Catfish Ponds.” *Water Science and Technology* 25 (Copyright (C) 2015 American Chemical Society (ACS). All Rights Reserved.): 315–21. https://doi.org/10.2166/wst.1992.0067.

Medsker, Lloyd L., David Jenkins, Jerome F. Thomas, and C. Koch. 1969. “Odorous Compounds in Natural Waters. 2-Exo-Hydroxy-2-Methylbornane, the Major Odorous Compound Produced by Several Actinomycetes.” *Environmental Science & Technology* 3 (5): 476–77. https://doi.org/10.1021/es60028a008.

Meyer, Andre S., Howard A. Schneiderman, Edith Hanzmann, and Jane H. Ko. 1968. “The Two Juvenile Hormones from the Cecropia Silk Moth*.” *Proceedings of the National Academy of Sciences* 60 (3): 853–60. https://doi.org/10.1073/pnas.60.3.853.

Miller, David J., Fanglei Yu, and Rudolf K. Allemann. 2007. “Aristolochene Synthase-Catalyzed Cyclization of 2-Fluorofarnesyl-Diphosphate to 2-Fluorogermacrene A.” *ChemBioChem* 8 (15): 1819–25. https://doi.org/10.1002/cbic.200700219.

Morgan, E. D., K. Parry, and R. C. Tyler. 1979. “The Chemical Composition of the Dufour Gland Secretion of the Ant *Myrmica Scrabrinodis*.” *Insect Biochemistry* 9 (1): 117–21. https://doi.org/10.1016/0020-1790(79)90036-2.

Nagaki, Masahiko, Shunsuke Sato, Yuji Maki, Tokuzo Nishino, and Tanetoshi Koyama. 2000. “Artificial Substrates for Undecaprenyl Diphosphate Synthase from *Micrococcus Luteus* B-P 26.” *Journal of Molecular Catalysis B: Enzymatic* 9 (1): 33–38. https://doi.org/10.1016/S1381-1177(99)00081-8.

Nagaki, Masahiko, Hiroto Yamamoto, Ayumi Takahashi, Yuji Maki, Junji Ishibashi, Tokuzo Nishino, and Tanetoshi. Koyama. 2002. “Substrate Specificity of Thermostable Farnesyl Diphosphate Synthase with Respect to 4-Alkyl Group Homologs of Isopentenyl Diphosphate.” *Journal of Molecular Catalysis B: Enzymatic* 17 (Copyright (C) 2016 American Chemical Society (ACS). All Rights Reserved.): 81–89. https://doi.org/10.1016/S1381-1177(02)00012-7.

Nishino, Tokuzo, Kyozo Ogura, and Shuichi Seto. 1973. “Comparative Specificity of Prenyltransferase of Pig Liver and Pumpkin with Respect to Artificial Substrates.” *Biochimica et Biophysica Acta, Enzymology* 302 (1): 33. https://doi.org/10.1016/0005-2744(73)90005-3.

Ogura, Kyozo, Tokuzo Nishino, Tanetoshi Koyama, and Shuichi Seto. 1970. “Enzymic Condensation of 3-Methyl-2-Alkenyl Pyrophosphates with Isopentenyl Pyrophosphate.” *Journal of the American Chemical Society* 92 (20): 6036. https://doi.org/10.1021/ja00723a037.

Ohnuma, Shin-ichi, Hisashi Hemmi, Tanetoshi Koyama, Kyozo Ogura, and Tokuzo Nishino. 1998. “Recognition of Allylic Substrates in Sulfolobus Acidocaldarius Geranylgeranyl Diphosphate Synthase: Analysis Using Mutated Enzymes and Artificial Allylic Substrates.” *Journal of Biochemistry* 123 (6): 1036–40. https://doi.org/10.1093/oxfordjournals.jbchem.a022040.

Ohnuma, Shinichi, Michio Ito, Tanetoshi Koyama, and Kyozo. Ogura. 1989. “Undecaprenyl Diphosphate Synthase Reaction with Artificial Substrate Homologs: Novel Behavior in the Termination of Prenyl Chain Elongation.” *Tetrahedron* 45 (Copyright (C) 2016 American Chemical Society (ACS). All Rights Reserved.): 6145–60. https://doi.org/10.1016/S0040-4020(01)85127-9.

Ozaki, Taro, Sandip S. Shinde, Lei Gao, Ryo Okuizumi, Chengwei Liu, Yasushi Ogasawara, Xiaoguang Lei, Tohru Dairi, Atsushi Minami, and Hideaki Oikawa. 2018. “Enzymatic Formation of a Skipped Methyl-Substituted Octaprenyl Side Chain of Longestin (KS-505a): Involvement of Homo-IPP as a Common Extender Unit.” *Angewandte Chemie, International Edition* 57 (22): 6629–32. https://doi.org/10.1002/anie.201802116.

Pazouki, Leila, and Ülo Niinemets. 2016. “Multi-Substrate Terpene Synthases: Their Occurrence and Physiological Significance.” *Frontiers in Plant Science* 7. https://doi.org/10.3389/fpls.2016.01019.

Piechulla, Birgit, Chi Zhang, Daniela Eisenschmidt-Bönn, Feng Chen, and Nancy Magnus. 2021. “Non-Canonical Substrates for Terpene Synthases in Bacteria Are Synthesized by a New Family of Methyltransferases.” *FEMS Microbiology Reviews*, no. fuab024 (April). https://doi.org/10.1093/femsre/fuab024.

Piriou, P., R. Devesa, M. De Lalande, and K. Glucina. 2009. “European Reassessment of MIB and Geosmin Perception in Drinking Water.” *Journal of Water Supply: Research and Technology - Aqua* 58 (8): 532–38. https://doi.org/10.2166/aqua.2009.124.

Polito, Alan, G. Popjak, and Thomas Parker. 1972. “Artificial Substrate in Squalene and Sterol Biosynthesis.” *Journal of Biological Chemistry* 247 (11): 3464. https://doi.org/10.1016/S0021-9258(19)45163-6.

Popják, G., and J. W. Cornforth. 1960. “The Biosynthesis of Cholesterol.” In *Advances in Enzymology and Related Areas of Molecular Biology*, 281–335. John Wiley & Sons, Ltd. https://doi.org/10.1002/9780470122679.ch7.

Popják, G., J. L. Rabinowitz, and J. M. Baron. 1969. “Artificial Substrates for Prenyltransferase.” *Biochemical Journal* 113 (5): 861–68. https://doi.org/10.1042/bj1130861.

Poulter, C. D., J. C. Argyle, and E. A. Mash. 1978. “Farnesyl Pyrophosphate Synthetase. Mechanistic Studies of the 1’-4 Coupling Reaction with 2-Fluorogeranyl Pyrophosphate.” *The Journal of Biological Chemistry* 253 (20): 7227–33. https://doi.org/10.1016/S0021-9258(17)34489-7.

Rabe, Patrick, Thomas Schmitz, and Jeroen S. Dickschat. 2016. “Mechanistic Investigations on Six Bacterial Terpene Cyclases.” *Beilstein Journal of Organic Chemistry* 12 (1): 1839–50. https://doi.org/10.3762/bjoc.12.173.

Radhika, Venkatesan, Nanae Ueda, Yuuri Tsuboi, Mikiko Kojima, Jun Kikuchi, Takuji Kudo, and Hitoshi Sakakibara. 2015. “Methylated Cytokinins from the Phytopathogen *Rhodococcus Fascians* Mimic Plant Hormone Activity.” *Plant Physiology* 169 (2): 1118–26. https://doi.org/10.1104/pp.15.00787.

Reuss, Stephan von, Dajana Domik, Marie Chantal Lemfack, Nancy Magnus, Marco Kai, Teresa Weise, and Birgit Piechulla. 2018. “Sodorifen Biosynthesis in the Rhizobacterium *Serratia Plymuthica* Involves Methylation and Cyclization of MEP-Derived Farnesyl Pyrophosphate by a SAM-Dependent C-Methyltransferase.” *Journal of the American Chemical Society* 140 (37): 11855–62. https://doi.org/10.1021/jacs.8b08510.

Ritter, F. J., I. E. M. Brüggemann-Rotgans, P. E. J. Verwiel, C. J. Persoons, and E. Talman. 1977. “Trail Pheromone of the Pharaoh’s Ant, *Monomorium Pharaonis*: Isolation and Identification of Faranal, a Terpenoid Related to Juvenile Hormone II.” *Tetrahedron Letters* 18 (30): 2617–18. https://doi.org/10.1016/S0040-4039(01)83835-1.

Rohmer, Michel. 1999. “The Discovery of a Mevalonate-Independent Pathway for Isoprenoid Biosynthesis in Bacteria, Algae and Higher Plants†.” *Natural Product Reports* 16 (5): 565–74. https://doi.org/10.1039/A709175C.

Rohmer, Michel, M Knani, P Simonin, B Sutter, and H Sahm. 1993. “Isoprenoid Biosynthesis in Bacteria: A Novel Pathway for the Early Steps Leading to Isopentenyl Diphosphate.” *Biochemical Journal* 295 (2): 517–24. https://doi.org/10.1042/bj2950517.

Röller, H., K. H. Dahm, C. C. Sweely, and B. M. Trost. 1967. “The Structure of the Juvenile Hormone.” *Angewandte Chemie International Edition in English* 6 (2): 179–80. https://doi.org/10.1002/anie.196701792.

Rossoni, Luca, Stephen J. Hall, Graham Eastham, Peter Licence, and Gill Stephens. 2015. “The Putative Mevalonate Diphosphate Decarboxylase from *Picrophilus Torridus* Is in Reality a Mevalonate-3-Kinase with High Potential for Bioproduction of Isobutene.” *Applied and Environmental Microbiology* 81 (7): 2625–34. https://doi.org/10.1128/AEM.04033-14.

Rudolf, Jeffrey D., Tyler A. Alsup, Baofu Xu, and Zining Li. 2021. “Bacterial Terpenome.” *Natural Product Reports* 38 (5): 905–80. https://doi.org/10.1039/D0NP00066C.

Ružička, Leopold. 1953. “The Isoprene Rule and the Biogenesis of Terpenic Compounds.” *Experientia* 9 (10): 357–67. https://doi.org/10.1007/BF02167631.

———. 1963. “Perspektiven Der Biogenese Und Der Chemie Der Terpene.” *Pure and Applied Chemistry* 6 (4): 493–524. https://doi.org/10.1351/pac196306040493.

Rynkiewicz, Michael J., David E. Cane, and David W. Christianson. 2001. “Structure of Trichodiene Synthase from *Fusarium Sporotrichioides* Provides Mechanistic Inferences on the Terpene Cyclization Cascade.” *Proceedings of the National Academy of Sciences* 98 (24): 13543–48. https://doi.org/10.1073/pnas.231313098.

Sandmann, Gerhard. 2015. “Carotenoids of Biotechnological Importance.” In *Biotechnology of Isoprenoids*, edited by Jens Schrader and Jörg

Bohlmann, 449–67. Advances in Biochemical Engineering/Biotechnology. Cham: Springer International Publishing. https://doi.org/10.1007/10_2014_277.

Schempp, Florence M., Laura Drummond, Markus Buchhaupt, and Jens Schrader. 2018. “Microbial Cell Factories for the Production of Terpenoid Flavor and Fragrance Compounds.” *Journal of Agricultural and Food Chemistry* 66 (10): 2247–58. https://doi.org/10.1021/acs.jafc.7b00473.

Schmidt, Ruth, Victor de Jager, Daniela Zühlke, Christian Wolff, Jörg Bernhardt, Katarina Cankar, Jules Beekwilder, et al. 2017. “Fungal Volatile Compounds Induce Production of the Secondary Metabolite Sodorifen in *Serratia Plymuthica* PRI-2C.” *Scientific Reports* 7 (1): 862. https://doi.org/10.1038/s41598-017-00893-3.

Schönherr, Heike, and Tim Cernak. 2013. “Profound Methyl Effects in Drug Discovery and a Call for New C-H Methylation Reactions.” *Angewandte Chemie International Edition* 52 (47): 12256–67. https://doi.org/10.1002/anie.201303207.

Schooley, David A., Kenneth J. Judy, B. John Bergot, M. Sharon Hall, and John B. Siddall. 1973. “Biosynthesis of the Juvenile Hormones of *Manduca Sexta*: Labeling Pattern from Mevalonate, Propionate, and Acetate.” *Proceedings of the National Academy of Sciences* 70 (10): 2921–25. https://doi.org/10.1073/pnas.70.10.2921.

Seemann, Myriam, Guangzhi Zhai, Jan-Willem de Kraker, Chiana M. Paschall, David W. Christianson, and David E. Cane. 2002. “Pentalenene Synthase. Analysis of Active Site Residues by Site-Directed Mutagenesis.” *Journal of the American Chemical Society* 124 (26): 7681–89. https://doi.org/10.1021/ja026058q.

Shinka, Toshihiro, Kyozo Ogura, and Shuichi Seto. 1975. “Comparative Specificity of Geranylgeranyl Pyrophosphate Synthetase of *Micrococcus Lysodeikticus* and Pumpkin.” *Journal of Biochemistry* 78 (6): 1177. https://doi.org/10.1093/oxfordjournals.jbchem.a131014.

Sommer, Svenja, Leon M. Lang, Laura Drummond, Markus Buchhaupt, Marco A. Fraatz, and Holger Zorn. 2022. “Odor Characteristics of Novel Non-Canonical Terpenes.” *Molecules* 27 (12): 3827. https://doi.org/10.3390/molecules27123827.

Staddon, B. W., A. Abdollahi, J. Parry, M. Rossiter, and D. W. Knight. 1994. “Major Component in Male Sex Pheromone of Cereal Pest *Eurygaster Integriceps* Puton (Heteroptera: Scutelleridae) Identified as a Homosesquiterpenoid.” *Journal of Chemical Ecology* 20 (10): 2721–31. https://doi.org/10.1007/BF02036203.

Starks, Courtney M., Kyoungwhan Back, Joseph Chappell, and Joseph P. Noel. 1997. “Structural Basis for Cyclic Terpene Biosynthesis by Tobacco 5-Epi-Aristolochene Synthase.” *Science* 277 (5333): 1815–20. https://doi.org/10.1126/science.277.5333.1815.

Stönner, Christof, and Jonathan Williams. 2016. “Goals Change Crowd Air Chemistry.” *Nature* 535 (7612): 355–355. https://doi.org/10.1038/535355a.

Tabachek, Jo-Anne L., and M. Yurkowski. 1976. “Isolation and Identification of Blue-Green Algae Producing Muddy Odor Metabolites, Geosmin, and 2-Methylisoborneol, in Saline Lakes in Manitoba.” *Journal of the Fisheries Research Board of Canada* 33 (1): 25–35. https://doi.org/10.1139/f76-004.

Takagi, Chiharu, Momoka Abe, Yumi Kaneko, Akane Sasaki, Ayumi Ito, Yuka Sakemi, Takashi Maoka, Jürgen Breitenbach, Gerhard Sandmann, and Kazutoshi Shindo. 2020. “Structures of New C41 Carotenoids Produced Using Recombinant *Escherichia Coli* Expressing Genes Encoding Isopentenyl Pyrophosphate, Methyltransferase, and Carotenoid Biosynthetic Enzymes.” *Tetrahedron Letters* 61 (51): 152633. https://doi.org/10.1016/j.tetlet.2020.152633.

Tansey, Terese R, and Ishaiahu Shechter. 2000. “Structure and Regulation of Mammalian Squalene Synthase.” *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, Cholesterol in the year 2000, 1529 (1): 49–62. https://doi.org/10.1016/S1388-1981(00)00137-2.

Tarshis, L. C., Mujing Yan, C. Dale Poulter, and James C. Sacchettini. 1994. “Crystal Structure of Recombinant Farnesyl Diphosphate Synthase at 2.6-.ANG. Resolution.” *Biochemistry* 33 (36): 10871–77. https://doi.org/10.1021/bi00202a004.

Tetali, Sarada D. 2019. “Terpenes and Isoprenoids: A Wealth of Compounds for Global Use.” *Planta* 249 (1): 1–8. https://doi.org/10.1007/s00425-018-3056-x.

Tholl, Dorothea. 2015. “Biosynthesis and Biological Functions of Terpenoids in Plants.” In *Biotechnology of Isoprenoids*, edited by Jens Schrader and Jörg Bohlmann, 63–106. Advances in Biochemical Engineering/Biotechnology. Cham: Springer International Publishing. https://doi.org/10.1007/10_2014_295.

Thulasiram, Hirekodathakallu V., Hans K. Erickson, and C. Dale Poulter. 2007. “Chimeras of Two Isoprenoid Synthases Catalyze All Four Coupling Reactions in Isoprenoid Biosynthesis.” *Science (New York, N.Y.)* 316 (5821): 73–76. https://doi.org/10.1126/science.1137786.

Toyota, Masao, Yoshinori Asakawa, and Jan-Teter Frahm. 1990. “Homomono- and Sesquiterpenoids from the Liverwort *Lophocolea Heterophylla*.” *Phytochemistry* 29 (7): 2334–37. https://doi.org/10.1016/0031-9422(90)83066-A.

Tsutsumi, Hayama, Yoshitaka Moriwaki, Tohru Terada, Kentaro Shimizu, Kazuo Shin-ya, Yohei Katsuyama, and Yasuo Ohnishi. 2022. “Structural and Molecular Basis of the Catalytic Mechanism of Geranyl Pyrophosphate C6-Methyltransferase: Creation of an Unprecedented Farnesyl Pyrophosphate C6-Methyltransferase.” *Angewandte Chemie* 134 (1): e202111217. https://doi.org/10.1002/ange.202111217.

Vedula, L. Sangeetha, Yuxin Zhao, Robert M. Coates, Tanetoshi Koyama, David E. Cane, and David W. Christianson. 2007. “Exploring Biosynthetic Diversity with Trichodiene Synthase.” *Archives of Biochemistry and Biophysics* 466 (2): 260–66. https://doi.org/10.1016/j.abb.2007.06.016.

Velikova, Violeta, Andrea Ghirardo, Elisa Vanzo, Juliane Merl, Stefanie M. Hauck, and Jörg-Peter Schnitzler. 2014. “Genetic Manipulation of Isoprene Emissions in Poplar Plants Remodels the Chloroplast Proteome.” *Journal of Proteome Research* 13 (4): 2005–18. https://doi.org/10.1021/pr401124z.

Vinci, Leonardo da. 1503. *Mona Lisa (La Gioconda)*. Öl auf Pappelholz. Louvre Museum. https://commons.wikimedia.org/wiki/File:Mona_Lisa,_by_Leonardo_da_Vinci,_from_C2RMF_retouched.jpg.

Vinokur, Jeffrey M., Tyler P. Korman, Zheng Cao, and James U. Bowie. 2014. “Evidence of a Novel Mevalonate Pathway in Archaea.” *Biochemistry* 53 (25): 4161–68. https://doi.org/10.1021/bi500566q.

Wallach, O. 1887. “Zur Kenntniss Der Terpene Und Der Ätherischen Oele; Fünfte Abhandlung.” *Justus Liebigs Annalen Der Chemie* 239 (1): 1–54. https://doi.org/10.1002/jlac.18872390102.

Wang, Chieh-Mei, and David E Cane. 2008. “Biochemistry and Molecular Genetics of the Biosynthesis of the Earthy Odorant Methylisoborneol in *Streptomyces Coelicolor*.” *Journal of the American Chemical Society* 130 (28): 8908–9. https://doi.org/10.1021/ja803639g.

Wang, Kevin C, and Shin-ichi Ohnuma. 2000. “Isoprenyl Diphosphate Synthases.” *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, Cholesterol in the year 2000, 1529 (1): 33–48. https://doi.org/10.1016/S1388-1981(00)00136-0.

Wang, Z., I. H. Suffet, and Al-Samarrai. 2006. “Sensory and Chemical Analysis Methods for Earthy and Musty Odours in Drinking Water Caused by Geosmin and 2-Methylisoborneol.” *Water Science and Technology: Water Supply* 6 (3): 147–55. https://doi.org/10.2166/ws.2006.732.

Wendt, K. Ulrich, Karl Poralla, and Georg E. Schulz. 1997. “Structure and Function of a Squalene Cyclase.” *Science* 277 (5333): 1811–15. https://doi.org/10.1126/science.277.5333.1811.

Wendt, K. Ulrich, and Georg E Schulz. 1998. “Isoprenoid Biosynthesis: Manifold Chemistry Catalyzed by Similar Enzymes.” *Structure* 6 (2): 127–33. https://doi.org/10.1016/S0969-2126(98)00015-X.

Went, F. W. 1960. “Blue Hazes in the Atmosphere.” *Nature* 187 (4738): 641–43. https://doi.org/10.1038/187641a0.

Williams, C. Greville. 1860. “On Isoprene and Caoutchine.” *Philosophical Transactions of the Royal Society of London* 150 (January): 241–55. https://doi.org/10.1098/rstl.1860.0016.

Xia, Chen-Yang, Bo-Wen Lu, Ji-Yun Cui, Bai-Yang Wang, Yue-Yang Sun, and Fei Gan. 2023. “Engineered Geranyl Diphosphate Methyltransferase Produces 2-Methyl-Dimethylallyl Diphosphate as a Noncanonical C6 Unit for Terpenoid Biosynthesis.” *Synthetic and Systems Biotechnology* 8 (1): 107–13. https://doi.org/10.1016/j.synbio.2022.12.002.

Xu, Houchao, and Jeroen S. Dickschat. 2022. “Mechanistic Investigations on Microbial Type I Terpene Synthases through Site-Directed Mutagenesis.” *Synthesis* 54 (06): 1551–65. https://doi.org/10.1055/a-1675-8208.

Zulak, Katherine G., and Jörg Bohlmann. 2010. “Terpenoid Biosynthesis and Specialized Vascular Cells of Conifer Defense.” *Journal of Integrative Plant Biology* 52 (1): 86–97. https://doi.org/10.1111/j.1744-7909.2010.00910.x.

5 Anhang

5.1 Publikationen

5.1.1 Heterologous expression of 2-methylisoborneol / 2-methylenebornane biosynthesis genes in *Escherichia coli* yields novel C11-terpenes

Erklärung zu den Autorenanteilen an der Publikation / an dem Manuskript (Titel):
Heterologous expression of 2-methylisoborneol / 2 methylenebornane biosynthesis genes in *Escherichia coli* yields novel C11-terpenes
Status: Published

Name der Zeitschrift: PLOS ONE

Beteiligte Autoren:
Max J. Kschowak (Promovierender), Hannah Wortmann (HW), Jeroen S. Dickschat (JD), Jens Schrader (JS), Markus Buchhaupt (MB)

Was hat der Promovierende bzw. was haben die Koautoren beigetragen?*[3]

(1) zu Entwicklung und Planung

Promovierender: 60 %
MB: 30 %
JS: 10 %

(2) zur Durchführung der einzelnen Untersuchungen und Experimente

Plasmid and Strain Construction
Promovierender: 100 %

De novo terpenoid production in *E. coli*
Promovierender: 80 %
HW: 20 %

HS-SPME GC-MS analysis
Promovierender: 95 %
JD: 5 %

SBSE GC-MS analysis:
Promovierender: 50 %
HW: 50 %

(3) zur Erstellung der Datensammlung und Abbildungen

Fig. 1, 2, 3, 5, Tab. 1, 2, 3:
Promovierender: 100 %

Fig.4:
Promovierender: 80%
HW: 20%

Fig. 6.:
Promovierender: 70 %
MB 30 %

Supporting informations:
Promovierender: 80%
HW: 10 %
MB 10 %

(4) zur Analyse und Interpretation der Daten

C11-Terpen Datenanalyse und Interpretation
Promovierender: 75 %
MB: 15 %
JD: 10 %

[a] *Aus Gründen der Lesbarkeit wird im gesamten Text die männliche Schreibweise verwendet. Frauen sind natürlich inbegriffen.*

Monoterpen Datenanalyse und Interpretation
Promovierender: 80 %
MB: 20 %

Sesquiterpen und C16-Terpen Datenanalyse und Interpretation:
Promovierender: 65 %
HW: 10 %
MB: 20 %
JD: 5 %

(5) zum Verfassen des Manuskripts

Promovierender: 75 %
MB: 20 %
JD: 5 %

Zustimmende Bestätigungen der oben genannten Angaben:

Datum/Ort	Unterschrift Promovend
Datum/Ort	Unterschrift Betreuer
Datum/Ort	Ggfs. Unterschrift *corresponding author*

[*1] Der Name der Zeitschrift darf erst genannt werden, wenn die Arbeit akzeptiert, im Druck oder erschienen ist.

[*2] Als Autoren werden solche Personen bezeichnet, die an der Arbeit in Bezug auf die genannten Punkte in einer Weise mitgewirkt haben, dass sie für die ausgewiesenen Passagen (mit-) verantwortlich sind. Personen, die an der Arbeit mitgewirkt haben, jedoch nicht in diese Kategorie fallen, sollten in der Danksagung Erwähnung finden.

[*3] Bei (2), (3) und (4) bitte prozentuale und dazu kurze inhaltliche Angaben machen, bei (1) und (5) reichen prozentuale Angaben.

Wenn es sich um ein Review handelt, notieren Sie das bitte in Klammern hinter den Titel der Publikation. Zudem ist in diesem Fall (2) hinfällig, bitte notieren Sie dort „n/a“. Unter (3) geht es dann um die „Literatursammlung“ anstelle der „Datensammlung“. Bitte ändern Sie dies im Formular!

[a] *Aus Gründen der Lesbarkeit wird im gesamten Text die männliche Schreibweise verwendet. Frauen sind natürlich inbegriffen.*

PLOS ONE

RESEARCH ARTICLE

Heterologous expression of 2-methylisoborneol / 2 methylenebornane biosynthesis genes in *Escherichia coli* yields novel C11-terpenes

Max J. Kschowak[1,2], Hannah Wortmann[1], Jeroen S. Dickschat[3], Jens Schrader[1], Markus Buchhaupt[1]*

1 DECHEMA Research Institute, Industrial Biotechnology, Frankfurt am Main, Germany, **2** Faculty of Biological Sciences, Goethe University, Frankfurt am Main, Germany, **3** Kekulé-Institute of Organic Chemistry and Biochemistry, Rheinische Friedrich Wilhelms University, Bonn, Germany

* buchhaupt@dechema.de

Check for updates

OPEN ACCESS

Citation: Kschowak MJ, Wortmann H, Dickschat JS, Schrader J, Buchhaupt M (2018) Heterologous expression of 2-methylisoborneol / 2 methylenebornane biosynthesis genes in *Escherichia coli* yields novel C11-terpenes. PLoS ONE 13(4): e0196082. https://doi.org/10.1371/journal.pone.0196082

Editor: Björn Hamberger, Michigan State University, UNITED STATES

Received: January 11, 2018

Accepted: April 5, 2018

Published: April 19, 2018

Copyright: © 2018 Kschowak et al. This is an open access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This work was funded by the German Federal Ministry of Food and Agriculture (https://international.fnr.de/; FKZ 22008713). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

The structural diversity of terpenoids is limited by the isoprene rule which states that all primary terpene synthase products derive from methyl-branched building blocks with five carbon atoms. With this study we discover a broad spectrum of novel terpenoids with eleven carbon atoms as byproducts of bacterial 2-methylisoborneol or 2-methylenebornane synthases. Both enzymes use 2-methyl-GPP as substrate, which is synthesized from GPP by the action of a methyltransferase. We used *E. coli* strains that heterologously produce different C11-terpene synthases together with the GPP methyltransferase and the mevalonate pathway enzymes. With this *de novo* approach, 35 different C11-terpenes could be produced. In addition to eleven known compounds, it was possible to detect 24 novel C11-terpenes which have not yet been described as terpene synthase products. Four of them, 3,4-dimethylcumene, 2-methylborneol and the two diastereomers of 2-methylcitronellol could be identified. Furthermore, we showed that an *E. coli* strain expressing the GPP-methyltransferase can produce the C16-terpene 6-methylfarnesol which indicates the condensation of 2-methyl-GPP and IPP to 6-methyl-FPP by the *E. coli* FPP-synthase. Our study demonstrates the broad range of unusual terpenes accessible by expression of GPP-methyltransferases and C11-terpene synthases in *E. coli* and provides an extended mechanism for C11-terpene synthases.

Introduction

Terpenoids constitute the largest class of natural products with more than 70,000 compounds that have been discovered so far [1]. The diversity of structures is reflected in its various applications as nutrients (carotenoids), drugs (artemisinin and taxol), potential biofuels (farnesane), and flavor and fragrance compounds ((-)-menthol, sclareol) (for a comprehensive overview see [2], in particular part 3).

However, the structural diversity is limited by the isoprene rule, which states that terpenes have a methyl-branched carbon chain with a length of a multiple of five carbons. Already in

Competing interests: The authors have declared that no competing interests exist.

1887 Otto Wallach concluded during his comprehensive work on structures of terpenoids that terpenes are formal oligomers of isoprene [3]. In 1953 Leopold Ružička extended the isoprene rule and described non-canonical terpenoids as the products of subsequent reactions on canonical terpenes [4]. The cellular C5 precursors of all terpenes are isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). They are produced by two independently evolved pathways: the mevalonate pathway and the later discovered 2-C-methyl-d-erythritol 4-phosphate pathway. They proceed *via* different intermediates, but culminate in the same end products.

In 2007 and 2008 the elucidation of the biosynthetic pathway to the non-canonical terpene and off-flavor 2-methylisoborneol (2-MIB) in different microorganisms was reported independently by three research groups, based on feeding experiments with isotopically labelled precursors [5] or on *in vitro* experiments with recombinant enzymes [6,7]. In a first step the S-adenosyl-l-methionine-dependent geranyl diphosphate C-methyltransferase (GPP-MTase) catalyzes the electrophilic methylation at the C-2 position of geranyl diphosphate (GPP). The 2-methyl-GPP serves as substrate for the 2-MIB synthase, which catalyzes the cyclization to the bicyclic terpene alcohol 2-MIB (Fig 1). The crystal structures of both involved enzymes have been determined and catalytic mechanisms of the GPP-MTase and 2-MIB synthase were proposed [8,9].

2-MIB has a muddy smell with a low odor and flavor threshold ($<$ 5 ng/L) [10,11] and is a contaminant of drinking water, wine [12] and fish [13]. Moreover it is related to the musty-earthy aroma in camembert and brie [14]. Since its isolation from streptomycetes and structure elucidation in 1969 [15,16], many other bacteria were identified as producers. Mainly actinomycetes and cyanobacteria [17], but also myxobacteria [5] and eukaryotes like fungi [14,18] and liverwort [19] were reported to release 2-MIB.

Besides 2-MIB synthases, two terpene synthases (TS) are described that produce the C11-terpene 2-methylenebornane (2-MB) as main product. Likewise, the 2-MB synthases of *Pseudomonas fluorescens* and *Micromonospora olivasterospora* use 2-methyl-GPP as substrate and are closely related to 2-MIB synthases [6,20].

The reaction from 2-methyl-GPP to 2-MIB or to 2-MB proceeds via a series of carbocationic intermediates. Corresponding to other terpene synthases these carbocations can be quenched by the addition of water or by deprotonation [21]. Early quenching reactions at the cationic intermediates of the 2-MIB cyclization cascade explain the previously identified side products of C11-terpene synthases (C11-TS) from different bacteria, including 2-methylgeraniol, 2-methylnerol, 2-methyllinalool, 2-methylmyrcene, 2-methyl-α-terpineol, 2-methyl-β-fenchol, 2-methyllimonene, 2-methyl-2-bornene and 1-methylcamphene [20,22,23]. The 2-methylbornyl cation is the last carbocation in the reaction mechanism catalyzed by C11-TSs and is the precursor for the two different main products 2-MIB or 2-MB.

In this study we heterologously expressed four different C11-TS genes, in each case together with the genes for a GPP-MTase and for the whole mevalonate pathway including an IPP-isomerase gene, in *E. coli* to produce C11-terpenes. Using headspace-solid phase micro extraction (HS-SPME) or stir bar sorptive extraction (SBSE) and subsequent gas chromatography-mass spectrometry (GC-MS) analysis we observed the production of many different compounds and were able to identify diverse mono-, C11-, sesqui- and C16-terpenoids from the *E. coli* strains containing such a non-canonical terpenoid biosynthesis pathway.

Materials and methods

E. coli strain DH5α (New England Biolabs) was used for plasmid construction. *E. coli* MG1655 Δ*endA* Δ*recA* (DE3) (hereinafter referred to as *E. coli* MG1655) [24] was constructed in the lab of Kristala Prather (Addgene #37854) and used for production experiments. pJBEI-6409 was

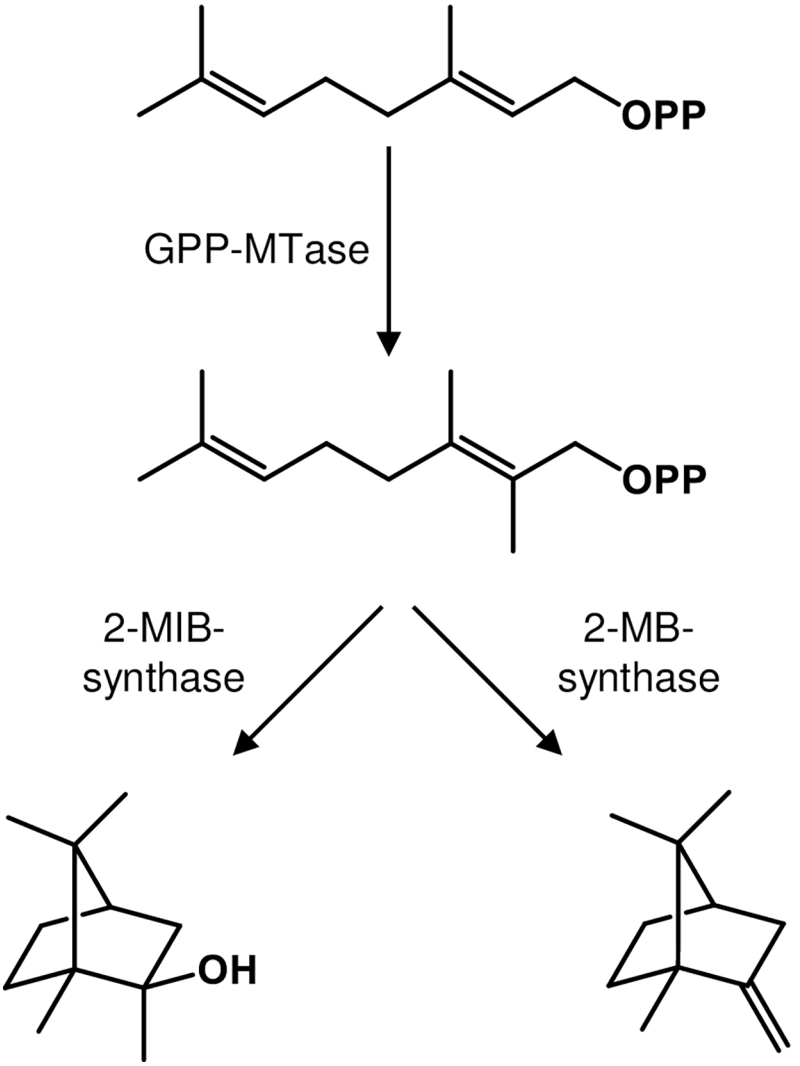


Fig 1. Biosynthesis of 2-methylisoborneol (2-MIB) and 2-methylenebornane (2-MB) GPP is methylated at the second C-atom by a GPP-MTase. 2-methyl-GPP serves as substrate for the 2-MIB synthase and the 2-MB synthase.

https://doi.org/10.1371/journal.pone.0196082.g001

constructed in the lab of Taek Soon Lee (Addgene plasmid # 47048). It contains the genes of the mevalonate pathway, of an IPP isomerase, of a GPP synthase and of a limonene synthase [25] and was used to construct a plasmid for provision of high GPP levels.

E. coli strains were cultivated routinely at 37˚C in lysogeny broth (LB, 10 g/L yeast extract, 10 g/L NaCl, 5 g/L tryptone [26]) containing the appropriate antibiotics (ampicillin, 100 μg/mL; chloramphenicol, 34 μg/mL; gentamicin, 20 μg/mL).

Genes encoding the 2-MB synthase from *Pseudomonas fluorescens* Pf0-1 (MBSp), the 2-MB synthase from *Micromonospora olivasterospora* (MBSm), the 2-MIB synthase from *Streptomyces griseus subsp. griseus* (MIBSg), the 2-MIB synthase from *Streptomyces coelicolor* A3(2) (MIBSc) and the GPP-MTase from *Streptomyces coelicolor* A3(2) were codon-optimized for *E. coli* and synthesized by Life Technology GmbH (Darmstadt, Germany). Optimal ribosomal binding sites (RBS, S1 Table) for those genes were designed with the RBS calculator V1.1 [27,28]. New RBSs of C11-TSs were synthesized together with the genes. The new RBS of the GPP-MTase was integrated via Gibson assembly.

2-Methylisoborneol and 2-Methylborneol were synthesized from camphor as previously described [16]. Other C11- and C16-terpene reference substances were synthesized by Enamine Ltd (Riga, Latvia).

Plasmid construction

The gene for chloramphenicol resistance in pJBEI-6409 were exchanged with the gene for gentamicin resistance via Gibson assembly [29]. The backbone was amplified with the primers mk20 and mk23 (S1 Table), the insert was amplified with the primer pair mk24 / mk25 and pBBR1MCS-5 [30] as template. From the new plasmid pMK-04 (Table 1) the gene encoding the limonene synthase was deleted via Gibson assembly for construction of pMK-05. The backbone was amplified with the primers mk7 and mk8. The oligo mk78x was used as insert. To provide the empty vector control pMK-06, a PCR with the primers mk8 and mk26, a restriction digest of the PCR product with *Bam*HI and self-ligation was done.

For integration of the GPP-MTase gene into the second multiple cloning site of pETDuet-1, it was amplified with the primers mk12 and mk13. The backbone was amplified with the primers mk10 and mk11. The products were combined to construct plasmid pMK-03 via Gibson assembly.

The genes encoding MBSp, MBSm, MIBSg and MIBSc were integrated in the first multiple cloning sites of pMK-09 and pETDuet-1 via standard restriction cloning (*Xba*I and *Not*I) to provide the plasmids pMK-08, -12, -13, -14 and -15.

Strains

To construct the production strains (11-p, 11-m, 11-g, 11-c; Table 2) which heterologously express the genes of the mevalonate pathway, of an IPP isomerase, of a GPP synthase, of the

Table 1. Plasmids used in this study.

Name	Description (origin of replication, antibiotic marker, promoter and genes)	Expressed proteins	Reference
pETDuet-1	colE1, Amp^r, P_{T7}	-	Novagen
pJBEI-6409	p15A, Cm^r, P_{lacUV5}, P_{trc} *atoB*, *mvaS*, *mvaA*, *ERG12*, *ERG8*, *MVD1*, *idi*, *trGPPS2*, *trLS*	Mevalonate pathway proteins, IPP-isomerase, GPP-synthase, limonene synthase	[25]
pMK-04	pJBEI-6409 with Gm^r instead of Cam^r	Mevalonate pathway proteins, IPP-isomerase, GPP-synthase, limonene synthase	This study
pMK-05	pMK-04, *trLS* deleted	Mevalonate pathway proteins, IPP-isomerase, GPP-synthase	This study
pMK-06	pMK-04, empty vector	-	This study
pMK-03	pETDuet-1, *gppmtase*[a]	GPP-MTase (NP_631739.1)	This study
pMK-08	pETDuet-1, *mbsp*[a]	MBSp (WP_011333305.1)	This study
pMK-12	pETDuet-1, *mbsp*, *gppmtase*	MBSp, GPP-MTase	This study
pMK-13	pETDuet-1, *mbsm*[a], *gppmtase*	MBSm (BAK26793.1), GPP-MTase	This study
pMK-14	pETDuet-1, *mibsg*[a], *gppmtase*	MIBSg (WP_012378420.1), GPP-MTase	This study
pMK-15	pETDuet-1, *mibsc*[a], *gppmtase*	MIBSc (NP_733742.1), GPP-MTase	This study

[a] Optimized gene sequences can be found in S1–S5 Figs

https://doi.org/10.1371/journal.pone.0196082.t001

Table 2. Strains used in this study.

Strain	Genotype / harboring plasmids	Reference
E. coli MG1655	F$^-$, λ$^-$, *ilvG- rfb-50 rph-1*, Δ*endA*, Δ*recA*	[24]
E. coli DH5α	F$^-$, Φ80*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deoR*, *recA1*, *endA1*,*hsdR17*(r$^-$, m$^+$), *phoA*, *sup*E44, λ$^-$, *thi-1*	[31]
0–0	*E. coli* MG1655 + pMK-04 + pETDuet-1	This study
10–0	*E. coli* MG1655 + pMK-05 + pETDuet-1	This study
11–0	*E. coli* MG1655 + pMK-05 + pMK-03	This study
11-p	*E. coli* MG1655 + pMK-05 + pMK-12	This study
11-m	*E. coli* MG1655 + pMK-05 + pMK-13	This study
11-g	*E. coli* MG1655 + pMK-05 + pMK-14	This study
11-c	*E. coli* MG1655 + pMK-05 + pMK-15	This study

https://doi.org/10.1371/journal.pone.0196082.t002

GPP-MTase and one of four different C11-TS genes, the respective plasmids were transformed into *E. coli* MG1655. Furthermore, control strains lacking a C11-TS gene (11–0), the C11-TS gene and the GPP-MTase gene (10–0) and a negative control with empty vectors (0–0) were constructed.

De novo terpenoid production in *E. coli*

Pre-cultures in reaction tubes containing 5 mL LB medium with appropriate antibiotics were incubated overnight at 37˚C and 180 rpm. Main cultures in 15 ml 2x YT medium (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl, pH 7,0 [32]) with 2% (v/v) glycerol and appropriate antibiotics in 100 ml baffled shake flasks were inoculated from pre-cultures to an OD_{600} value of 0.1. After cultivation at 37˚C to an OD_{600} value of 1, gene expression was induced with isopropyl-β-D-1-thiogalactopyranoside (IPTG, 100 μM). Induced cultures had terpenoids extracted after 24 hours of incubation at 30˚C and 180 rpm.

HS-SPME GC-MS analysis

Volatile compounds in the headspace of each culture were analyzed by extraction with an 85 μm SPME stableflex fiber composed of polydimethylsiloxane and Carboxen (Supelco, Bellefonte, USA). The SPME fibers were exposed for 30 minutes and then inserted into the injection port of a GC-MS-QP2010 (Shimadzu) containing a DB-5 column (30 m x 0.25 mm x 0.25 μm, Agilent, Santa Clara, USA). 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 as follows: 40˚C for 1.5 minutes, 10˚C/min up to 250˚C followed by 20˚C/min up to 300˚C.

Compounds were identified via comparison of mass spectra and retention indexes (RI) to the ones of reference substances or mass spectra of the NIST mass spectral library (v14) and RIs published by Adams [33]. Identity was assumed if the similarity index was equal to or higher than 90 and the RI was ± 10 compared to the published data.

To identify 2-methyl-α-terpineol ion trace analyses were done. For this purpose, the proportions of the peak areas of the fragment ions at m/z = 107, 135, 150 and 93 were compared

to the proportions of the signal intensities of the same fragment ions of the reference compound.

Structural conversions of analytes during the procedure were excluded by HS-SPME GC-MS analysis with the mono- and C11-terpenoids.

SBSE GC-MS analysis

For extraction of less volatile terpenoids, a twister (10 x 0.5 mm PDMS; Gerstel, Mühlheim an der Ruhr, Germany) was fixated in a shake flask by an external magnet in order to cover it completely with culture. After the production phase the twister was removed from the culture, rinsed with ddH_2O, dried with lint free wipes and transferred to the inlet of a GC-vial, which was filled with 250 μL n-pentane. After 15 minutes of incubation in an ultrasonic bath (Merck eurolab, Darmstadt, Germany) 5 μL of the pentane were used for GC-MS analysis. Besides the injection mode (split ratio = 10) the same settings and equipment described for the HS-SPME GC-MS analysis were used.

Results

C11-terpenoids

Volatiles of four different production strains (11-p, 11-m, 11-g and 11-c) heterologously expressing the genes of the mevalonate pathway, an IPP isomerase gene, a GPP synthase gene, the GPP-MTase gene and one of four different C11-TS genes were analyzed with HS-SPME GC-MS.

The resulting total ion chromatograms (Fig 2) and the corresponding mass spectra (S6 Fig) show a complex and diverse mixture of volatiles in the culture headspaces.

From mass spectral data, 35 C11-terpenes could be identified (Table 3). They show typical fragment ions for C11-terpenes e.g. at *m*/*z* 69, 93, 107, 121 and 135 (S6 Fig). Ten of them have a molecular ion of a C11-terpene alcohol at *m*/*z* 168 as observed for 2-MIB, suggesting the structures of methylated monoterpene alcohols, while 22 compounds showed like 2-MB a molecular ion at *m*/*z* 150 in the mass spectra, in agreement with the structure of a methylated monoterpene hydrocarbon. The main products 2-MIB and 2-MB were detected in the headspace of all four production strains. Furthermore, the previously described products 2-methyl-2-bornene, 1-methylcamphene, 2-methylmyrcene, 2-methyllimonene, 2-methyl-β-fenchol, 2-methyllinalool, 2-methyl-α-terpineol, 2-methylgeraniol and 2-methylnerol were produced by the different strains in strain-specific proportions. 2-Methyl-α-terpineol (RI = 1291) coelutes with indole, which shows high signals in the total ion chromatograms of all investigated *E. coli* strains and has a similar retention index of 1294. However, via ion trace analyses it was possible to identify 2-methyl-α-terpineol as products of the production strains harboring the C11-terpene synthases of *P. fluorescens* and *S. griseus*.

Four identified compounds have not been described as C11-TS products or as natural products in general. 2-Methylborneol (*exo*-isomer of 2-MIB), 3,4-dimethylcumene and both diastereomers of 2-methylcitronellol were detected. In contrast to the other identified C11-terpenes, 3,4-dimethylcumene exhibit the molecular ion at *m*/*z* = 148 and the two diastereomers of 2-methylcitronellol at *m*/*z* = 170 (Fig 3), requiring an additional oxidation step for 3,4-dimethylcumene and a reduction step for 2-methylcitronellol.

In addition to previously described C11-TS products and those four newly found compounds, 20 new C11-terpenes with unknown structures were detected.

From a total of 35 identified C11-terpenes, 2-methylmyrcene, 2-methyllinalool, 2-methylgeraniol and two C11-terpenes with unknown structures are produced by strain 11–0 (Table 3

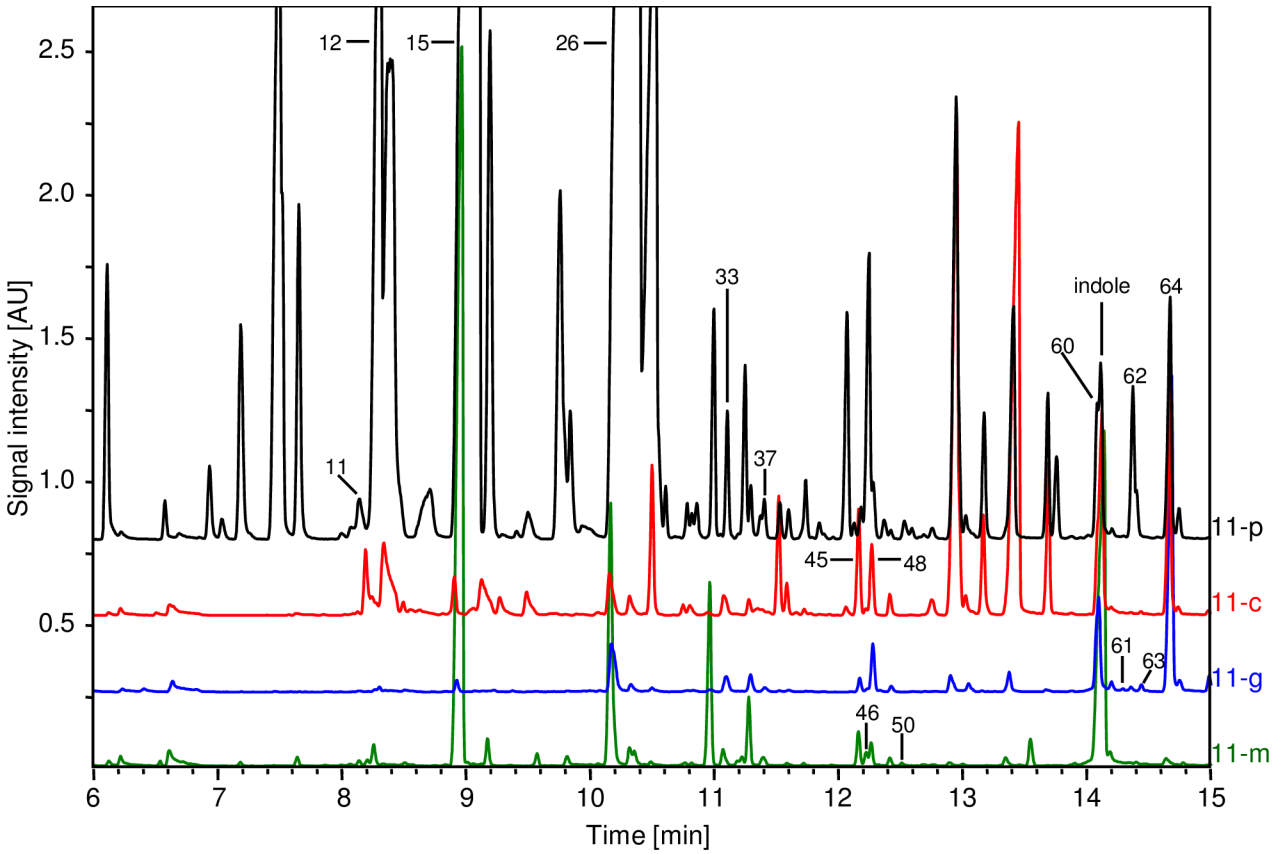


Fig 2. Total ion chromatograms of HS-SPME-GCMS analyses of four production strains expressing four different C11-TSs. The peaks of all identified and mentioned compounds are labeled. Compound names are listed in Table 3.

https://doi.org/10.1371/journal.pone.0196082.g002

and S8 Fig), which does not express a terpene synthase. This indicates that they are formed from 2-methyl-GPP without participation of a TS.

Monoterpenoids

In the headspace of four production strains also 29 monoterpenoids have been detected. We were able to identify hydrocarbons (cyclofenchene, 2-bornene, tricyclene, α-thujene, α- and β-pinene, camphene, sabinene, β-myrcene, α-phellandrene, limonene, (Z)- and (E)-β-ocimene, γ-terpinene, alloocimene), alcohols (linalool, isopulegol, borneol, terpinen-4-ol, γ-isogeraniol, β-citronellol, geraniol) and aldehydes (citronellal, isoneral, isogeranial, neral, geranial). A couple of them only occur in strains expressing one of the C11-TS (S2 Table), showing the acceptance of GPP as substrate of the investigated C11-TS as reported previously [7,34]. However, 16 monoterpenoids are produced as well by strains not expressing any TS, indicating that that they are formed from GPP without participation of a TS.

Sesquiterpenoids and C16-terpenoids

The formation of less volatile terpenoids by the production and the control strains was investigated using SBSE GC-MS analyses. With nerolidol, 2,3-dihydrofarnesol, (*Z*,*E*)-farnesol, (*E*,*E*)-farnesol and farnesal, five sesquiterpenoids were identified by comparison of their mass spectra to known spectral data and RIs. Furthermore, the production of 6-methylfarnesol could be

Table 3. C11-compounds detected in the headspace of four C11-terpene production strains (11-p, 11-m, 11-g, 11-c) and one control strain without TS (11–0).

		Detected in culture of strain						
#	Compound[b]	11-p	11-c	11-g	11-m	11–0	Identified by[a]	RI
8	C11-terpene	x			x			958
11	2-methyl-2-bornene	x	x		x		Ref	980
12	1-methylcamphene	x	x	x	x		Ref	986
15	2-methylenebornane	x	x	x	x		Ref	1018
17	C11-terpene				x			1032
19	C11-terpene			x				1041
20	C11-terpene	x						1044
22	C11-terpene				x			1052
24	C11-terpene	x			x			1064
25	C11-terpene			x				1073
26	2-methylmyrcene	x	x	x	x	x	Lit	1081
27	C11-terpene				x			1091
28	C11-terpene				x			1095
30	C11-terpene	x						1099
31	C11-terpene	x		x	x			1115
32	C11-terpene	x	x	x		x		1125
33	2-methyllimonene	x	x	x	x		Ref	1128
35	C11-terpene	x			x			1138
36	C11-terpene	x	x	x	x	x		1139
37	3,4-dimethylcumene	x	x	x	x		Ref	1146
41	C11-terpene	x		x	x			1164
45	2-methylisoborneol	x	x	x	x		Ref	1186
46	2-methyl-β-fenchol		x		x			1189
47	C11-terpene	x		x				1191
48	2-methyllinalool	x	x	x	x	x	Ref	1193
49	C11-terpene	x						1194
50	2-methylborneol				x		Ref	1204
51	C11-terpene alcohol	x						1207
55	C11-terpene alcohol			x				1234
59	C11-terpene alcohol	x						1275
60	2-methyl-α-terpineol	x		x			Ref	1293
61	2-methylcitronellol, diastereomer 1			x			Ref	1306
62	2-methylnerol	x					Ref	1308
63	2-methylcitronellol, diastereomer 2		x	x			Ref	1310
64	2-methylgeraniol	x	x	x	x	x	Ref	1327

[a] Compounds were identified via comparison of mass spectra and RIs of reference compounds (Ref) or of literature data (Lit, [22]).

[b] Compounds with unknown structures were named regarding their highest *m/z* value as C11-terpene (*m/z* = 150) or C11-terpene alcohol (*m/z* = 168)

https://doi.org/10.1371/journal.pone.0196082.t003

shown by comparison of the mass spectrum and the RI to the ones of the reference substance (Fig 4).

Like the sesquiterpenoids, this C16-terpene was produced by the production strains and 11–0 indicating that the TS is not involved in the formation of these compounds. Though the control strain 10–0 without the GPP-MTase and TS produced the sesquiterpenoids, it did not produce 6-methylfarnesol, revealing the participation of the GPP-MTase in the production of 6-methyfarnesol. Presumably, the endogenous *E. coli* farnesyl diphosphate (FPP) synthase,

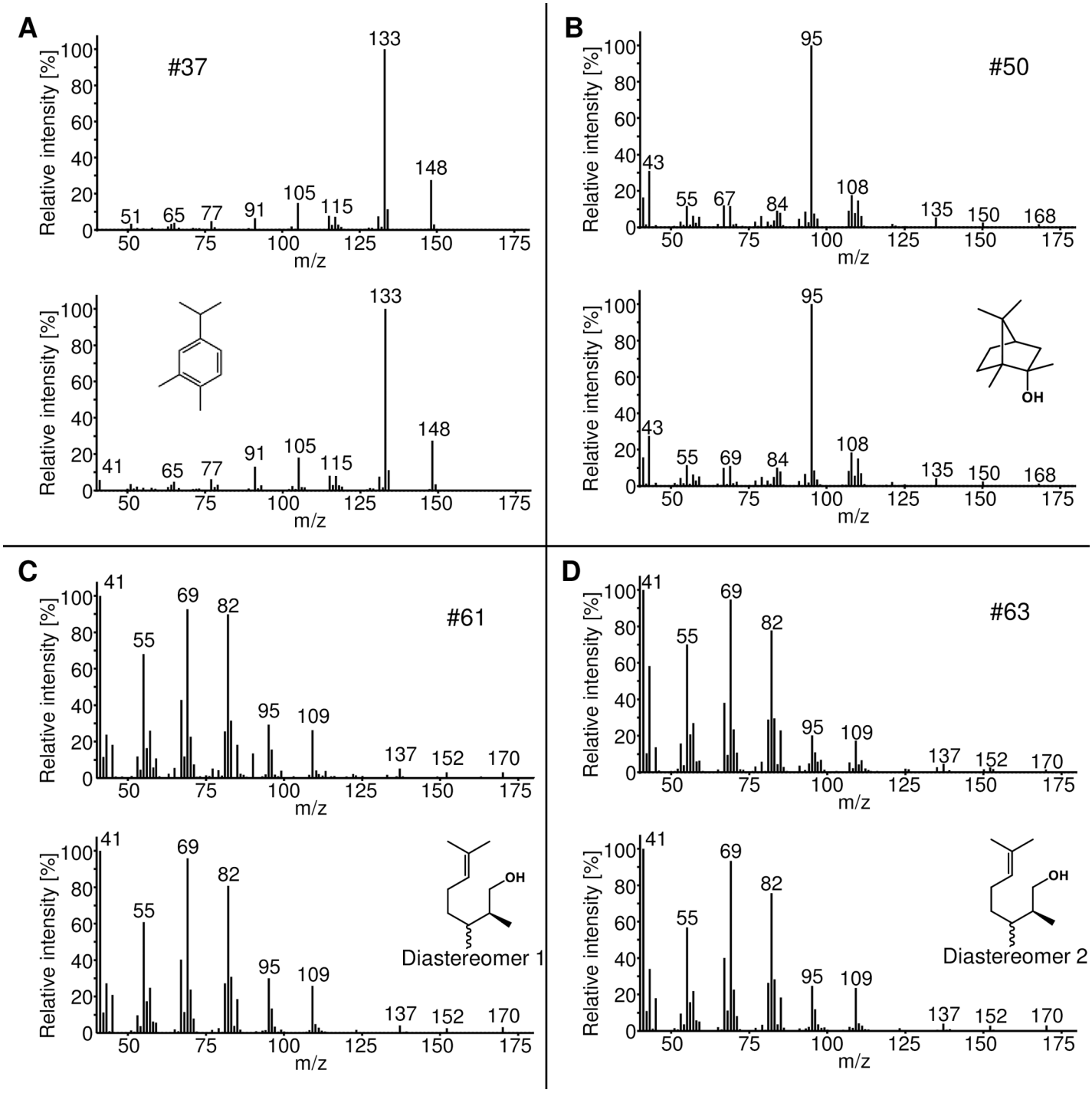


Fig 3. Mass spectra and structures of the four novel C11-TS products 3,4-dimethylcumene (A), 2-methylborneol (B), 2-methylcitronellol diastereomer 1 (C) and 2-methylcitronellol diastereomer 2 (D) The mass spectra obtained from the analyses of the production strains (above) are compared with those of reference compounds (below).The mass spectra of the diastereomers of 2-methylcitronellol are almost identical.

https://doi.org/10.1371/journal.pone.0196082.g003

encoded by *ispA*, catalyzes the condensation of 2-methyl-GPP with IPP to 6-methyl-FPP. This is probably dephosphorylated or hydrolyzed to 6-methylfarnesol in the next step, either spontaneously or by another endogenous enzyme (Fig 5).

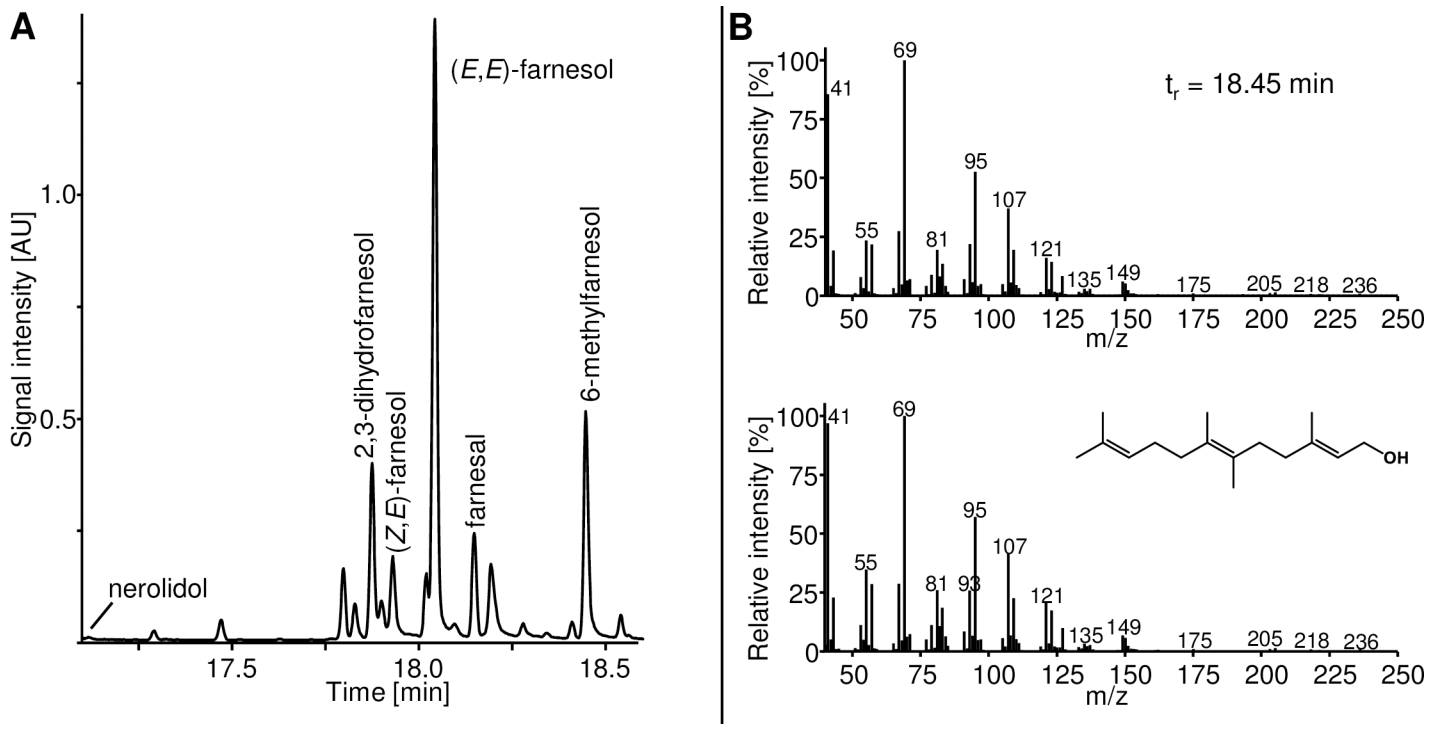


Fig 4. Total ion chromatogram of SBSE-GCMS analysis of the control strain 11–0 (A) and the structure and mass spectra of 6-methylfarnseol (B) The spectra of the 6-methylfarnesol peak of the shown chromatogram (above) is compared with that of the reference compound (below).

https://doi.org/10.1371/journal.pone.0196082.g004

Discussion

After the elucidation of the biosynthetic pathway of 2-MIB and 2-MB [5–7] and the description of eleven C11-terpenes as products of specific bacterial strains or C11-TSs [20,22,23], we could produce 35 different C11-terpenoids via a *de novo* approach and heterologous expression with *E. coli*. This enormous product variety originating from only four different C11-TSs reveals that there is a high potential to discover even more C11-terpenes by investigating other C11-TSs or through protein engineering.

Besides the eleven previously described C11-terpenes, 24 novel compounds could be detected in the headspace of four different production strains. From those, the structure of four substances could be identified. Thereby, 3,4-dimethylcumene and the two diastereomers of 2-methylcitronellol are downstream metabolites of further conversions of primary C11-terpene synthase products that are formed by spontaneous or enzymatically catalyzed reactions. 3,4-Dimethylcumene is likely the product of a spontaneous oxidation of 2-methyl-γ-terpinene, in analogy to the reported spontaneous oxidation of the corresponding monoterpene γ-terpinene to p-cymene [35,36]. Since no reference substance is available for 2-methyl-γ-terpinene the presence of the probable precursor of 3,4-dimethylcumene could not be proven in this study.

2-Methylcitronellol is probably produced by hydrogenation of 2-methylgeraniol. A spontaneous reaction is unlikely, but in *Saccharomyces cerevisiae*, for example, the reduction of geraniol to citronellol by endogenous enzymes has been described [37]. The proposed reaction mechanisms of described and potential C11-terpenes as well as the suggested subsequent reactions are shown in Fig 6.

The phenomenon of the occurrence of various monoterpenoids in *E. coli* strains producing a high level of GPP has already been demonstrated [38]. Fischer et al. showed that different

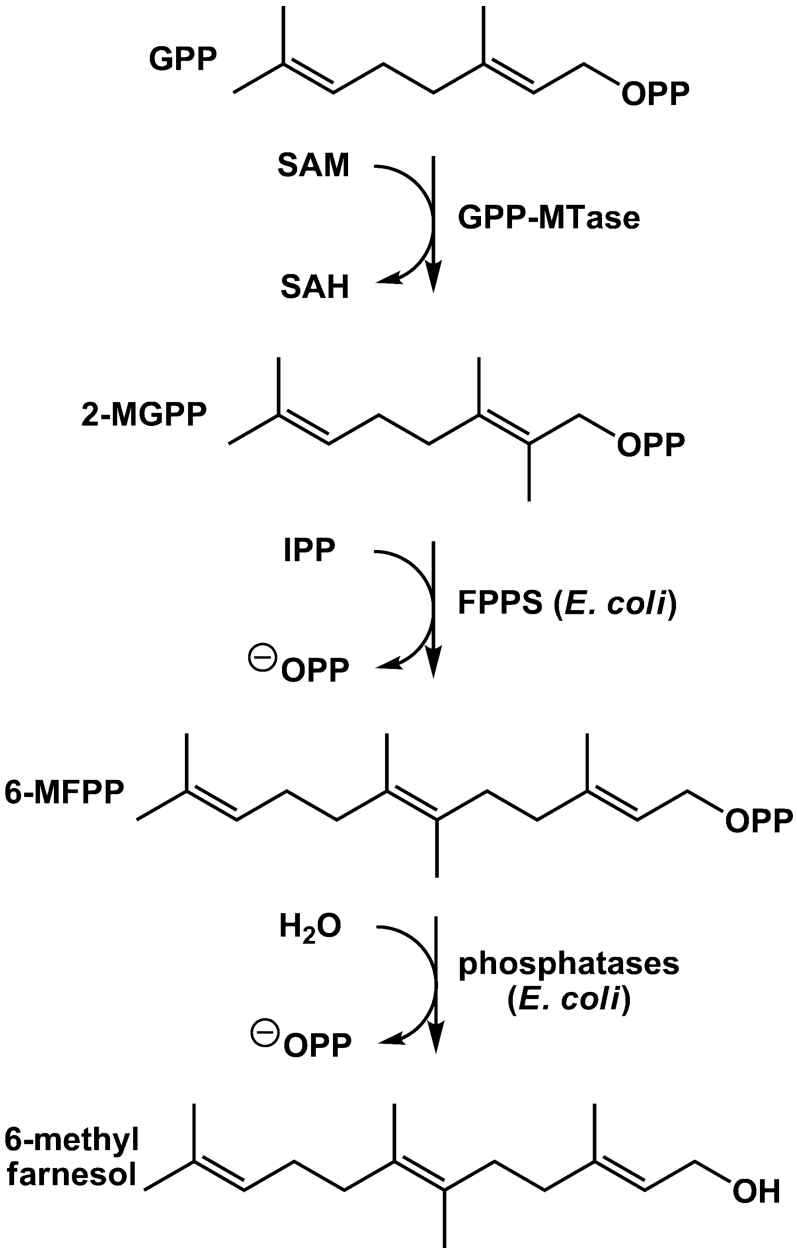


Fig 5. Proposed biosynthesis of 6-methylfarnesol in *E.coli* expressing the GPP-MTase of *S. coelicolor*. The elongation of 2-MGPP with IPP by an endogenous FPP synthase (FPPS) to 6-methylfarnesyldiphosphate (6-MFPP) and its dephosphorolation by endogenous phosphatases (probably PgpB and YbjG) are assumed.

https://doi.org/10.1371/journal.pone.0196082.g005

monoterpenes like nerol, linalool and even the cyclic α-terpineol can derive from GPP or geraniol, when GPP is overproduced in *Saccharomyces cerevisae*. Furthermore, the *E. coli* enzyme YjgB is known to convert geraniol to nerol, geranial and neral [39]. These findings provide an explanation for the variety of 16 monoterpenes that occur in all strains harboring the pMK-05 plasmid, even in those that do not express any terpene synthase. Nevertheless, we could unequivocally establish that the studied C11-TSs produce further monoterpenes from GPP.

A highly interesting additional product of *E. coli* strains expressing the GPP-MTase is the C16-terpene 6-methylfarnesol. Previous studies showed that different prenyltransferases

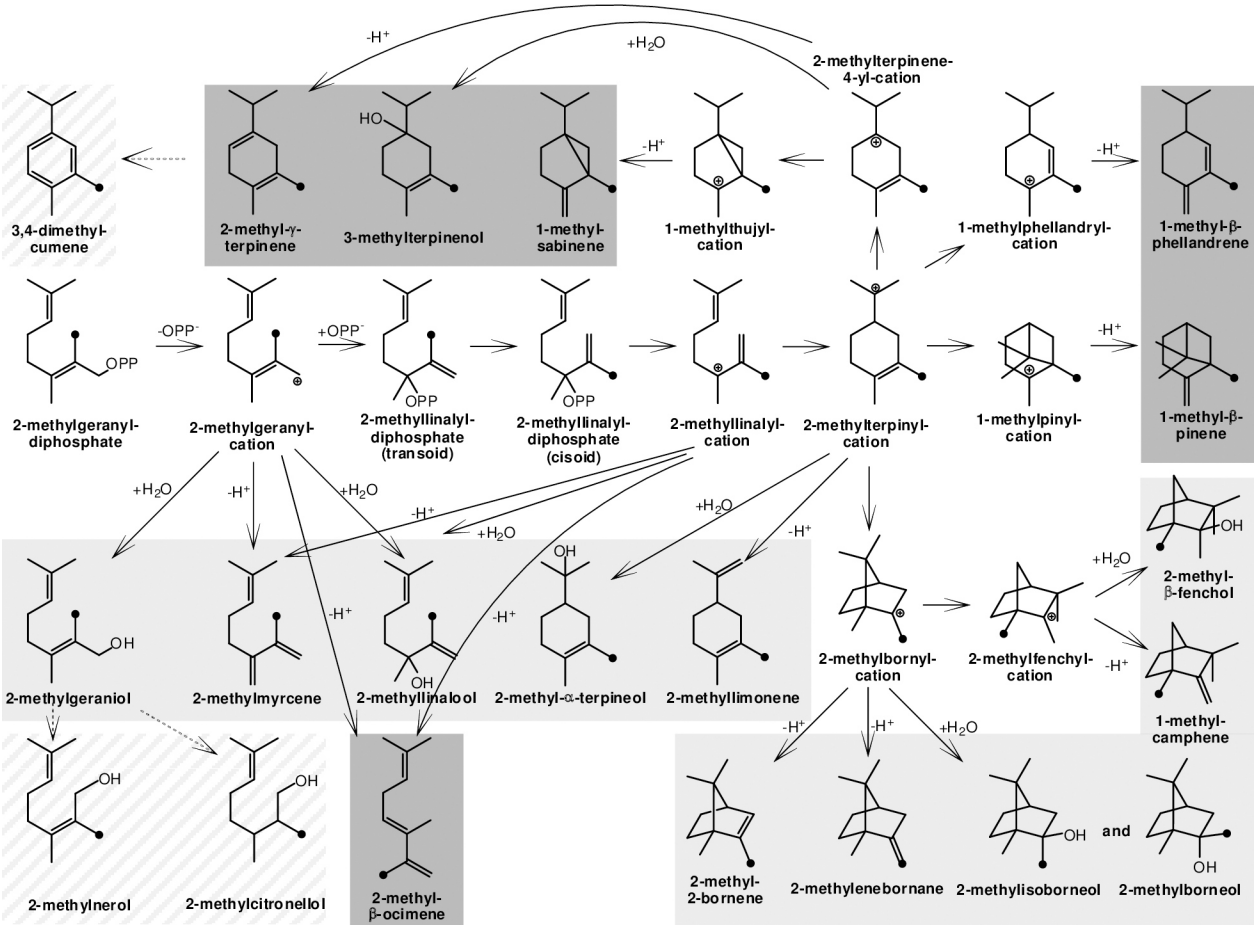


Fig 6. Proposed reaction mechanism of C11-terpenes catalyzed by C11-TSs based on Brock et al. [22]. Methyl groups introduced by the GPP-MTase are labeled with a black dot. Potential, but not detected products have a dark gray background. Products detected in this or other studies have a light gray background. Compounds that have been detected, but are no direct terpene synthase products have a hatched background.

https://doi.org/10.1371/journal.pone.0196082.g006

accept various substrate analogs. For example, pig liver FPP synthase can convert various methylated GPP derivatives and C6 homologs of IPP [40], while undecaprenyl diphosphate synthase from *Micrococcus luteus* accepts the C6 compound 3-ethylbut-3-enyl diphosphate and also the C4 analog of IPP, but-3-enyl diphosphate, for the elongation of FPP [41]. Therefore, it can be assumed that 6-methyl-FPP is derived from elongation of the GPP-MTase product 2-methyl-GPP with IPP by the *E. coli* FPP synthase encoded by *ispA*. Dephosphorylation of 6-methyl-FPP to 6-methylfarnesol could be a spontaneous reaction or catalyzed by an endogenous phosphatase, e. g. by one of the two integral membrane phosphatases PgpB and YbjG that are known to hydrolyze FPP to farnesol [42].

Our study provides a detailed view on the product diversity accessible by conversion of 2-methyl-GPP with different C11-terpene synthases and provides a cellular synthesis route for 6-methyl-FPP.

Supporting information

S1 Table. Oligos and RBS sequences used in this study (overhangs of primers are underlined).
(PDF)

S2 Table. Monoterpenoids detected in the headspace of four C11-terpene production strains (11-p, 11-m, 11-g, 11-c) and one control strain without GPP-MTase and TS (10–0).
(PDF)

S1 Fig. *gppmtase* sequence optimized in codon usage for *E. coli*.
(PDF)

S2 Fig. *mbsp* sequence optimized in codon usage for *E. coli*.
(PDF)

S3 Fig. *mbsm* sequence optimized in codon usage for *E. coli*.
(PDF)

S4 Fig. *mibsg* sequence optimized in codon usage for *E. coli*.
(PDF)

S5 Fig. *mibsc* sequence optimized in codon usage for *E. coli*.
(PDF)

S6 Fig. Mass spectra of all compounds with unknown structure listed in Table 3 or S2 Table.
(PDF)

S7 Fig. Mass spectra of C11- and C16-reference substances.
(PDF)

S8 Fig. Total ion chromatograms of HS-SPME-GCMS analyses of the control strains 0–0, 10–0 and 11–0 compared to the one of the production strain 11-p.
(PDF)

Author Contributions

Conceptualization: Max J. Kschowak, Jens Schrader, Markus Buchhaupt.

Data curation: Max J. Kschowak.

Formal analysis: Max J. Kschowak, Jeroen S. Dickschat, Markus Buchhaupt.

Funding acquisition: Jens Schrader, Markus Buchhaupt.

Investigation: Max J. Kschowak, Hannah Wortmann, Jeroen S. Dickschat.

Methodology: Max J. Kschowak, Markus Buchhaupt.

Project administration: Max J. Kschowak, Jens Schrader, Markus Buchhaupt.

Supervision: Markus Buchhaupt.

Validation: Max J. Kschowak, Markus Buchhaupt.

Visualization: Max J. Kschowak.

Writing – original draft: Max J. Kschowak.

Writing – review & editing: Jeroen S. Dickschat, Markus Buchhaupt.

References

1. Buckingham J. Dictionary of Natural Products 26.1. London: Chapman and Hall/CRC Press. Available at: https://dnp.chemnetbase.com; last accessed 08 November 2017.

2. Schrader J, Bohlmann J, editors. Biotechnology of Isoprenoids. Cham (Switzerland): Springer International Publishing; 2015.
3. Wallach O. Zur Kenntniss der Terpene und der ätherischen Oele; Fünfte Abhandlung. Justus Liebigs Ann Chem. 1887; 239: 1–54. https://doi.org/10.1002/jlac.18872390102
4. Ružička L. The isoprene rule and the biogenesis of terpenic compounds. Experientia. 1953; 9: 357–367. https://doi.org/10.1007/BF02167631 PMID: 13116962
5. Dickschat JS, Nawrath T, Thiel V, Kunze B, Mueller R, Schulz S. Biosynthesis of the off-flavor 2-methylisoborneol by the myxobacterium *Nannocystis exedens*. Angew Chem Int Ed. 2007; 46: 8287–8290. https://doi.org/10.1002/anie.200702496 PMID: 17899580
6. Komatsu M, Tsuda M, Ōmura S, Oikawa H, Ikeda H. Identification and functional analysis of genes controlling biosynthesis of 2-methylisoborneol. Proc Natl Acad Sci. 2008; 105: 7422–7427. https://doi.org/10.1073/pnas.0802312105 PMID: 18492804
7. Wang C-M, Cane DE. Biochemistry and molecular genetics of the biosynthesis of the earthy odorant methylisoborneol in *Streptomyces coelicolor*. J Am Chem Soc. 2008; 130: 8908–8909. https://doi.org/10.1021/ja803639g PMID: 18563898
8. Köksal M, Chou WK, Cane DE, Christianson DW. Structure of geranyl diphosphate C-methyltransferase from *Streptomyces coelicolor* and implications for the mechanism of isoprenoid modification. Biochemistry (Mosc). 2012; 51: 3003–3010. https://doi.org/10.1021/bi300109c PMID: 22455498
9. Köksal M, Chou WK, Cane DE, Christianson DW. Structure of 2-methylisoborneol synthase from *Streptomyces coelicolor* and implications for the cyclization of a noncanonical C-methylated monoterpenoid substrate. Biochemistry (Mosc). 2012; 51: 3011–3020. https://doi.org/10.1021/bi201827a PMID: 22455514
10. Wang Z, Suffet IH, Al-Samarrai. Sensory and chemical analysis methods for earthy and musty odours in drinking water caused by geosmin and 2-methylisoborneol. Water Sci Technol Water Supply. 2006; 6: 147–155. https://doi.org/10.2166/ws.2006.732
11. Piriou P, Devesa R, Lalande MD, Glucina K. European reassessment of MIB and geosmin perception in drinking water. J Water Supply Res Technol—Aqua. 2009; 58: 532–538. https://doi.org/10.2166/aqua.2009.124
12. Callejón RM, Ubeda C, Ríos-Reina R, Morales ML, Troncoso AM. Recent developments in the analysis of musty odour compounds in water and wine: A review. J Chromatogr A. 2016; 1428: 72–85. https://doi.org/10.1016/j.chroma.2015.09.008 PMID: 26372442
13. Martin JF, Fisher TH, Bennett LW. Musty odor in chronically off-flavored channel catfish: isolation of 2-methylenebornane and 2-methyl-2-bornene. J Agric Food Chem. 1988; 36: 1257–1260. https://doi.org/10.1021/jf00084a032
14. Karahadian C, Josephson DB, Lindsay RC. Volatile compounds from *Penicillium sp*. contributing musty-earthy notes to Brie and Camembert cheese flavors. J Agric Food Chem. 1985; 33: 339–343. https://doi.org/10.1021/jf00063a005
15. Gerber NN. A volatile metabolite of actinomycetes, 2-methylisoborneol. J Antibiot (Tokyo). 1969; 22: 508–509. https://doi.org/10.7164/antibiotics.22.508
16. Medsker LL, Jenkins D, Thomas JF, Koch C. Odorous compounds in natural waters. 2-exo-Hydroxy-2-methylbornane, the major odorous compound produced by several actinomycetes. Environ Sci Technol. 1969; 3: 476–477. https://doi.org/10.1021/es60028a008
17. Tabachek J-AL, Yurkowski M. Isolation and Identification of Blue-Green Algae Producing Muddy Odor Metabolites, Geosmin, and 2-Methylisoborneol, in Saline Lakes in Manitoba. J Fish Res Board Can. 1976; 33: 25–35. https://doi.org/10.1139/f76-004
18. Fravel DR, Connick William J, Grimm CC, Lloyd SW. Volatile Compounds Emitted by *Sclerotia* of *Sclerotinia minor*, *Sclerotinia sclerotiorum* and *Sclerotium rolfsii*. J Agric Food Chem. 2002; 50: 3761–3764. https://doi.org/10.1021/jf0117098 PMID: 12059156
19. Toyota M, Asakawa Y, Frahm J-T. Homomono- and sesquiterpenoids from the liverwort *Lophocolea heterophylla*. Phytochemistry. 1990; 29: 2334–2337. https://doi.org/10.1016/0031-9422(90)83066-A
20. Chou WK, Ikeda H, Cane DE. Cloning and characterization of Pfl_1841, a 2-methylenebornane synthase in *Pseudomonas fluorescens* PfO-1. Tetrahedron. 2011; 67: 6627–6632. https://doi.org/10.1016/j.tet.2011.05.084 PMID: 21804650
21. Dickschat JS. Bacterial terpene cyclases. Nat Prod Rep. 2016; 33: 87–110. https://doi.org/10.1039/c5np00102a PMID: 26563452
22. Brock NL, Ravella SR, Schulz S, Dickschat JS. A Detailed View of 2-Methylisoborneol Biosynthesis. Angew Chem Int Ed. 2013; 52: 2100–2104. https://doi.org/10.1002/anie.201209173 PMID: 23316002

23. Chou WK, Gould CA, Cane DE. Incubation of 2-methylisoborneol synthase with the intermediate analog 2-methylneryl diphosphate. J Antibiot (Tokyo). 2017; 70: 625–631. https://doi.org/10.1038/ja.2017.24 PMID: 28246382

24. Tseng H-C, Harwell CL, Martin CH, Prather KL. Biosynthesis of chiral 3-hydroxyvalerate from single propionate-unrelated carbon sources in metabolically engineered *E. coli*. Microb Cell Factories. 2010; 9: 96–107. https://doi.org/10.1186/1475-2859-9-96 PMID: 21110891

25. Alonso-Gutierrez J, Chan R, Batth TS, Adams PD, Keasling JD, Petzold CJ, et al. Metabolic engineering of *Escherichia coli* for limonene and perillyl alcohol production. Metab Eng. 2013; 19: 33–41. https://doi.org/10.1016/j.ymben.2013.05.004 PMID: 23727191

26. Bertani G. Studies on Lysogenesis I. J Bacteriol. 1951; 62: 293–300. PMID: 14888646

27. Espah Borujeni A, Channarasappa AS, Salis HM. Translation rate is controlled by coupled trade-offs between site accessibility, selective RNA unfolding and sliding at upstream standby sites. Nucleic Acids Res. 2014; 42: 2646–2659. https://doi.org/10.1093/nar/gkt1139 PMID: 24234441

28. Salis HM, Mirsky EA, Voigt CA. Automated design of synthetic ribosome binding sites to control protein expression. Nat Biotechnol. 2009; 27: 946–950. https://doi.org/10.1038/nbt.1568 PMID: 19801975

29. Gibson DG, Young L, Chuang R-Y, Venter JC, Hutchison CA, Smith HO. Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat Methods. 2009; 6: 343–345. https://doi.org/10.1038/nmeth.1318 PMID: 19363495

30. Kovach ME, Elzer PH, Steven Hill D, Robertson GT, Farris MA, Roop II RM, et al. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene. 1995; 166: 175–176. https://doi.org/10.1016/0378-1119(95)00584-1 PMID: 8529885

31. Hanahan D. Techniques for transformations of E. coli. In: Glover DM, editor. DNA cloning: A practical approach. Oxford, United Kingdom: IRL Press; 1985. pp. 109–135.

32. Ausubel F, Brent R, Kingston R, Moore D, Seidman J, Smith J, et al., editors. Current Protocols in Molecular Biology. New York: Wiley-Interscience; 1987.

33. Adams RP. Identification of Essential Oil Components by Gas Chromatography/ Mass Spectroscopy. 4th ed. Illinois, USA: Allured Publishing Corporation; 2007.

34. Citron CA, Gleitzmann J, Laurenzano G, Pukall R, Dickschat JS. Terpenoids are widespread in actinomycetes: a correlation of secondary metabolism and genome data. ChemBioChem. 2012; 13: 202–214. https://doi.org/10.1002/cbic.201100641 PMID: 22213220

35. Granger R, Passet J, Verdier R. Gamma-Terpinene, Precursor of p-Cymene in *Thymus vulgaris*. Comptes Rendus L'Académie Sci. 1964; 258: 5539–5541.

36. Asikainen M, Jauhiainen O, Aaltonen O, Harlin A. Continuous catalyst-free aromatization of γ-terpinene using air as an oxidant. Green Chem. 2013; 15: 3230–3235. https://doi.org/10.1039/C3GC41224E

37. Steyer D, Erny C, Claudel P, Riveill G, Karst F, Legras J-L. Genetic analysis of geraniol metabolism during fermentation. Food Microbiol. 2013; 33: 228–234. https://doi.org/10.1016/j.fm.2012.09.021 PMID: 23200656

38. Fischer MJC, Meyer S, Claudel P, Bergdoll M, Karst F. Metabolic engineering of monoterpene synthesis in yeast. Biotechnol Bioeng. 2011; 108: 1883–1892. https://doi.org/10.1002/bit.23129 PMID: 21391209

39. Zhou J, Wang C, Yoon S-H, Jang H-J, Choi E-S, Kim S-W. Engineering *Escherichia coli* for selective geraniol production with minimized endogenous dehydrogenation. J Biotechnol. 2014; 169: 42–50. https://doi.org/10.1016/j.jbiotec.2013.11.009 PMID: 24269531

40. Koyama T, Saito A, Ogura K, Seto S. Substrate specificity of farnesylpyrophosphate synthetase. Application to asymmetric synthesis. J Am Chem Soc. 1980; 102: 3614–18. https://doi.org/10.1021/ja00530a050

41. Nagaki M, Sato S, Maki Y, Nishino T, Koyama T. Artificial substrates for undecaprenyl diphosphate synthase from *Micrococcus luteus* B-P 26. J Mol Catal B Enzym. 2000; 9: 33–38. https://doi.org/10.1016/S1381-1177(99)00081-8

42. Wang C, Park J-E, Choi E-S, Kim S-W. Farnesol production in *Escherichia coli* through the construction of a farnesol biosynthesis pathway–application of PgpB and YbjG phosphatases. Biotechnol J. 2016; 11: 1291–1297. https://doi.org/10.1002/biot.201600250 PMID: 27440491

5.1.2 Expanding the Isoprenoid Building Block Repertoire with an IPP Methyltransferase from *Streptomyces monomycini*

Reprinted with permission from *ACS Synth. Biol.* 2019, 8, 6, 1303–1313. Copyright © 2019 American Chemical Society.
https://doi.org/10.1021/acssynbio.8b00525

Erklärung zu den Autorenanteilen an der Publikation / an dem Manuskript (Titel):
Expanding the Isoprenoid Building Block Repertoire with an IPP Methyltransferase from *Streptomyces monomycini*

Status (*submitted, under review, in revision, accepted*): Accepted

Name der Zeitschrift: ACS Synthetic Biology

Beteiligte Autoren:
Laura Drummond (LD)*, Max J. Kschowak (Promovierender)*, Jürgen Breitenbach (JB), Hendrik Wolff (HW), Yi-Ming Shi (YS), Jens Schrader (JS), Helge B. Bode (HB), Gerhard Sandmann (GS), Markus Buchhaupt (MB)
*Diese Autoren haben zu gleichen Teilen beigetragen

Was hat der Promovierende bzw. was haben die Koautoren beigetragen?*[3]

(1) zu Entwicklung und Planung

Promovierender: 27,5 %
LD: 27,5 %
JB / GS: 10 %
HW / YS / HB: 5 %
JS: 5 %
MB: 20 %

(2) zur Durchführung der einzelnen Untersuchungen und Experimente

Identification and Characterization of an IPP Methyltransferase from *Streptomyces monomycini*
Promovierender: 50 %
LD: 50 %

In Vivo Production of Noncanonical Prenyl Pyrophosphates by the IPP Methyltransferase
Promovierender: 45 %
LD: 55 %

In Vivo Incorporation of Noncanonical Prenyl Pyrophosphates Enables Biosynthesis of New-to-Nature Carotenoids
Promovierender: 15 %
LD: 10 %
JB / GS: 50 %
HW / YS / HB: 25 %

(3) zur Erstellung der Datensammlung und Abbildungen

Figure1
MB: 100 %

Figure 2
Promovierender: 100 %

Figure 3
MB 67 %
Promovierender: 33 %

Table 1
LD: 100 %

Figure 4
LD: 50 %

[a] *Aus Gründen der Lesbarkeit wird im gesamten Text die männliche Schreibweise verwendet. Frauen sind natürlich inbegriffen.*

MK: 30 %
MB: 20 %

Figure 5
JB / GS: 33 %
MB: 67 %

Table MM1 - MM3:
Promovierender: 50 %
LD: 50 %

Figure S1 - S17:
Promovierender: 50 %
LD: 50 %

Figure S18:
JB / GS: 100 %

Table S1 and Figure S19:
HW / YS / HB: 100 %

Figure S20:
MB: 100 %

Figure S21:
LD: 100 %

(4) zur Analyse und Interpretation der Daten

Identification and Characterization of an IPP Methyltransferase from *Streptomyces monomycini*
Promovierender: 40 %
LD: 40 %
MB: 20

In Vivo Production of Noncanonical Prenyl Pyrophosphates by the IPP Methyltransferase
Promovierender: 40 %
LD: 40 %
MB: 20 %

In Vivo Incorporation of Noncanonical Prenyl Pyrophosphates Enables Biosynthesis of New-to-Nature Carotenoids
Promovierender: 5 %
LD: 5 %
MB: 5 %
JB / GS: 35 %
HW / YS / HB: 50 %

(5) zum Verfassen des Manuskripts

Promovierender: 20 %
LD: 25 %
JB / GS: 15 %
HW / YS / HB: 10 %
JS: 5 %
MB: 25 %

---

[a] *Aus Gründen der Lesbarkeit wird im gesamten Text die männliche Schreibweise verwendet. Frauen sind natürlich inbegriffen.*

Zustimmende Bestätigungen der oben genannten Angaben:

Datum/Ort	Unterschrift Promovend
Datum/Ort	Unterschrift Betreuer
Datum/Ort	Ggfs. Unterschrift *corresponding author*

[*1] Der Name der Zeitschrift darf erst genannt werden, wenn die Arbeit akzeptiert, im Druck oder erschienen ist.

[*2] Als Autoren werden solche Personen bezeichnet, die an der Arbeit in Bezug auf die genannten Punkte in einer Weise mitgewirkt haben, dass sie für die ausgewiesenen Passagen (mit-) verantwortlich sind. Personen, die an der Arbeit mitgewirkt haben, jedoch nicht in diese Kategorie fallen, sollten in der Danksagung Erwähnung finden.

[*3] Bei (2), (3) und (4) bitte prozentuale und dazu kurze inhaltliche Angaben machen, bei (1) und (5) reichen prozentuale Angaben.

Wenn es sich um ein Review handelt, notieren Sie das bitte in Klammern hinter den Titel der Publikation. Zudem ist in diesem Fall (2) hinfällig, bitte notieren Sie dort „n/a“. Unter (3) geht es dann um die „Literatursammlung“ anstelle der „Datensammlung“. Bitte ändern Sie dies im Formular!

[a] *Aus Gründen der Lesbarkeit wird im gesamten Text die männliche Schreibweise verwendet. Frauen sind natürlich inbegriffen.*

ACS Synthetic Biology
Cite This: ACS Synth. Biol. 2019, 8, 1303−1313
Research Article
pubs.acs.org/synthbio

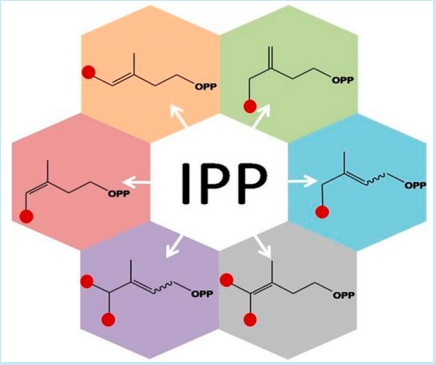
Expanding the Isoprenoid Building Block Repertoire with an IPP Methyltransferase from *Streptomyces monomycini*

Laura Drummond,[§,†,#] Max J. Kschowak,[§,†,#] Jürgen Breitenbach,[†] Hendrik Wolff,[†] Yi-Ming Shi,[†] Jens Schrader,[§] Helge B. Bode,[†,‡] Gerhard Sandmann,[†] and Markus Buchhaupt*[,§]

[§]Industrial Biotechnology, DECHEMA Research Institute, Theodor-Heuss-Allee 25, 60486 Frankfurt am Main, Germany
[†]Institute for Molecular Bioscience, Johann Wolfgang Goethe University, Max-von-Laue-Straße 9, 60438 Frankfurt am Main, Germany
[‡]Buchmann Institute for Molecular Life Sciences (BMLS), Johann Wolfgang Goethe University, Max-von-Laue-Straße 15, 60438 Frankfurt am Main, Germany

Supporting Information

ABSTRACT: Many synthetic biology approaches aim at expanding the product diversity of enzymes or whole biosynthetic pathways. However, the chemical structure space of natural product forming routes is often restricted by the limited cellular availability of different starting intermediates. Although the terpene biosynthesis pathways are highly modular, their starting intermediates are almost exclusively the C_5 units IPP and DMAPP. To amplify the possibilities of terpene biosynthesis through the modification of its building blocks, we identified and characterized a SAM-dependent methyltransferase converting IPP into a variety of C_6 and C_7 prenyl pyrophosphates. Heterologous expression in *Escherichia coli* not only extended the intracellular prenyl pyrophosphate spectrum with mono- or dimethylated IPP and DMAPP, but also enabled the biosynthesis of C_{11}, C_{12}, C_{16}, and C_{17} prenyl pyrophosphates. We furthermore demonstrated the general high promiscuity of terpenoid biosynthesis pathways toward uncommon building blocks by the *E. coli*-based production of polymethylated C_{41}, C_{42}, and C_{43} carotenoids. Integration of the IPP methyltransferase in terpene synthesis pathways enables an expansion of the terpenoid structure space beyond the borders predetermined by the isoprene rule which indicates a restricted synthesis by condensation of C_5 units.



KEYWORDS: *terpene biosynthesis, IPP methyltransferase, carotenoids, isoprene rule, Streptomyces monomycini*

Downloaded via UNIV FRANKFURT on April 16, 2023 at 21:19:37 (UTC).
See https://pubs.acs.org/sharingguidelines for options on how to legitimately share published articles.

Terpenoids are the largest class of natural products, of which more than 70 000 compounds are currently known.[1] Among them, many substances are used as pharmaceuticals (e.g., artemisinin and taxol), flavor and fragrance compounds (e.g., menthol, nootkatone, and ambrox) or structural materials (e.g., polyisoprene). This immense structural variety is the result of the modular arrangement of terpenoid biosynthesis. Furthermore, the carbocationic reaction mechanism employed by all terpene synthases can lead to a multitude of different structures due to combinations of hydride shifts, deprotonations, Wagner−Meerwein-rearrangements, hydrations, and ring-closure reactions.

Despite the extremely high structural diversity, all terpene backbones originate from two building blocks, isopentenyl pyrophosphate (IPP, **1a**) and dimethylallyl pyrophosphate (DMAPP, **2a**) (Figure 1). The utilization of these building blocks is reflected in the isoprene rule established by Otto Wallach, who proposed the C_5 isoprene molecule as the basic structural element of all terpenes,[2] thereby determining their carbon atom number to be a multiple of 5. This concept was extended by Leopold Ružička, who postulated in his biogenetic isoprene rule the biochemical synthesis of terpenoids from C_5 intermediates, but also the possibility of subsequent degradation or other modifications leading to noncanonical terpenoids.[3]

In accordance with the isoprene rule, the synthesis of terpenes follows a very specific modular concept, with the successive addition of five carbon units to a prenyl chain (Figure 1). The structural diversity of terpenoids and thereby the biotechnological exploitation of terpenoid biosynthesis would be greatly expanded if modifications could be added to the canonical building blocks. Such a strategy would take advantage of the generally high substrate promiscuity of prenyltransferases[4] and terpene synthases,[5−10] and would lead to a biocatalytic access to novel prenyl pyrophosphate building blocks.

Some terpene building blocks with a number of carbon atoms different than a multiple of 5 have been described previously. During the elucidation of the biosynthetic pathway of the long-known noncanonical terpenoid 2-methylisoborneol (2-MIB) in bacteria, the unusual C_{11} prenyl pyrophosphate 2-

Received: December 16, 2018
Published: May 6, 2019

© 2019 American Chemical Society

DOI: 10.1021/acssynbio.8b00525
ACS Synth. Biol. 2019, 8, 1303−1313

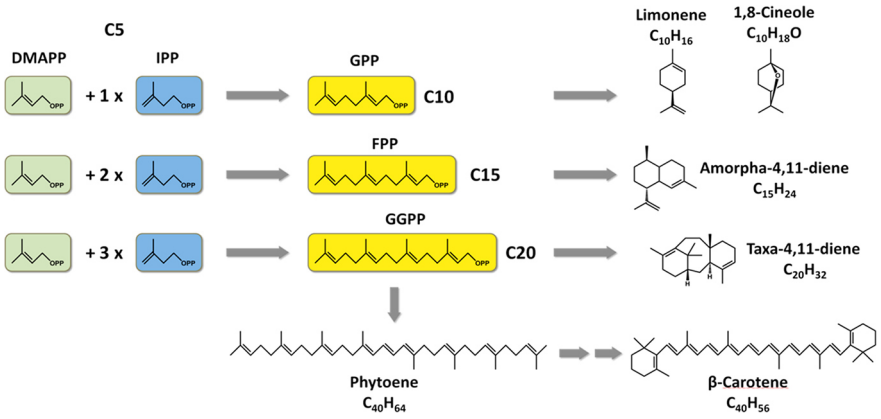


Figure 1. Modular biosynthesis of terpenoids including the central intermediates IPP and DMAPP (C_5), GPP (C_{10}), FPP (C_{15}), or GGPP (C_{20}).

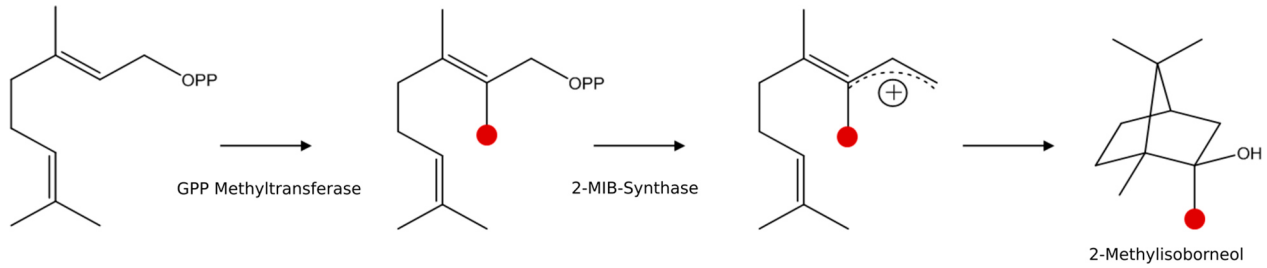


Figure 2. Biosynthesis of C_{11} compound 2-methylisoborneol

methyl-GPP was identified as the substrate of 2-MIB-forming terpene synthases (Figure 2).[11−13] The *S*-adenosyl-methionine (SAM)-dependent methyl transfer to GPP is catalyzed by the enzyme GPP methyltransferase. By analyzing the volatiles of actinomycete cultures or of recombinant *E. coli* strains coexpressing a GPP methyltransferase and one of several 2-methyl-GPP-accepting bacterial terpene synthases, more than 30 different 2-methyl-GPP-derived C_{11} terpenes could be observed.[14,15] In a recent report, Ignea and colleagues furthermore demonstrated the conversion of 2-methyl-GPP by different plant terpene synthases, when coexpressed with a GPP methyltransferase in bakers yeast.[16]

A recent publication described the identification of a methyltransferase in *Serratia plymuthica* catalyzing a methyl transfer to carbon 10 of FPP, which is followed by cyclization to a cyclic prenyl pyrophosphate named pre-sodorifen.[17] This final product of the methyltransferase reaction could be shown to be the substrate of a terpene synthase which forms the unusual bicyclic C_{16} hydrocarbon sodorifen and some minor byproducts.

During the elucidation of biosynthetic pathways for mono- and dimethylated cytokinines in *Rhodococcus fascians* and longestin in *Streptomyces argenteolus*, IPP-methyltransferases have been identified and partially characterized.[18,19] MT2 from *R. fascians* was shown to synthesize 4-methyl-DMAPP, while the incubation of IPP with MT1 yielded an uncharacterized C6 prenyl pyrophosphate, which was converted to 4,5-dimethyl-DMAPP by MT2. An enzyme from *S. argenteolus* was demonstrated to synthesize (*Z*)-4-methyl-IPP, which was proposed to be introduced at two positions during the prenyltransferase-catalyzed formation of the longestin precursor 4,12-dimethyl-GGPP.

Moreover, methylated IPP derivatives have been shown to be incorporated in prenyl chains in different terpene biosynthesis pathways of some insects (reviewed by Morgan, 2007[20]). In contrast to methyl transfer by SAM-dependent methyltransferases, a so-called homomevalonate pathway was demonstrated to provide different C_6 prenyl pyrophosphates after introduction of propionyl-CoA instead of acetyl-CoA in the ketothiolase-catalyzed reaction.

In the present work, we describe the detailed characterization and application of a newly identified methyltransferase from *Streptomyces monomycini*, which catalyzes the methylation of the C_5 key intermediate of terpenoid biosynthesis, IPP, thereby forming different C_6 and C_7 prenyl pyrophosphates. Using this enzyme and engineered variants to design synthetic metabolic pathways allows an enormous expansion of the chemical structure space of terpenoids beyond the borderlines defined so far by the isoprene rule. Here, we exemplarily demonstrate the possibilities of this approach by synthesizing a variety of methylated carotenoids in engineered *E. coli* cells.

RESULTS AND DISCUSSION

Identification and Characterization of an IPP Methyltransferase from *Streptomyces monomycini*. We started out looking for enzymes capable of generating new terpene building blocks. For this purpose, the sequence of the above-mentioned GPP methyltransferase from *Streptomyces coelicolor* was used as query on a BLAST search for genes encoding related methyltransferases. A gene in an operon of *S. monomycini* attracted our attention, as the operon not only encoded a putative terpene synthase, but also a putative prenyltransferase (Figure 3a), which might be responsible for further condensation of 2-methyl-GPP with another IPP unit. On the search for the natural product of this pathway, we analyzed compounds produced by the strain via headspace SPME-GC−MS analysis. Although several terpenes could be identified, feeding experiments with ^{13}C-labeled methionine did not reveal the synthesis of a terpenoid compound

DOI: 10.1021/acssynbio.8b00525
ACS Synth. Biol. 2019, 8, 1303−1313

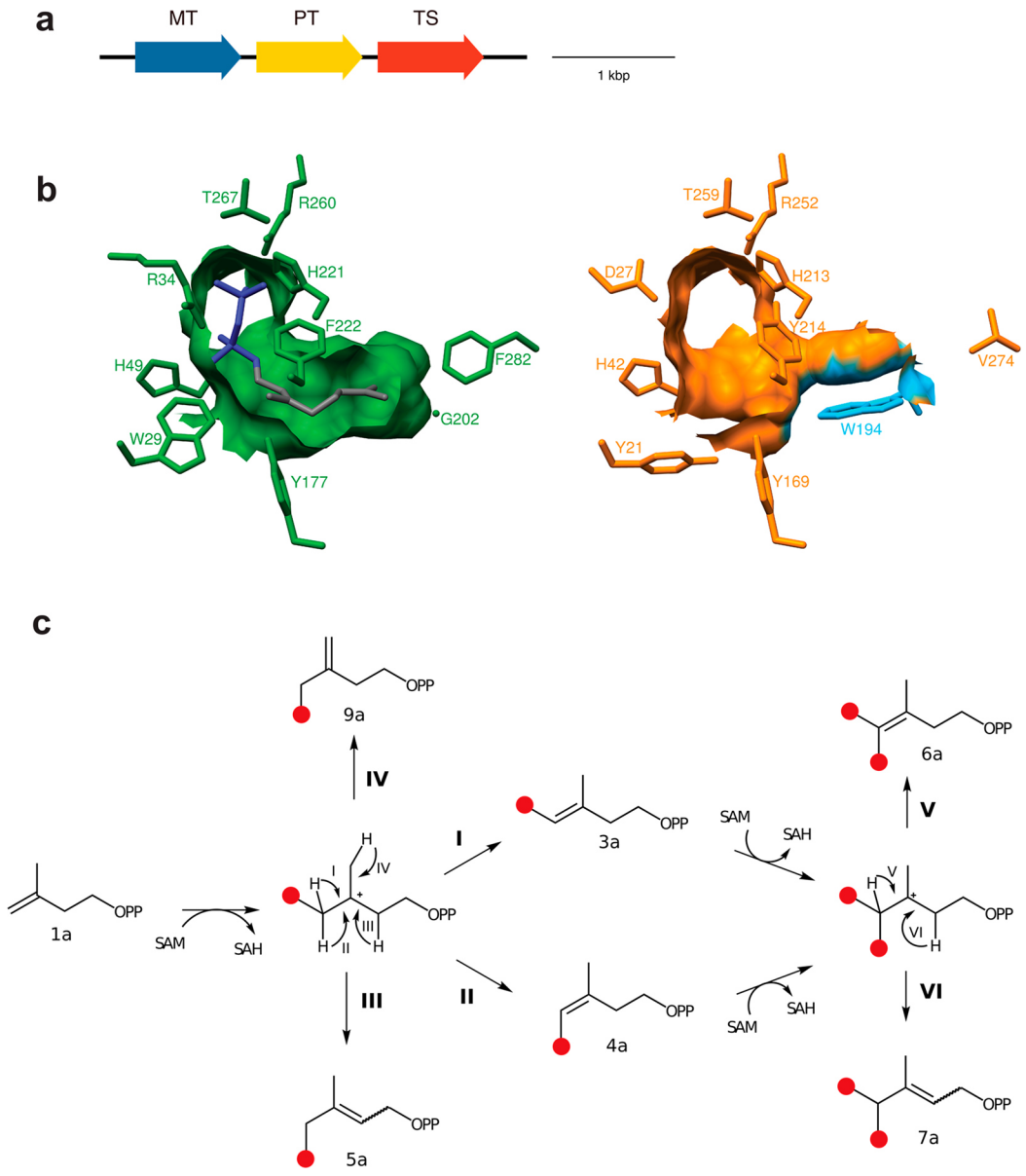


Figure 3. Identification and characterization of an IPP methyltransferase. (a) Biosynthesis gene cluster identified in *S. monomycini*, encoding a methyltransferase (MT), a prenyltransferase (PT), and a terpene synthase (TS). (b) Comparison of substrate binding pockets of prenyl pyrophosphate methyltransferases from *S. coelicolor* (left image, utilizing GPP as substrate shown in gray) and *S. monomycini* (right image). The W194 residue marked in light blue is responsible for a smaller pocket in the *S. monomycini* protein. (c) The proposed reaction pathway of the IPP methyltransferase proceeds via proton abstraction from the initially formed methylated carbocation intermediates leading to the main product **3a** by deprotonation at carbon atom 4 (deprotonation pathway I). 5-Methyl-IPP (**9a**) could only be detected in *in vivo* experiments and might be formed during the IPP-methyltransferase-catalyzed reaction by deprotonation at carbon atom 5 or by a reaction catalyzed by an endogenous *E. coli* enzyme. Dots indicate the transferred methyl groups.

containing an additional methyl group (Figure S1 and Note S1). To investigate the substrate binding site of the *S. monomycini* methyltransferase, a homology-based structure model was created using the crystal structure of the GPP methyltransferase from *Streptomyces coelicolor*. The comparative analysis of both proteins identified a smaller active site in the *S. monomycini* enzyme (Figure 3b), pointing to a C_5 prenyl pyrophosphate as physiological substrate instead of GPP.

To elucidate its catalytic function, we isolated the methyltransferase after expression in *E. coli* and used it in a series of *in vitro* assays. We found the MTase enzyme to accept only IPP as substrate, for which a number of different conversion products were identified in the form of corresponding alcohols after incubation and subsequent dephosphorylation with phosphatase (Table 1 and Figures 3c, 4a,b). The main product after a reaction time of 20 h is the C_6 compound (*E*)-4-methyl-IPP (**3a**), but appearance of the minor product (*Z*)-4-methyl-IPP (**4a**) was also observed. Furthermore, 4-methyl-DMAPP (**5a**) as another C_6 compound besides the two 4-methyl-IPP isomers was also formed. The formation of **5a**, together with the two main products **3a** and **4a**, indicates the reaction proceeds via a methyl-group transfer from SAM followed by one of three different proton abstraction possibilities from the carbocation intermediate (Figure 3c). Such a two-step mechanism has been proposed for the GPP methyltransferase reaction,[21,22] for which, however,

DOI: 10.1021/acssynbio.8b00525
ACS Synth. Biol. 2019, 8, 1303−1313

Table 1. Conversion of IPP (1a) into Mono- and Dimethylated IPP Derivatives by the *S. monomycini* IPP Methyltransferase *in Vitro* Using SAM as Cofactor[a]

prenyl pyrophosphate		dephosphorylated form		relative product amounts 20 h	relative product amounts 40 h
3a	(*E*)-4-methyl-IPP	**3b**	(*E*)-4-methyl-isoprenol	86.6 ± 8.4	0
4a	(*Z*)-4-methyl-IPP	**4b**	(*Z*)-4-methyl-isoprenol	2.9 ± 1.2	0
5a	4-methyl-DMAPP	**5b**	4-methyl-prenol	10.5 ± 7.5	17.0 ± 0.5
6a	4,4-dimethyl-IPP	**6b**	4,4-dimethyl-isoprenol	0	80.0 ± 4.2
7a	4,4-dimethyl-DMAPP	**7b**	4,4-dimethyl-prenol	0	3.0 ± 1.7

[a]Products were identified by comparison with reference compounds via GC−MS (Figures S2−S7) and product amounts are given as relative peak area ratios.

only one product could be identified.[12,23,24] Surprisingly, after an incubation time of 40 h, compounds containing seven carbon atoms were observed: 4,4-dimethyl-IPP (**6a**) and 4,4-dimethyl-DMAPP (**7a**) (Table 1 and Figure 4a,b). These C_7 structures must be formed by a second methylation of the monomethylated products **3a** and **4a** (Figure 3c). This is possible due to the remaining double bond between carbons 3 and 4, which enables the insertion of an additional methyl group attached to carbon 4. Within the second methylation reaction, again a carbocation can be formed at carbon 3, which is then quenched through deprotonation at adjacent carbon atoms. With deprotonation at carbon 4, compound **6a** is formed, whereas deprotonation at carbon 2 leads to release of **7a** (Figure 3c). An additional peak of an unknown structure was observed during *in vitro* assays, which seems to be specific for this reaction and might correspond to a third isomer (**8**) from double methylated IPP (see mass spectrum in Figure S8). The relative enrichment of **5a** after 40 h indicates that this intermediate is not accepted as substrate for a second methylation (Table 1 and Figure 4a,b).

The appearance of methylated IPP or DMAPP derivatives in secondary metabolite pathways is not exclusive for *S. monomycini*, but probably widespread at least in actinobacteria, showing that this kind of molecules may play important biological roles. Many protein sequences with identities higher than 45% were analyzed with respect to the amino acid at the position that is equivalent to W194 in the *S. monomycini* protein (Figure S20). Different protein sequences that contain a tryptophan at the respective position were identified, and we propose a C_5 prenyl pyrophosphate methyltransferase activity for all of them. The enzyme sequence from *Streptomyces argenteolus* (BAF98640) is contained in the operon encoding the biosynthetic pathway of longestin and was recently shown to possess IPP methyltransferase activity.[19] It was found to synthesize only (*Z*)-4-methyl-IPP, which is proposed to be incorporated at two positions of a dimethyl-GGPP by a prenyltransferase also encoded in the respective operon. The two methyltransferases encoded in a cytokinin biosynthesis operon of *Rhodococcus fascians* (WP_037174549 and WP_037174547) have been shown to convert IPP into a dimethylated DMAPP intermediate in a concerted action, although the exact biochemical functions must be clarified.[18] Moreover, the occurrence of these noncanonical prenyl pyrophosphates is not restricted to microorganisms. 4-Methyl-IPP, 4-methyl-DMAPP, and 5-methyl-IPP are also intermediates in terpenoid biosynthesis pathways of certain insects and a number of different linear and cyclic mono- and sesquiterpenoids containing additional methyl groups have been structurally elucidated.[20] The possibility of methyltransferase participations in the respective pathways has been excluded.[25−27] Instead, the C_6 prenyl pyrophosphates are formed from propionyl-CoA and acetyl-CoA via a pathway analogous to the mevalonate pathway involving the intermediate homomevalonate.[20]

***In Vivo* Production of Noncanonical Prenyl Pyrophosphates by the IPP Methyltransferase.** After successfully demonstrating the enzymatic formation of different C_6 and C_7 prenyl pyrophosphates *in vitro*, we aimed at an implementation of this synthesis route in a cellular environment. Therefore, we expressed the *S. monomycini* IPP methyltransferase in *E. coli* cells, which were engineered toward high IPP and DMAPP production. The headspace of the culture was analyzed via SPME-GC−MS. Using this setup we were able to verify the *in vivo* synthesis of all C_6 and C_7 compounds also produced in the *in vitro* reaction (Figure 4c). Moreover, we identified 5-methyl-IPP (**9a**) through the release of the alcohol 5-methyl-isoprenol (**9b**), in whose biosynthesis an isomerization reaction catalyzed by an endogenous *E. coli* enzyme might be involved. However, this compound could also be formed through deprotonation at carbon 5 of the carbocation intermediate in the IPP methyltransferase, which would result in the release of **9a** (deprotonation pathway IV in Figure 3c) even though we could not identify **9b** in the *in vitro* reactions.

After establishing the efficient *in vivo* production of the noncanonical C_6 and C_7 prenyl pyrophosphates, we asked the question, if these building blocks are also incorporated into longer prenyl pyrophosphates via prenyltransferase-catalyzed reactions. This would allow the generation of a multitude of different terpenoid building blocks of different length. The synthesis of 4-methyl-GPP (**10a**) and 4-methyl-FPP (**11a**), which we identified by detection of the corresponding alcohol derivatives 4-methyl-geraniol (**10b**) and 4-methyl-farnesol (**11b**) (Figure 4d), must be the result of endogenous *E. coli* FPP synthase-catalyzed[28] 4-methyl-IPP transfer to DMAPP and GPP, respectively (Figure 4e). Synthesis of 8-methyl-GPP (**12a**), demonstrated by the detection of 8-methyl-geraniol, (**12b**, Figure 4d), furthermore indicates the incorporation of 4-methyl-DMAPP (Figure 4e).

To identify which peaks from the chromatogram corresponded to methylated products, we performed a feeding experiment with methyl-^{13}C labeled methionine. Using this approach, we were able to identify each methyltransferase-catalyzed incorporation of methyl groups by comparing mass spectra from cultures fed with unlabeled methionine or ^{13}C-labeled methionine. Thus, the presence of not only C_{11} and C_{16}, but also C_{12} (**13**) and C_{17} (**14**) terpenoids (Figures S9−S17) within the respective *E. coli* cultures could be unambiguously proven. A generally high substrate promiscuity could be demonstrated already for different prenyltransferases by replacement of their physiological substrates by methylated derivatives.[29] We expected a high specificity of the prenyltransferase encoded in the respective *S. monomycini* operon for 4-methyl-IPP as substrate. However, coexpression of the IPP methyltransferase and the prenyltransferase from *S. monomycini* in *E. coli* cells did not result in higher amounts of

DOI: 10.1021/acssynbio.8b00525
ACS Synth. Biol. 2019, 8, 1303−1313

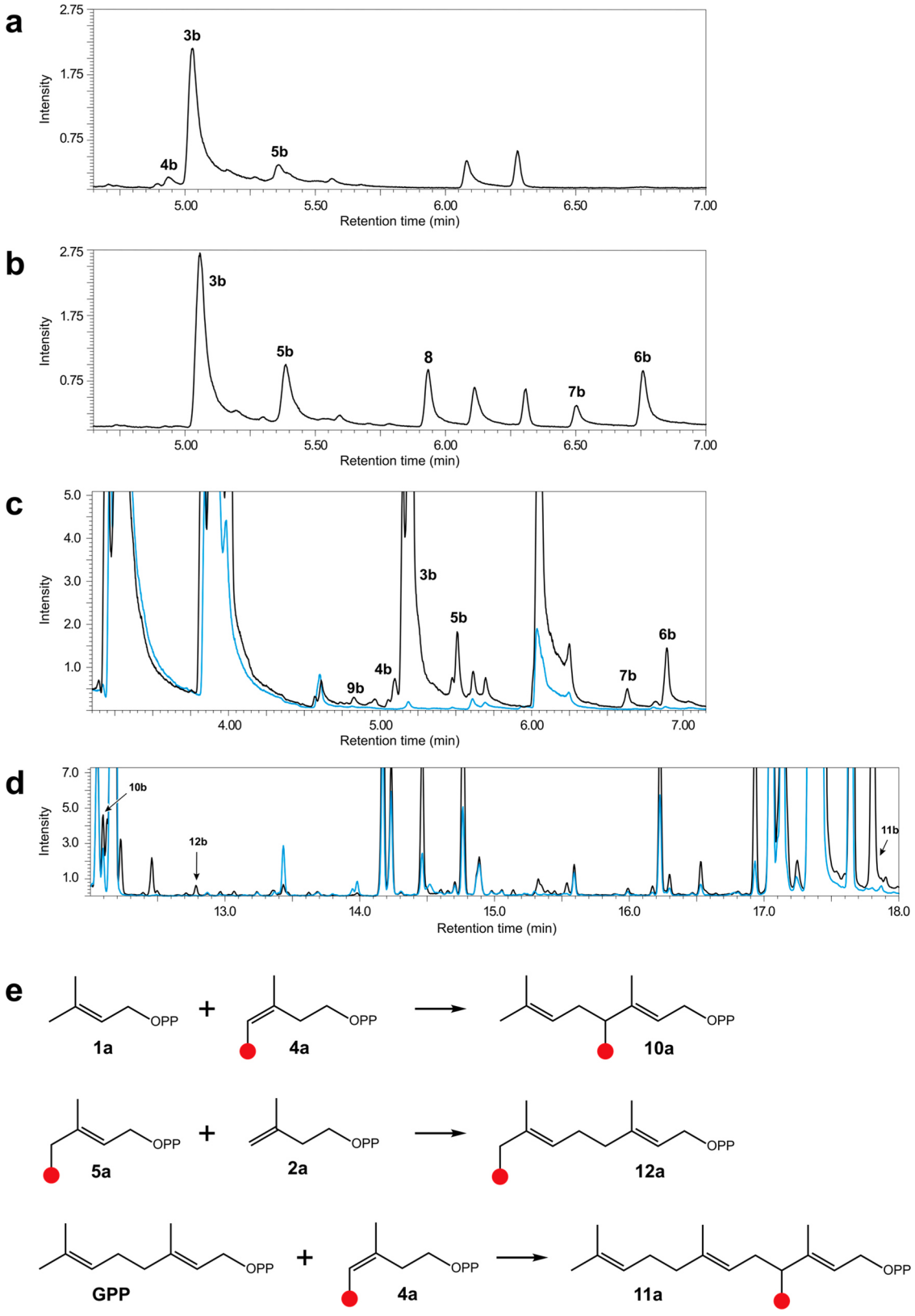


Figure 4. (a,b) Product analysis after *in vitro* incubation of *S. monomycini* IPP methyltransferase with IPP and SAM. Volatile products of the reaction were analyzed via SPME-GC−MS after an incubation time of 20 h (a) and 40 h (b) as described in the methods section. The methyltransferase reaction was followed by a phosphatase treatment to dephosphorylate the prenyl pyrophosphate compounds. The chromatograms are representative of three independent experiments. Numbered peaks correspond to identified compounds: (**4b**) (*Z*)-4-methyl-isoprenol; (**3b**) (*E*)-4-methyl-isoprenol; (**5b**) 4-methyl-prenol; (**7b**) 4,4-dimethyl-prenol; (**6b**) 4,4-dimethyl-isoprenol; (**8**) C_7 compound of unknown structure. Products were identified by comparison of the obtained mass spectra with mass spectra and retention time of reference compounds (Figures S2−S7). (c) *In vivo* synthesis of noncanonical prenyl pyrophosphates. Production of noncanonical C_6 and C_7 prenyl alcohols

DOI: 10.1021/acssynbio.8b00525
ACS Synth. Biol. 2019, 8, 1303−1313

Figure 4. continued

as products of *E. coli* cells expressing the *S. monomycini* IPP methyltransferase. Blue line refers to empty vector control. The alcohol derivatives of respective prenyl pyrophosphates were detected by headspace-SPME-GC−MS analyses, and products were identified by comparison of the obtained mass spectra with mass spectra and retention time of reference compounds (Figures S2−S7). (d) Identification of C_{11}, and C_{16} isoprenoid intermediates in *E. coli* cells expressing the *S. monomycini* IPP methyltransferase. Total ion chromatogram of *in vivo* SBSE-extracted medium sample of strain *E. coli* MG1655 Δ*endA* Δ*recA* (DE3) containing plasmids pLD-03 and pMK-17. Blue line refers to empty vector control. Arrows indicate peaks corresponding to compounds 4-methyl-geraniol (**10b**); 8-methyl-geraniol (**12b**); 4-methyl-farnesol (**11b**). (e) Schematic presentation of proposed biosynthetic pathways. 4-Methyl-GPP (**10a**), 8-methyl-GPP (**12a**) and 4-methyl-FPP (**11a**) are formed by incorporation of 4-methyl-IPP or 4-methyl-DMAPP during prenyl transferase-catalyzed elongation of the prenyl chain. Red dots indicate the transferred methyl groups.

certain noncanonical prenyl pyrophosphates if compared to the strain expressing only the methyltransferase (data not shown).

Interestingly, a chiral center is created during elongation reactions with 4-methyl-IPP and its configuration depends on the *E*- or *Z*-configuration of the C_6 substrate.[29,30] The introduction of chirality into the so far nonchiral terpenoid building block repertoire adds another level of complexity to terpene biosynthesis pathways.

***In Vivo* Incorporation of Noncanonical Prenyl Pyrophosphates Enables Biosynthesis of New-to-Nature Carotenoids.** Besides the chain-elongating prenyltransferases, also terpene synthases possess a high substrate promiscuity,[5−10] which would be a prerequisite for our structure space expansion strategy via the supply of novel building blocks. Recently, the combination of a bacterial GPP methyltransferase and different monoterpene synthases in yeast cells led to a variety of previously unknown C_{11} terpene structures.[31] To demonstrate the general tolerance of terpenoid biosynthesis pathways toward the methyl-IPP and methyl-DMAPP intermediates and thereby the application possibilities of the IPP methyltransferase, we tested the incorporation of the noncanonical prenyl pyrophosphates into different carotenoids along the pathway to zeaxanthin catalyzed by five different enzymes (Figure S18). The possible methylation sites of a mono- or multimethylated carotenoid are shown in Figure 5a. *E. coli* cells that were engineered to synthesize phytoene and lycopene produced at least three methylated carotenoids containing one (C_{41} derivatives), two (C_{42} derivatives), or three additional methyl groups (C_{43} derivatives), if the IPP methyltransferase was coexpressed (Figure 5b and Table S1). Strains expressing the whole β-carotene or zeaxanthin pathway together with the IPP methyltransferase showed products with one or two additional methyl groups in each case (Figure 5b, further details in Table S1). Due to the possibility of incorporation of different building blocks at different positions, each peak of methylated carotenoids may contain several mono-methyl-carotenoid positional isomers with the methyl groups at any positions of the molecule indicated in Figure 5a. To get further information on the methylation pattern of, for example, the monomethyl β-carotene (peak E-Mb1) the MS−MS fragmentation was analyzed in detail (Figure S19). Figure 5c presents selected MS−MS fragment ions from the monomethyl β-carotene peak of the HPLC trace in Figure 5b. Multiple asterisks in every fragment indicate possible positions of a single additional methyl group. Among the methylated molecular ion **26m** other extra monomethylated fragment ions **14m** and **17m** from the ionone end group were detected. Ion **25n** results from monomethyl β-carotene, but does not contain the additional methyl group and originates from the elimination of trimethylcyclohexene, leaving a remaining C_{32} ion.[17] This indicates an additional methyl group in the polyene chain of one of the C_{41} compounds of the E-Mb1 peak. In contrast, methylated ion **24m** formed during the elimination of xylene exists. In addition, also different fragment ions without any methylation (Figure S19) exist from all regions of the β-carotene molecule in this E-Mb1 fraction. These results demonstrate the promiscuity of the carotenoid pathway for slightly modified substrates. The possibility to synthesize many different slighty modified derivatives of known functional terpenoids can simplify the search for molecules with improved properties, for example, in the flavor and fragrance area. Special attention should be given to pharma-relevant compounds, as additional methyl groups often modify binding affinity and selectivity to the pharmacological target, side activities or half-life time, a phenomenon described as "magic methyl effect".[32,33]

In summary, we identified and characterized a methyltransferase converting IPP into a variety of C_6 and C_7 prenyl pyrophosphates and demonstrated its application for biotechnological terpene production approaches. The development of two completely different pathways leading to the same C_6 building blocks in bacteria and insects illustrates the importance of such terpenoid structure variation. It is known that terpenes are important compounds for chemical interactions between organisms, but the exact physiological function of the different noncanonical terpenes is still to be unraveled. As the noncanonical terpenoid building blocks formed by the *S. monomycini* enzyme are accepted as substrates by the *E. coli* FPP synthase, implementation of the enzyme created biosynthetic routes toward different C_{11}, C_{12}, C_{16}, and C_{17} prenyl pyrophosphates. Our synthetic biology approach combining the IPP methyltransferase with carotenoid biosynthesis pathways led to the generation of a variety of C_{41}, C_{42}, and C_{43} carotenoids, which exemplifies the structural plasticity of terpenoid biosynthesis toward the implementation of additional methyl groups. The application of this and other prenyl pyrophosphate methyltransferases will enable an enormous expansion of the terpenoid structure space beyond the borders predetermined by the isoprene rule, enabling the formation of innumerous so far inaccessible structures.

MATERIALS AND METHODS

Bioinformatic Analyses. The protein sequence of the *S. coelicolor* GPP methyltransferase (NP_631739.1) was used as query for a BLAST search using the NCBI nonredundant protein database. The operon structures of resulting methyltransferase sequences were screened for prenyltransferase-encoding genes, which yielded the *S. monomycini* terpene biosynthesis operon shown in Figure 1a.

Protein Model Generation. A protein model of the prenyl pyrophosphate methyltransferase of *S. monomycini* was built with SWISS-MODEL (ProMod version 3.70).[34−36] The crystal structure of the GPP-methyltransferase of *S. coelicolor*

DOI: 10.1021/acssynbio.8b00525
ACS Synth. Biol. 2019, 8, 1303−1313

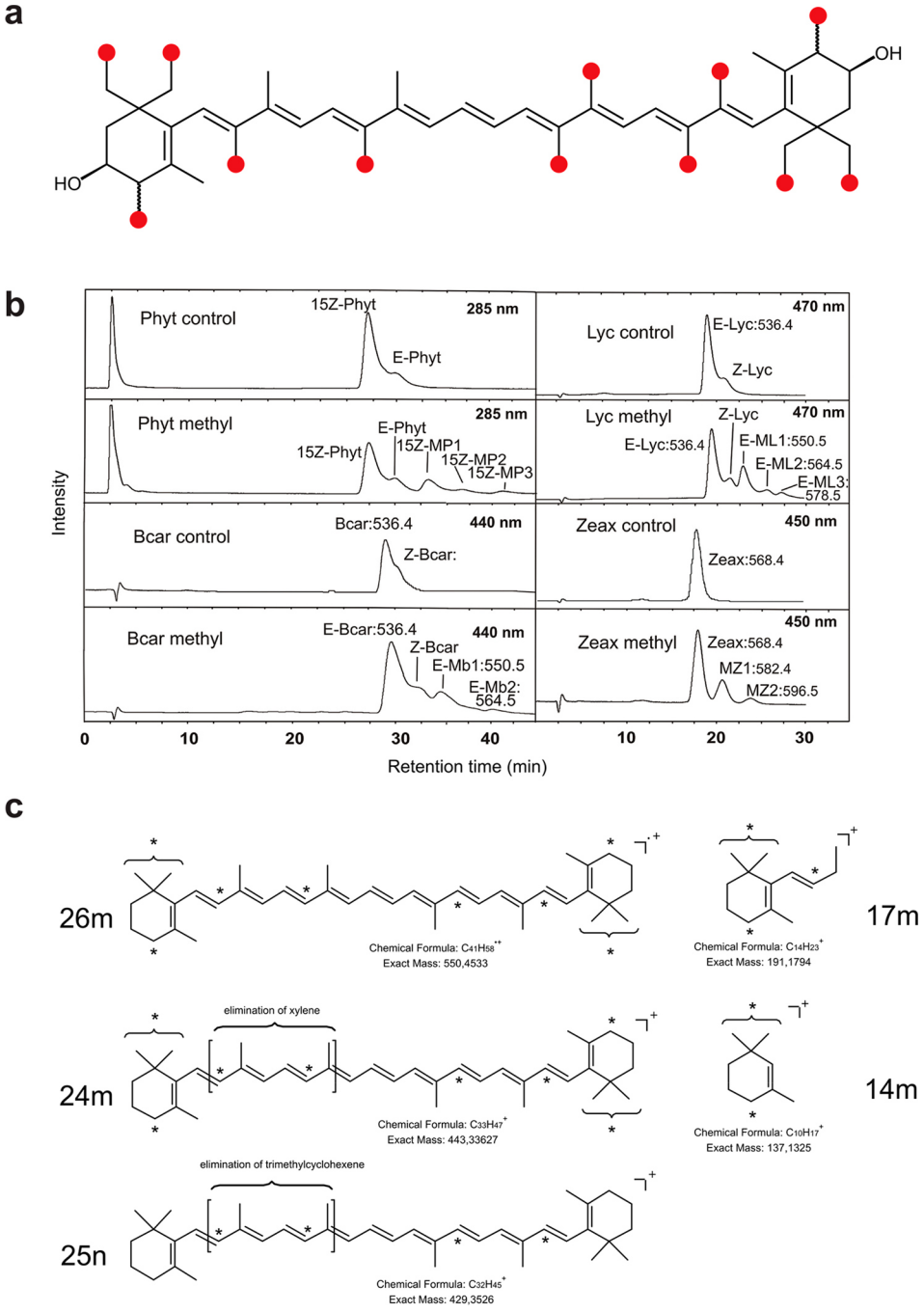


Figure 5. Formation of methylated carotenoids from noncanonical prenyl pyrophosphates in *Escherichia coli*. (a) Possible modifications of zeaxanthin caused by incorporation of 4-methyl prenyl pyrophosphates during carotenoid biosynthesis. Red circles indicate possible sites for methylation at earlier steps of the pathway from incorporated 4-methyl-IPP or 4-methyl-DMAPP. The wavy bond indicates a chiral center. (b) HPLC separation of mono-, di-, and trimethylated carotenoids produced with *Escherichia coli* strains. Genes of the carotenoid biosynthesis pathways were expressed sequentially to yield phytoene, lycopene, β-carotene, or zeaxanthin as controls and by coexpression of the IPP methyltransferase gene also their methylated products. Molecular masses are indicated at the peaks; more detailed values and absorbance maxima are given in Supplementary Table 1. (c) Molecular and fragment MS−MS ions from monomethyl β-carotenes of the E-Mb1 peak, selected from Supplementary Figure 19. Owing to the incorporation of 4-methyl prenyl pyrophosphate, the additional methyl group occurs in different regions of the carotenoid molecules. Alternative sites for methyl groups in the β-carotenes are marked with an asterisk.

(PDP ID: 3vc2.1.A) was used as template. Figures of the crystal and model structure were created using UCSF Chimera.[37]

Chemicals, Media, and Bacterial Strains. *E. coli* strain DH5α (New England Biolabs) was used for plasmid construction. *E. coli* MG1655 (*E. coli* MG1655 Δ*endA* Δ*recA* (DE3)),[38] constructed in the lab of Kristala Prather (Addgene #37854) was used for prenyl alcohol production experiments. *E. coli* BL21(DE3) (Novagen) was used for His-tag protein expression and carotenoid production.

E. coli strains DH5α, MG1655, and BL21 were grown in LB medium,[39] 2x YT medium,[40] with 2% glycerol, or TB medium[40] containing the appropriate antibiotics (ampicillin at 100 μg/L and kanamycin at 50 μg/L), at 37 °C and 180

DOI: 10.1021/acssynbio.8b00525
ACS Synth. Biol. 2019, 8, 1303−1313

rpm. Solid medium was prepared by the addition of 1.5% agar−agar (m/v). *Streptomyces monomycini* DSM 41801 was obtained from DSMZ (Leibniz Institut—German Collection of Microorganisms and Cell Cultures, Braunschweig). The strain was grown in GYM medium (glucose 4.0 g/L, yeast extract 4.0 g/L, malt extract 10.0 g/L, pH 7.2) as liquid culture. Agar plates were made by adding 1.2% agar−agar (m/v) to GYM medium.

For protein purification, buffers with adapted composition[24,41] were prepared. Assay buffer (pH 6.7) consisted of PIPES 50 mM, NaCl 100 mM, $MgCl_2$15 mM, and DTT 5 mM in ultrapure water. Lysis, wash, and elution buffers had a pH of 7.4 and consisted of $NaH_2PO_4$50 mM, NaCl 300 mM, DTT 5 mM with increasing concentrations of imidazol through each step: 10 mM (lysis), 30 mM (wash), and 250 mM (elution).

Glycerol, PIPES, NaCl, Imidazol, NaH_2PO_4 ampicillin and kanamycin were purchased from Carl Roth GmbH. Solid-phase microextraction (SPME) fibers, DTT, acid phosphatase, IPP, *S*-adenosyl-methionine, RNase, DNase, and protease inhibitor were purchased from Sigma-Aldrich. Reference compounds 5-methyl-isoprenol, 4-methyl-prenol, 4,4-dimethyl-prenol, 4,4-dimethyl-isoprenol, 4-methyl-geraniol, 8-methyl-geraniol, and 4-methyl-farnesol were synthesized by Enamine (Riga, Latvia); 4-methyl-isoprenol with *Z* or *E* configuration were synthesized by Akos (Steinen, Germany). All reference compounds were more than 95% pure and had their identities confirmed by NMR and GC−MS.

Plasmid Construction. The plasmid pJBEI-6409, constructed in the lab of Taek Soon Lee (Addgene plasmid no. 47048), contains genes of the mevalonate pathway, of an IPP isomerase, of a GPP synthase, and of a limonene synthase[42] and it was used to construct a plasmid for provision of high IPP and DMAPP levels.

The constructions of plasmids were done via Gibson assembly[43] or standard restriction-cloning. Ribosomal binding sites (RBS) were designed with the RBS calculator v1.1.[44,45]pMK-17 including the gene of the IPP-methyltransferase (accession number WP_033037353) and a new RBS with the sequence TAAGATTAAATAAGGAGGTTACCA and a maximal translation ignition rate (TIR) of 399634 was constructed via Gibson assembly. The insert was amplified from the genomic DNA of *S. monomycini* (DSM No. 41801) via PCR with the primers mk34 and mk36. The backbone was amplified from pETDuet-1 via PCR with the primers mk32 and mk33.

For the construction of pLD-03 the antibiotic resistance gene of pJBEI-6409 was exchanged to a kanamycin resistance gene via Gibson Assembly. The insert was amplified via PCR from pET28a(+) with the primers mk42 and mk44. The backbone was amplified via PCR from pJBEI-6409 with the primers mk20 and mk23. To delete the genes of the limonene-synthase and GPP-synthase from the assembled product, a PCR with the primers mk8 and mk31, a restriction digest with *Bam*HI and self-ligation was done.

For construction of pMK-24 the gene of the IPP-methyltransferase (SEQ ID no. 1) was amplified via PCR with the primers mk52 and mk53 and pMK-17 as template. The PCR product and the vector pET28a(+) were digested with NdeI and *Bam*HI. The digested fragments were then ligated. Successful construction of the plasmid constructs was validated by Sanger sequencing.

Production of Alkylated Isoprenoids. *E. coli* strain MG1655 was transformed with pLD-03 and pMK-17. Main cultures with 15 mL of 2xYT medium in 100 mL of baffled shake flasks were inoculated from LB preculture to an OD_{600} of 0.1. Where indicated, ^{13}C-methyl-L-methionine or L-methionine was added to the medium at 3 g/L. After cultivation for 2 h at 37 °C (OD_{600} of 1.0), IPTG was added to yield a final concentration of 100 μM for induction. Induced cultures had terpenes extracted after 24 h of incubation at 30 °C and 180 rpm with exposure of SPME fiber on the headspace for 30 min or SBSE devices in the medium during the cultivation for 24 h.

Carotenoid production was carried out in *E. coli* BL21-DE3 using plasmids pACCRT-EB for phytoene, pACCRT-EBI for lycopene, pACCAR16ΔcrtX for β-carotene, and pAC-Car25ΔcrtX for zeaxanthin as controls[46] and each one in combination with the IPP methyl transferase gene carrying plasmid pMK-17. Cells were grown for 48 h in LB medium at 28 °C with added antibiotics ampicillin (100 mg/mL), kanamycin (34 mg/mL), and chloramphenicol (34 mg/mL), according to their plasmids.

S. monomycini 15 mL main cultures were inoculated with cells from GYM-agar plates. Liquid cultures were incubated at 28 °C and shaken at 220 rpm. The analysis of volatiles was made between days four and six of the cultivation cycle, with exposure of SPME fiber to culture headspace for 24 h.

Enzyme Expression and Purification. *E. coli* strain BL21 (DE3) was transformed with pMK-24. LB precultures were used to inoculate 400 mL of TB medium main cultures. After the cells were cultivated for 2 h at 37 °C (OD_{600} of 0.6), IPTG was added to yield a final concentration of 100 μM for induction. After cultivation at 18 °C, 110 rpm overnight, cells were harvested by centrifugation for 20 min at 4000*g* and 4 °C followed by resuspension in 15 mL of lysis buffer. Ultrasound sonication was performed on ice with 20% amplitude (0.5 s pulse, 1 s pause, 4 min pulse time), followed by digestion with RNase (10 μg/mL) and DNase (5 μg/mL) on ice for 15 min. After centrifugation for 20 min at 4000*g* and 4 °C, enzymes were purified using His-Pur Ni-NTA Spin Columns (Thermo Fischer Scientific) according to the manufacturer's recommendations using buffers described above. The size of the purified protein was confirmed through SDS-PAGE (Figure S21).

***In Vitro* Incubations.** Purified protein at 25 μM was added to 1 mL of assay buffer containing 60 μM of IPP and 120 μM SAM. The enzymatic reaction was incubated at 30 °C overnight. Then 1 mL of 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 SPME fiber for 10 min.

HS-SPME-GC/MS Analysis. Volatile compounds in the headspace of each assay and culture were analyzed by extraction with an 85 μm SPME composed of PDMS and Carboxen (Stableflex). The SPME fiber 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 min sampling time, and column flow of 1.1 mL/min. The column temperature was programmed as follows: 40 °C for 1.5 min, 10 °C/min until 250 °C, and then 20 °C/min until 300 °C.

Compounds were identified via comparison of mass spectra and retention indexes (RI) to the ones of reference substances

DOI: 10.1021/acssynbio.8b00525
ACS Synth. Biol. 2019, 8, 1303−1313

or mass spectra of the NIST mass spectral library (v14) and RIs published elsewhere.[47]

SBSE GC-MS Analysis. The extraction of less volatile terpenoids was made with a twister (10 × 0.5 mm PDMS; Gerstel, Mühlheim an der Ruhr, Germany) submerged in the culture. After cultivation, the twister was placed in a Thermal Desorption Unit (TDU) glass liner and desorbed at 250 °C for 15 min. This glass liner mates directly with the glass inlet liner of the cooled injection system (CIS) in which the analytes were trapped by cooling to −100 °C. The CIS was rapidly heated to 250 °C and analytes were transferred onto the GC column with a split of 1:100.

Carotenoid Analysis. Carotenoids were extracted from freeze-dried *E. coli* cells with methanol containing 6% KOH for 15 min at 60 °C and partitioned into 30% ether in petrol (bp 40−60 °C). Initial HPLC analysis was performed first on a 25 × 0.4 cm Nucleosil 100 C18, 3 μm column and as mobile phase acetonitrile (ACN)/methanol/2-propanol (85:10:5, by volume) plus variable amounts of H_2O, none for the separation of phytoene, lycopene, and β-carotene, and 7% for zeaxanthin. A flow rate of 0.8 mL/min at 10 °C was used. Carotenoid identification by HPLC was carried out with authentic standards generated in *E. coli* by the combination of different crt genes as previously described.[48]

UPLC-ESI-HRMS/MS analysis was performed with an UltiMate 3000 system (Thermo Fisher) coupled to an Impact II qToF mass spectrometer (Bruker) with solvent A, MeCN/methanol (70:30, v/v) + 0.1% formic acid, and solvent B, H_2O 100% + 0.1% formic acid using the following gradient: 0−2 min 85% A, 2−3 min 100% A, hold to 14 min, 14−15 min 85% A, flow 0.8 μL/min, 40 °C column temperature. Separation was carried out on a 2.1 mm × 50 mm ACQUITY UPLC BEH C18, 1.7 μm column (Waters). The Impact II qTof mass spectrometer was initially calibrated using 10 mM sodium formate solution prior to data acquisition in positive measurement mode from m/z 100−1200. The MS acquisition method was split into two segments: (1) 10 mM sodium formate solution was injected and served as an internal calibrant for mass data acquired in the subsequent segment, and (2) analytes of UPLC separation were detected. The following MS settings were used for data acquisition. Source settings: capillary voltage, 4500 V; nebulizer gas pressure (nitrogen), 3 bar; ion source temperature, 200 °C; dry gas flow of 8 L min^{-1}. General scan settings: ion polarity positive; mass range, 100−2000 m/z; spectra rate, 3 Hz (MS and MS/MS). Tune parameters: transfer funnel, 1 RF 300 Vpp; funnel 2 RF, 300 Vpp; isCID off; hexapole RF, 60 Vpp. MS/MS settings: mass corresponding to analyzed carotenoid analogue included; threshold, 1000 cts (absolute); activated active exclusion after three spectra and 0.5 min release time; active precursor reconsidering factor, 4; smart exclusion 2 times; stepping 40%−120% normalized collisional energy.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.8b00525.

Plasmids, primers, and bacterial strains used in this study, as well as chromatography and mass spectrometry data (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: buchhaupt@dechema.de.

ORCID

Yi-Ming Shi: 0000-0001-6933-4971
Jens Schrader: 0000-0003-2235-6553
Helge B. Bode: 0000-0001-6048-5909
Markus Buchhaupt: 0000-0003-2720-5973

Author Contributions

#These authors contributed equally.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work is dedicated to Hendrik Wolff, who did excellent work in the analysis of unusual carotenoid structures and unfortunately passed away in 2018. The authors would like to thank Tobias Trapp, Marco Fraatz, and Holger Zorn from Justus Liebig University Giessen for helping with SSBE analyses and Taek Soon Lee for providing the plasmid pJBEI-6409. This work was supported by the German Federal Ministry of Food and Agriculture and Fachagentur Nachwachsende Rohstoffe e.V. (FKZ 22008713), by the LOEWE project AROMA plus of the State of Hessen and by the Brazilian National Council for Scientific and Technological Development (CNPq) with a scholarship for L.D.

■ REFERENCES

(1) Buckingham, J. *Dictionary of Natural Products*, version 26.1, Chapman Hall/CRC Press, London.

(2) Wallach, O. (1887) Zur Kenntniss der Terpene und ätherischen Oele - 5. Abhandlung. *Justus Liebigs Ann. Chem. 238*, 78−89.

(3) Ruzicka, L. (1953) The isoprene rule and the biogenesis of terpenic compounds. *Experientia 9*, 357−367.

(4) Nagaki, M., Yamamoto, H., Takahashi, A., Maki, Y., Ishibashi, J., Nishino, T., and Koyama, T. (2002) Substrate specificity of thermostable farnesyl diphosphate synthase with respect to 4-alkyl group homologs of isopentenyl diphosphate. *J. Mol. Catal. B: Enzym. 17*, 81−89.

(5) Miller, D. J., Yu, F., and Allemann, R. K. (2007) Aristolochene synthase-catalyzed cyclization of 2-fluorofarnesyl- diphosphate to 2-fluorogermacrene A. *ChemBioChem 8*, 1819−1825.

(6) Faraldos, J. A., Zhao, Y., O'Maille, P. E., Noel, J. P., and Coates, R. M. (2007) Interception of the enzymatic conversion of farnesyl diphosphate to 5-epi-aristolochene by using a fluoro substrate analogue: 1-Fluorogermacrene A from (2E,6Z)-6-fluorofarnesyl diphosphate. *ChemBioChem 8*, 1826−1833.

(7) Vedula, L. S., Zhao, Y., Coates, R. M., Koyama, T., Cane, D. E., and Christianson, D. W. (2007) Exploring biosynthetic diversity with trichodiene synthase. *Arch. Biochem. Biophys. 466*, 260−266.

(8) Subramanian, T., Pais, J. E., Liu, S., Troutman, J. M., Suzuki, Y., Leela Subramanian, K., Fierke, C. A., Andres, D. A., and Spielmann, H. P. (2012) Farnesyl diphosphate analogues with Aryl moieties are efficient alternate substrates for protein farnesyltransferase. *Biochemistry 51*, 8307−8319.

(9) Rising, K. A., Crenshaw, C. M., Koo, H. J., Subramanian, T., Chehade, K. A. H., Starks, C., Allen, K. D., Andres, D. A., Spielmann, H. P., Noel, J. P., and Chappell, J. (2015) Formation of a Novel Macrocyclic Alkaloid from the Unnatural Farnesyl Diphosphate Analogue Anilinogeranyl Diphosphate by 5-Epi-Aristolochene Synthase. *ACS Chem. Biol. 10*, 1729−1736.

(10) Demiray, M., Tang, X., Wirth, T., Faraldos, J. A., and Allemann, R. K. (2017) An Efficient Chemoenzymatic Synthesis of Dihydroartemisinic Aldehyde. *Angew. Chem., Int. Ed. 56*, 4347−4350.

DOI: 10.1021/acssynbio.8b00525
ACS Synth. Biol. 2019, 8, 1303−1313

(11) Dickschat, J. S., Nawrath, T., Thiel, V., Kunze, B., Müller, R., and Schulz, S. (2007) Biosynthesis of the off-flavor 2-methylisoborneol by the myxobacterium Nannocystis exedens. *Angew. Chem., Int. Ed. 46*, 8287−8290.
(12) Komatsu, M., Tsuda, M., Omura, S., Oikawa, H., and Ikeda, H. (2008) Identification and functional analysis of genes controlling biosynthesis of 2-methylisoborneol. *Proc. Natl. Acad. Sci. U. S. A. 105*, 7422−7427.
(13) Wang, C. M., and Cane, D. E. (2008) Biochemistry and molecular genetics of the biosynthesis of the earthy odorant methylisoborneol in Streptomyces coelicolor. *J. Am. Chem. Soc. 130*, 8908−8909.
(14) Rabe, P., Citron, C. A., and Dickschat, J. S. (2013) Volatile terpenes from actinomycetes: A biosynthetic study correlating chemical analyses to genome data. *ChemBioChem 14*, 2345−2354.
(15) Kschowak, M. J., Wortmann, H., Dickschat, J. S., Schrader, J., and Buchhaupt, M. (2018) Heterologous expression of 2-methylisoborneol/2 methylenebornane biosynthesis genes in Escherichia coli yields novel C11-terpenes. *PLoS One 13*, No. e0196082.
(16) Ignea, C., Pontini, M., Motawia, M. S., Maffei, M. E., Makris, A. M., and Kampranis, S. C. (2018) Synthesis of 11-carbon terpenoids in yeast using protein and metabolic engineering. *Nat. Chem. Biol. 14*, 1090−1098.
(17) Von Reuss, S., Domik, D., Lemfack, M. C., Magnus, N., Kai, M., Weise, T., and Piechulla, B. (2018) Sodorifen Biosynthesis in the Rhizobacterium Serratia plymuthica Involves Methylation and Cyclization of MEP-Derived Farnesyl Pyrophosphate by a SAM-Dependent C-Methyltransferase. *J. Am. Chem. Soc. 140*, 11855−11862.
(18) Radhika, V., Ueda, N., Tsuboi, Y., Kojima, M., Kikuchi, J., Kudo, T., and Sakakibara, H. (2015) Methylated Cytokinins from the Phytopathogen *Rhodococcus fascians* Mimic Plant Hormone Activity. *Plant Physiol. 169*, 1118−1126.
(19) Ozaki, T., Shinde, S. S., Gao, L., Okuizumi, R., Liu, C., Ogasawara, Y., Lei, X., Dairi, T., Minami, A., and Oikawa, H. (2018) Enzymatic formation of a skipped methyl-substituted octaprenyl side chain of longestin (KS-505a): Involvement of homo-IPP as a common extender unit. *Angew. Chem., Int. Ed. 57*, 1−5.
(20) Morgan, E. D. (2007) *Biosynthesis in Insects*, Royal Society of Chemistry. DOI: 10.1039/9781847550262.
(21) Köksal, M., Chou, W. K. W., Cane, D. E., and Christianson, D. W. (2012) Structure of geranyl diphosphate C -methyltransferase from Streptomyces coelicolor and implications for the mechanism of isoprenoid modification. *Biochemistry 51*, 3003−3010.
(22) Ariyawutthiphan, O., Ose, T., Minami, A., Sinde, S., Tsuda, M., Gao, Y. G., Yao, M., Oikawa, H., and Tanaka, I. (2012) Structure analysis of geranyl pyrophosphate methyltransferase and the proposed reaction mechanism of SAM-dependent C-methylation. *Acta Crystallogr., Sect. D: Biol. Crystallogr. 68*, 1558−1569.
(23) Dickschat, J. S., Nawrath, T., Thiel, V., Kunze, B., Müller, R., and Schulz, S. (2007) Biosynthese des Duftstoffes 2-Methylisoborneol durch das Myxobakterium Nannocystis exedens. *Angew. Chem. 119*, 8436−8439.
(24) Wang, C.-M., and Cane, D. E. (2010) Biochemistry and Molecular Genetics of the Biosynthesis of the Earthy Odorant Methylisoborneol in *Streptomyces coelicolor*. *J. Am. Chem. Soc. 132*, 9509−9509.
(25) Peter, M. G., and Dahm, K. H. (1975) Biosynthesis of Juvenile hormone in the Cecropia Moth. Labelling pattern from 1-[14C]-propionate through degradation to single carbon atom derivatives. *Helv. Chim. Acta 58*, 1037−1048.
(26) Jennings, R. C., Judy, K. J., and Schooley, D. A. (1975) Biosynthesis of the homosesquiterpenoid Juvenile Hormone JH II [Methyl (2E,6E, lOZ)-10,11 -epoxy-3,7,1l-trimethyltridecadienoate] from [5−3H] homomevalonate in Manduca Sexta. *J. Chem. Soc., Chem. Commun. 0*, 21−22.
(27) Baker, F. C., and Schooley, D. A. (1981) Biosynthesis of 3-hydroxy-3-methylglutaryl-CoA, 3-hydroxy-3-ethylglutaryl-CoA, mevalonate and homomevalonate by insect corpus allatum and mammalian hepatic tissues. *Biochim. Biophys. Acta, Lipids Lipid Metab. 664*, 356−372.
(28) Fujisaki, S., Nishino, T., and Katsuki, H. (1986) Isoprenoid Purification Synthesis of Four in Escherichia Enzymes Separation and Partial Involved in the Synthesis1 Isopentenyl pyrophosphate phate (UPP) synthetase were partially purified from Escherichia coli by DEAE- FPP synthetase catalyzed the conde. *J. Biochem. 99*, 1327−1337.
(29) Koyama, T., Saito, A., Ogura, K., and Seto, S. (1980) Substrate Specificity of Farnesylpyrophosphate Synthetase. Application to Asymmetric Synthesis. *J. Am. Chem. Soc. 102*, 3614−3618.
(30) Nagaki, M., Ichijo, T., Kobashi, R., Yagihashi, Y., Musashi, T., Kawakami, J., Ohya, N., Gotoh, T., and Sagami, H. (2012) Substrate specificities of E- and Z-farnesyl diphosphate synthases with substrate analogs. *J. Mol. Catal. B: Enzym. 80*, 1−6.
(31) Ignea, C., Pontini, M., Motawia, M. S., Maffei, M. E., Makris, A. M., and Kampranis, S. C. (2018) Synthesis of 11-carbon terpenoids in yeast using protein and metabolic engineering. *Nat. Chem. Biol. 14*, 1090−1098.
(32) Quancard, J., Bollbuck, B., Janser, P., Angst, D., Berst, F., Buehlmayer, P., Streiff, M., Beerli, C., Brinkmann, V., Guerini, D., Smith, P. A., Seabrook, T. J., Traebert, M., Seuwen, K., Hersperger, R., Bruns, C., Bassilana, F., and Bigaud, M. (2012) A potent and selective S1P1antagonist with efficacy in experimental autoimmune encephalomyelitis. *Chem. Biol. 19*, 1142−1151.
(33) Schönherr, H., and Cernak, T. (2013) Profound methyl effects in drug discovery and a call for new C-H methylation reactions. *Angew. Chem., Int. Ed. 52*, 12256−12267.
(34) Biasini, M., Bienert, S., Waterhouse, A., Arnold, K., Studer, G., Schmidt, T., Kiefer, F., Cassarino, T. G., Bertoni, M., Bordoli, L., and Schwede, T. (2014) SWISS-MODEL: Modelling protein tertiary and quaternary structure using evolutionary information. *Nucleic Acids Res. 42*, 252−258.
(35) Arnold, K., Bordoli, L., Kopp, J., and Schwede, T. (2006) The SWISS-MODEL workspace: A web-based environment for protein structure homology modelling. *Bioinformatics 22*, 195−201.
(36) Benkert, P., Biasini, M., and Schwede, T. (2011) Toward the estimation of the absolute quality of individual protein structure models. *Bioinformatics 27*, 343−350.
(37) Pettersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C., and Ferrin, T. E. (2004) UCSF Chimera - A visualization system for exploratory research and analysis. *J. Comput. Chem. 25*, 1605−1612.
(38) Tseng, H.-C., Harwell, C. L., Martin, C. H., and Prather, K. L. (2010) Biosynthesis of chiral 3-hydroxyvalerate from single propionate-unrelated carbon sources in metabolically engineered E. coli. *Microb. Cell Fact. 9*, 96.
(39) Bertani, G. (1951) Studies on lysogenesis. I. The mode of phage liberation by lysogenic Escherichia coli. *J. Bacteriol. 62*, 293−300.
(40) Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. a, Struhl, K., Wiley, C. J., Allison, R. D., Bittner, M., and Blackshaw, S. (Current Protocols in Molecular Biology2003) *Mol. Biol.*, John Wiley & Sons.
(41) Chou, W. K. W., Ikeda, H., and Cane, D. E. (2011) Cloning and characterization of Pfl_1841, a 2-methylenebornane synthase in Pseudomonas fluorescens PfO-1. *Tetrahedron 67*, 6627−6632.
(42) Alonso-Gutierrez, J., Chan, R., Batth, T. S., Adams, P. D., Keasling, J. D., Petzold, C. J., and Lee, T. S. (2013) Metabolic engineering of Escherichia coli for limonene and perillyl alcohol production. *Metab. Eng. 19*, 33−41.
(43) Gibson, D. G., Young, L., Chuang, R.-Y., Venter, J. C., Hutchison, C. A., and Smith, H. O. (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat. Methods 6*, 343−345.
(44) Espah Borujeni, A., Channarasappa, A. S., and Salis, H. M. (2014) Translation rate is controlled by coupled trade-offs between site accessibility, selective RNA unfolding and sliding at upstream standby sites. *Nucleic Acids Res. 42*, 2646−2659.

DOI: 10.1021/acssynbio.8b00525
ACS Synth. Biol. 2019, 8, 1303−1313

(45) Salis, H. M., Mirsky, E. A., and Voigt, C. A. (2009) Automated design of synthetic ribosome binding sites to control protein expression. *Nat. Biotechnol. 27*, 946−950.
(46) Misawa, N., Satomi, Y., Kondo, K., Yokoyama, A., Kajiwara, S., Saito, T., Ohtani, T., and Miki, W. (1995) Structure and functional analysis of a marine bacterial carotenoid biosynthesis gene cluster and astaxanthin biosynthetic pathway proposed at the gene level. *J. Bacteriol. 177*, 6575−6584.
(47) Adams, R. P. (Identification of essential oil components by gas chromatography/mass spectrometry2007) *Allured Publishing Corporation, Carol Stream.*, Allured Publishing Corporation, Carol Stream.
(48) Sandmann, G. (2002) Combinatorial biosynthesis of carotenoids in a heterologous host: A powerful approach for the biosynthesis of novel structures. *ChemBioChem 3*, 629−635.

DOI: 10.1021/acssynbio.8b00525
ACS Synth. Biol. 2019, 8, 1303−1313

5.1.3 Analyzing and engineering the product selectivity of a 2-methylenebornane synthase

Reprinted with permission from *ACS Synth. Biol.* 2020, 9, 5, 981–986.
Copyright © 2020 American Chemical Society.
https://doi.org/10.1021/acssynbio.9b00432

Erklärung zu den Autorenanteilen an der Publikation / an dem Manuskript (Titel):
Analyzing and Engineering the Product Selectivity of a 2-Methylenebornane Synthase
Status: Printed
Name der Zeitschrift: ACS Synthetic Biology

Beteiligte Autoren:
Max J. Kschowak (Promovierender), Felix Meier (FM), Hannah Wortmann (HW), Markus Buchhaupt (MB)

Was hat der Promovierende bzw. was haben die Koautoren beigetragen?*[3]

(1) zu Entwicklung und Planung

Promovierender: 75 %
MB: 25 %

(2) zur Durchführung der einzelnen Untersuchungen und Experimente

Klonierungen, Sequenzanalyse, Protein-3D-Modell-Erstellung
Promovierender: 100 %

De novo terpenoid production in *E. coli*
Promovierender: 30 %
FM: 60 %
HW: 10 %

SBSE GC-MS analysis:
Promovierender: 25 %
FM: 60 %
HW: 15 %

(3) zur Erstellung der Datensammlung und Abbildungen

Fig. 1:
Promovierender: 50 %
MB 50 %

Fig.2, 3 :
Promovierender: 100%

Fig. 4.:
Promovierender: 40 %
FM: 20 %
MB 40 %

Supporting informations:
Promovierender: 50 %
FM: 20 %
MB 30 %

(4) zur Analyse und Interpretation der Daten

Sequenzanalyse, Protein-3D-Modell-Analyse:
Promovierender: 100 %

Alaninscan:
Promovierender: 80 %
MB: 20 %

Sättigungsmutagenese:
Promovierender: 60%
MB: 40%

[a] *Aus Gründen der Lesbarkeit wird im gesamten Text die männliche Schreibweise verwendet. Frauen sind natürlich inbegriffen.*

(5) zum Verfassen des Manuskripts

Promovierender: 65 %
MB: 35 %

Zustimmende Bestätigungen der oben genannten Angaben:

Datum/Ort	Unterschrift Promovend
Datum/Ort	Unterschrift Betreuer
Datum/Ort	Ggfs. Unterschrift *corresponding author*

[*1] Der Name der Zeitschrift darf erst genannt werden, wenn die Arbeit akzeptiert, im Druck oder erschienen ist.

[*2] Als Autoren werden solche Personen bezeichnet, die an der Arbeit in Bezug auf die genannten Punkte in einer Weise mitgewirkt haben, dass sie für die ausgewiesenen Passagen (mit-) verantwortlich sind. Personen, die an der Arbeit mitgewirkt haben, jedoch nicht in diese Kategorie fallen, sollten in der Danksagung Erwähnung finden.

[*3] Bei (2), (3) und (4) bitte prozentuale und dazu kurze inhaltliche Angaben machen, bei (1) und (5) reichen prozentuale Angaben.

Wenn es sich um ein Review handelt, notieren Sie das bitte in Klammern hinter den Titel der Publikation. Zudem ist in diesem Fall (2) hinfällig, bitte notieren Sie dort „n/a“. Unter (3) geht es dann um die „Literatursammlung“ anstelle der „Datensammlung“. Bitte ändern Sie dies im Formular!

[a] *Aus Gründen der Lesbarkeit wird im gesamten Text die männliche Schreibweise verwendet. Frauen sind natürlich inbegriffen.*

ACS SyntheticBiology

pubs.acs.org/synthbio Letter

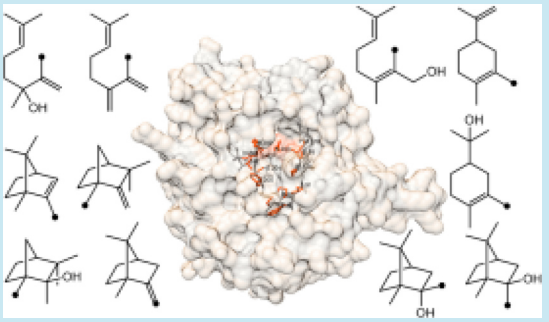
Analyzing and Engineering the Product Selectivity of a 2-Methylenebornane Synthase

Max J. Kschowak, Felix Maier, Hannah Wortmann, and Markus Buchhaupt*

Cite This: *ACS Synth. Biol.* 2020, 9, 981−986 Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: Terpenes constitute the largest class of natural products with more than 70 000 compounds. Many different terpenes find applications in the flavor and fragrance industry or can be used as fine chemicals or drugs. In some bacteria, noncanonical terpenes with 11 carbon atoms are synthesized via a GPP-C2-methyltransferase and the subsequent conversion of 2-methyl-GPP by certain terpene synthases into mainly 2-methylisoborneol and 2-methylenebornane. Many other C_{11}-terpenes were reported as side products, but they are synthesized only in minor amounts by the bacterial C_{11}-terpene biosynthesis pathway. To enable biotechnological synthesis of these largely unexplored natural products, we changed the product selectivity of the 2-methylenebornane synthase from *Pseudomonas fluorescens* by a semirational protein engineering approach. Active site amino acids with impact on the product selectivity were identified and variants with completely altered product spectra could be identified and characterized. The gathered data provide new insights into the structure−function relationship for C_{11}-terpene synthases and demonstrate the production of formerly inaccessible noncanonical terpenes.



KEYWORDS: *2-methylenebornane, C_{11}-terpenes, Pseudomonas fluorescens, 2-methyl-GPP, noncanonical terpenoids, terpene synthase*

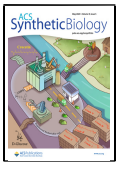
Downloaded via UNIV FRANKFURT on April 16, 2023 at 21:20:56 (UTC).
See https://pubs.acs.org/sharingguidelines for options on how to legitimately share published articles.

Terpenes are a natural product class with a large number of characterized structures, most of which were isolated from plants. While the underlying biosynthetic pathways and enzymes were initially elucidated in plants, the terpenoid-forming proteins in bacteria have been also discovered in more recent years.[1,2] The general biosynthetic blueprint of terpenes was found to be conserved in all organisms. This includes the two pathways for the synthesis of the universal terpene building blocks IPP (isopentenyl pyrophosphate) and DMAPP (dimethylallyl pyrophosphate), the mevalonate pathway and the deoxy-xylulose-phosphate pathway. Elongation of the prenyl pyrophosphate chain with IPP is catalyzed by prenyltransferases, which convert the C_5 pyrophosphates into GPP (geranyl pyrophosphate, C_{10}), FPP (farnesyl pyrophosphate, C_{15}), or GGPP (geranylgeranyl pyrophosphate, C_{20}).

The different prenyl pyrophosphates are substrates of terpene synthases that initiate a carbocationic reaction mechanism. The formation of the carbocations in class I terpene synthases is initiated by divalent metal ions and three amino acid residues called the effector triad. The latter includes the pyrophosphate-sensor, the linker, and the effector.[3] The metal ions are bound by an aspartate-rich motif (**DD**XX(XX)**D**) and the NSE or DTE motif, conserved as (**N,D**)D(L,I,V)X(**S,T**)XXX**E** (reviewed in ref 4). The resulting cationic intermediate can undergo hydride shifts, cyclizations, or rearrangements until the reaction sequence is terminated by deprotonation or addition of water or another nucleophile. The enormous diversity of terpenoid carbon backbones is the direct result of the large number of possible reaction sequences, which take place inside of terpene synthases and for which the stabilization of carbocationic intermediates, especially by aromatic residues is crucial.[5−7] Thereby the tryptophan of the conserved region WXXXXXRY at the C-terminus of terpene synthases also seems to be involved.[8] Whereas the majority of terpene synthases accepts only IPP, DMAPP, GPP, FPP, or GGPP as substrate, some of the enzymes show substrate promiscuity to some extent. Furthermore, terpene synthases with only one product exist, but most of them were found to release a certain pattern of multiple products, whereby also parameters such as pH value or the concentrations of magnesium and manganese ions can have strong influence on the product spectrum of a certain enzyme (reviewed in ref 9).

Although terpene biosynthesis pathways often include so-called decorating enzyme reactions which add hydroxyl, methyl, acetyl, or glycosyl groups, the primary products released by terpene synthases show a carbon atom number of 5, 10, 15, or 20 in nearly all cases. This restriction is the consequence of the modular assembly from C_5 building blocks. One exception to

Received: October 23, 2019
Published: May 4, 2020



ACS Publications © 2020 American Chemical Society

https://dx.doi.org/10.1021/acssynbio.9b00432
ACS Synth. Biol. 2020, 9, 981−986

this standardized construction plan was found in certain insects, where a so-called homomevalonate pathway produces C_6 prenyl pyrophosphates, which are known to be incorporated in many different terpenes (reviewed in ref 10). Another mechanism to introduce additional methyl groups into prenyl pyrophosphates is realized in certain bacteria by S-adenosylmethionine-dependent methyltransferases, which methylate IPP,[11−13] GPP,[14−16] or FPP.[17] The GPP-C2-methyltransferase was identified in bacteria, in which the resulting 2-methyl-GPP is converted to the C_{11}-compound 2-MIB (2-methylisoborneol) by a terpene synthase. Related 2-methyl-GPP-accepting terpene synthases in other bacteria were demonstrated to synthesize mainly the corresponding hydrocarbon 2-MB (2-methylenebornane).[18] Besides these two bicyclic compounds, a number of side products were shown to be released by wild type strains or *Escherichia coli* strains containing a GPP-methyltransferase and a C_{11}-terpene synthase[5,19,20] (Figure 1). However, so far, no 2-methyl-GPP-converting terpene synthase with a main product other than 2-MIB or 2-MB has been described.

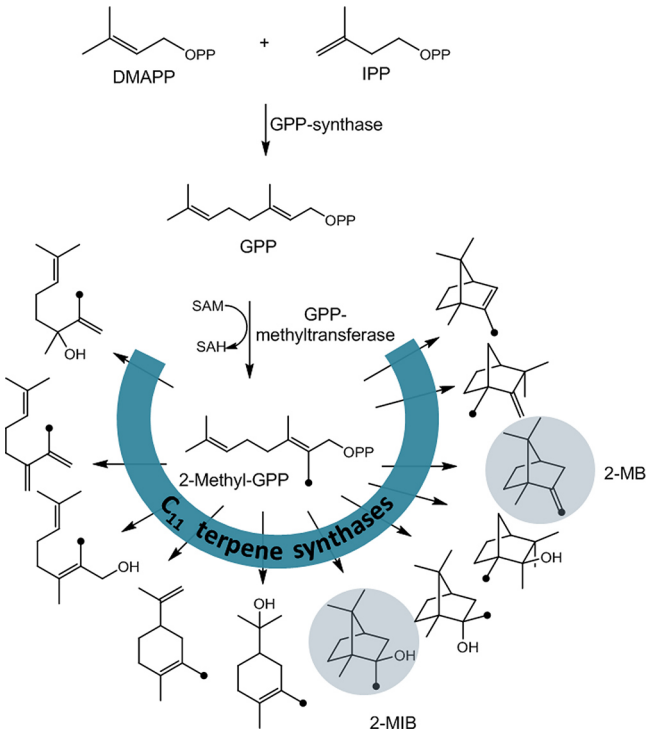


Figure 1. C_{11}-terpenes known to be synthesized from 2-methyl-GPP by bacterial terpene synthases.

A recent report about biotechnological C_{11}-terpene synthesis aimed at the use of plant monoterpene synthases, which normally convert GPP into C_{10} monoterpenes, to increase product diversity starting from 2-methyl-GPP.[21] Although their combination of a GPP-methyltransferase and a monoterpene synthase in yeast cells allowed the synthesis of 40 different C_{11}-compounds, GPP was concurrently converted into the physiological monoterpene products of the terpene synthases. The authors therefore identified a single amino acid exchange which increased the preference for 2-methyl-GPP in several monoterpene synthases. As the bacterial C_{11}-terpene synthases show at least 10-fold higher catalytic rates with 2-methyl-GPP compared to those with GPP,[16,18] we chose the 2-MB synthase from *P. fluorescens* and attempted to modify its product spectrum by semirational protein engineering.

RESULTS AND DISCUSSION

To identify the active site area of the 2-MB synthase from *Pseudomonas fluorescens*, its sequence was compared to the sequence of two other bacterial class I terpene synthases, the *Streptomyces coelicolor* 2-MIB synthase and the *Streptomyces pristinaspiralis* selinadiene synthase (SDS). The comparison (Figure 2a) showed the presence of the metal-binding aspartate-rich motif and the NSE motif, as well as the WXXXXXRY motif. In contrast to the SDS, both C_{11}-terpene synthases show an aspartate at the last position of the aspartate-rich motif instead of a glutamate. All three terpene synthases show an arginine as pyrophosphate sensor in the first position of the effector triad. At the linker position, the two C_{11}-terpene synthases contain asparagines instead of an aspartate at the respective position 181 in the selinadiene synthase. The residues at the effector position of the C_{11}-terpene synthases also differ from the respective amino acid G182 of the SDS, but the proposed key atom of the effector is the carbonyl oxygen atom of the amino acid.[3] A homology model of the 2-MB synthase (Figure 2b) based on the crystal structure of the 2-MIB synthase of *S. coelicolor* was created. It shows the same fold described as similar to a closed active site of a class I terpene synthase, but it is assumed to be incompletely closed due to the lack of one of the three MG^{2+} ions. A similar observation was found in the crystal structure of the diterpene synthase CotB2 bound to geranylgeranylthiophosphate.[22] Thirteen amino acids surrounding the active site were selected for an alanine scan approach. These include the pyrophosphate sensor (R200), the linker (N203), and the

a

	Asp-rich		triad		NSE		WXXXXXRY	
2-MBS *P. fluorescens*	95	DDHYCDD	200	RQVNSFW	245	NDLYSAYKE	326	WHKHSARY
2-MIBS *S. coelicolor*	197	DDCYCED	300	RQFNNFR	345	NDLYSYTKE	426	WHRTNTRY
SDS *S. pristinaspiralis*	82	DDGHCEE	178	RLYDGAT	224	NDIFSYHKE	288	WITSNKRY

b

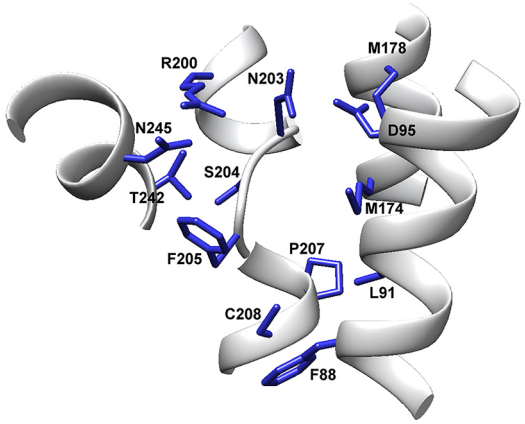


Figure 2. Identification of conserved sequence regions and active site lining amino acids by a sequence comparison and a structure model of the 2-MB synthase from *P. fluorescens*. (a) Comparison of the amino acid sequences of the aspartate-rich motif, the effector triad region, the NSE motif, and the WXXXXXRY motif of the 2-MB synthase from *P. fluorescens* (MBS), the 2-MIB synthase of *S. coelicolor* A3 (MIBS), and the selinadiene synthase of *S. pristinaspiralis* (SDS). (b) Homology-based model of the *P. fluorescens* 2-MB synthase based on the crystal structure of the 2-MIB synthase of *Streptomyces coelicolor* A3 as template (PDB ID: 3V1V). The blue amino acid side chains depict the positions targeted in the alanine scan.

https://dx.doi.org/10.1021/acssynbio.9b00432
ACS Synth. Biol. 2020, 9, 981−986

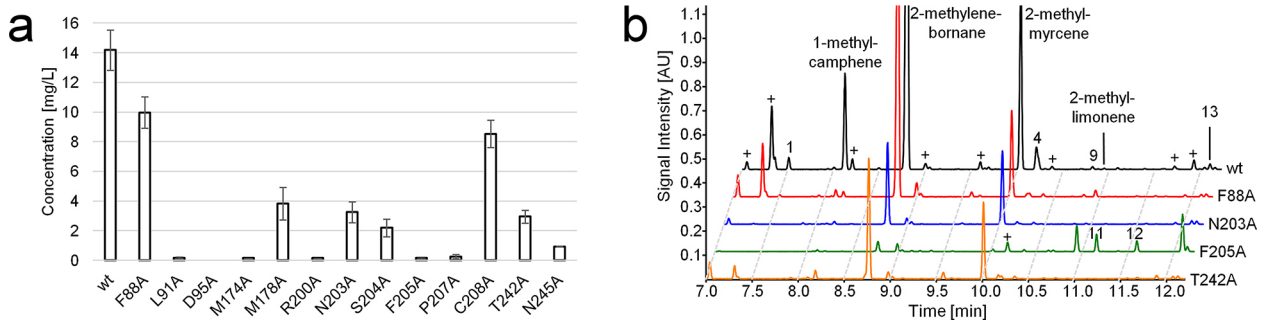


Figure 3. Identification of 2-MB synthase active site substitutions causing product composition variation. (a) Reduction or loss of 2-MB formation capability by different active site substitutions. 2-MB concentrations were determined in cultures of *E. coli* strains expressing the respective 2-MB synthase variant. The average of three replicates is shown; error bars represent standard deviations. (b) Alteration of product composition by alanine substitution of F88, N203, F205, and T242. TICs of SBSE analysis of *E. coli* strains expressing the *P. fluorescens* 2-MB synthase or respective variants are shown. Monoterpenes are labeled with a plus sign (+). C_{11}-terpenes with unknown structure are numbered (see Table S1).

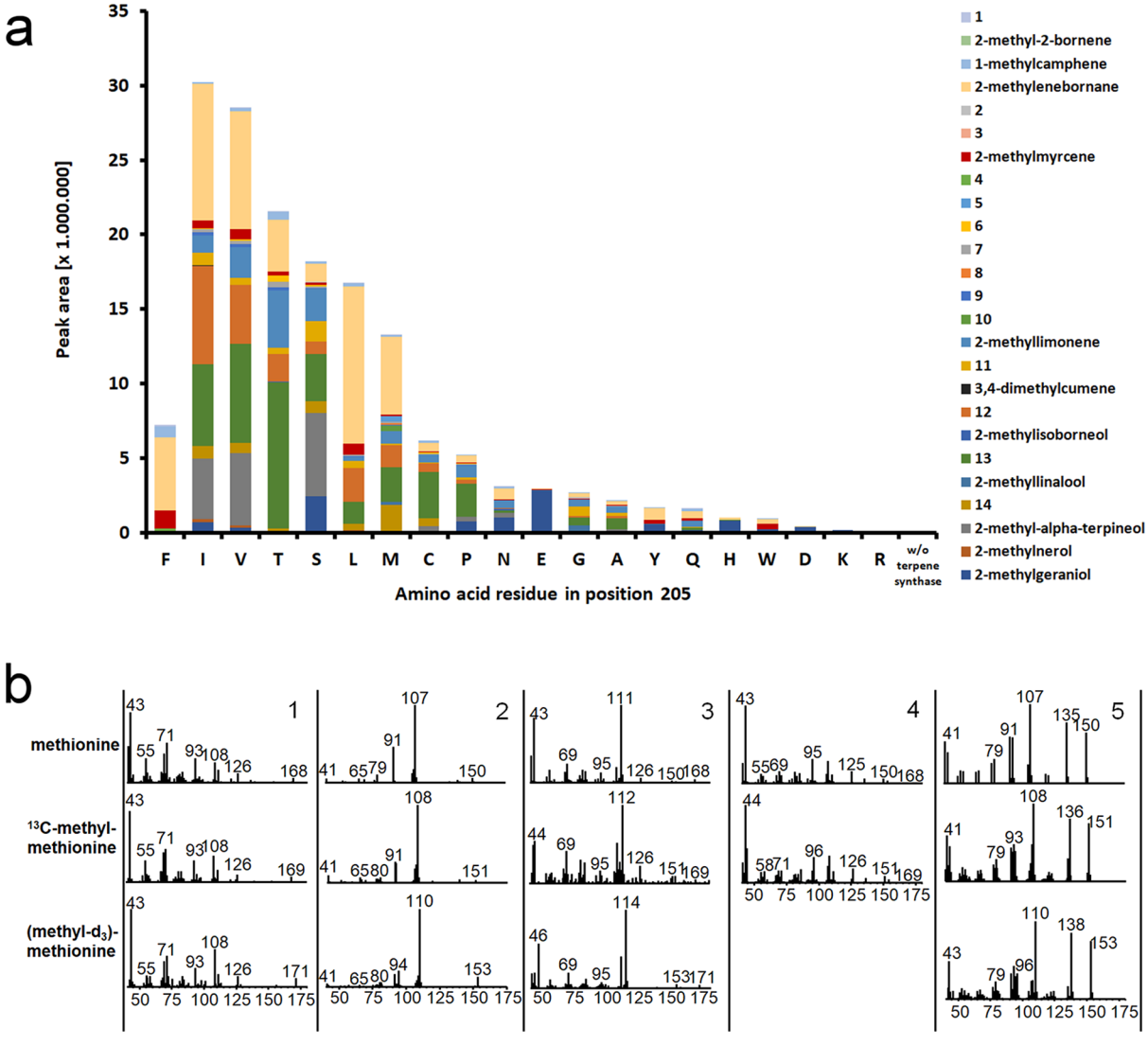


Figure 4. C_{11}-terpene product compositions of *E. coli* strains expressing *P. fluorescens* 2-MB synthase variants with different amino acids at position 205, which is occupied by phenylalanine (F) in the wild type enzyme. (a) *E. coli* strains containing the plasmid pMK-05 and one of the pETDuet1-MBSp-F205X-MTAse plasmids. The control strain (w/o terpene synthase) contained pMK-05 and plasmid pMK-03, the latter containing only the GPP-methyltransferase gene, but no terpene synthase gene. Product analysis using shake flask cultures was performed via SBSE-GC−MS and is described in detail in the material and methods section. Experiments were performed two times and showed highly similar results with the exception of the F205C sample. (b) Mass spectra of compounds 5, 6, 8, 10, and 13 from respective *E. coli* cultures supplemented with unlabeled methionine, ^{13}C-methyl-methionine or (methyl-d_3)-methionine are shown. In the case of compound 10, the respective deuterated compound coeluted with another substance, which did not allow further mass spectrum analysis of the deuterated compound.

https://dx.doi.org/10.1021/acssynbio.9b00432
ACS Synth. Biol. 2020, 9, 981−986

effector (S204), and one residue each of the metal-binding motifs (D95 and N245), as well as aromatic amino acids (F88 and F205).

All strains expressing one of the 2-MB synthase mutant genes showed clearly reduced 2-MB concentrations if compared to the strain expressing the wild type gene (Figure 3a and Supporting Information Figure S1). This result confirms the expected importance of the predicted active site amino acid residues in substrate binding or catalysis. Alanine substitution of D95 in the aspartate-rich motif resulted in a complete loss of 2-MB formation, which was expected due to its divalent cation complexation role in terpene synthases.[23] The almost complete loss of product formation in the strain with the R200A exchange is in accordance with the essential role of this highly conserved arginine in binding of the pyrophosphate moiety.[3] Hardly detectable 2-MB concentrations were furthermore determined in cultures of strains containing the L91A, M174A, F205A, and P207A exchange variants.

Alanine substitutions of amino acids F88, M178, N203, S204, C208, T242, or N245 resulted in reduced 2-MB product levels to varying degrees (Figure 3a). In the case of alanine substitution at positions F88, N203, and T242, clear alterations in the C_{11}-terpene product compositions of respective strains could be detected. All of these cultures showed clearly reduced levels of 1-methylcamphene, and for the strains with variants N203A and T242A, the ratios of 2-methylmyrcene and 2-MB to 1-methylcamphene were found to be increased (Figure 3b).

Closer inspection of the volatile product spectrum of the strain containing the F205A variant revealed a strongly altered C_{11}-terpene composition (Figure 3b). Whereas production of 2-MB and also 2-methylmyrcene was nearly eliminated, the previously described minor side products 2-methyllimonene and compounds 11, 12, and 13 of bacterial C_{11}-terpene synthases[5,19,20] were released. The relatively high retention indices observed for the main products of the F205A variant-containing strain point to linear or monocyclic structures. Therefore, an earlier termination of the reaction sequence probably takes place in the F205A enzyme variant and a carbocation-stabilizing role of F205 was assumed. This is also consistent with the results of studies with 2-MIB-synthase from *S. coelicolor*. This enzyme also has a phenylalanine residue (F305) at the corresponding position, for which a cation-stabilizing role through cation−π interactions is assumed.[5,24]

To further investigate the product-determining role of F205 in the 2-MB synthase from *P. fluorescens*, we performed a site saturation mutagenesis approach. Analysis of the volatile products released by *E. coli* strains expressing each of the 19 different F205 exchange variants of the *P. fluorescens* 2-MB synthase in addition to the GPP-methyltransferase and the mevalonate pathway was performed. The product compositions of all strains are shown in Figure 4, and the underlying chromatograms and a data table are shown in Figure S2 and Table S1. 2-Methylgeraniol and its isomer 2-methylnerol were not considered in the analysis, as they are also produced without participation of a terpene synthase, but by dephosphorylation of 2-methyl-GPP. The C_{11}-terpenes with unknown structure were numbered consecutively. In general, the 2-MB proportion of the total C_{11}-terpene product peak area is reduced in all strains with enzyme variants in comparison to the strain expressing the wild type enzyme. Furthermore, the product mixtures released by the variants with equal or higher total peak areas compared to the wild type enzyme products (F205I, F205V, F205T, F205S, F205L, F205M, F205C, F205P) show similar compositions with each other. In most of these samples, relatively high peak area values are observed for 2-MB and 2-methyllimonene and for the C_{11}-compounds 12 and 13. Substantial production of 2-methyl-α-terpineol in this group could be only observed with strains expressing the F205I, F205V, and F205S variants. Relatively low amounts of C_{11}-terpene synthase products were found in cultures of strains in which F205 was exchanged by one of the basic amino acids lysine, arginine, or histidine or the acidic amino acids aspartate and glutamate.

As aromatic amino acids often stabilize carbocationic intermediates in terpene synthase reactions,[4,25] we suspected F205 to fulfill such function during the reaction path toward 2-MB in the original enzyme. In a study with a hedycaryol synthase the carbocation-stabilizing effect of a phenylalanine residue could be confirmed by exchange with the aromatic residues tryptophane.[26] However, as also the amino acids leucine, isoleucine, and valine at position 205 cause the release of high amounts of 2-MB, carbocation stabilization by cation−π interactions with F205 is unlikely. Furthermore, substitution of F205 with the other aromatic amino acids tryptophane and tyrosine resulted in a clear reduction of C_{11}-terpene production. This effect has already been described with a limonene synthase, in which conversely the exchange of a tryptophane residue of the active site with phenylalanine leads to significantly lower limonene and therefore increased concentration of the termination product linalool.[27]

The production of novel terpenes by the exchange of aromatic residues in the active center has been reported with a class I terpene synthase.[28] Here we describe the appearance of four different C_{11}-compounds, which have not been produced by a terpene synthases before (Figure 4a,b). The probable alcohols 5 and 8 could be only detected in strains with the F205 M variant, whereas the probable alcohol 10 was found in strains expressing the F205 M or the F205S variant. Compound 6, which is presumably a hydrocarbon, was in turn not released if position 205 in the terpene synthase was occupied by methionine, but by alanine, leucine, isoleucine, valine, threonine, serine, cysteine, or proline. All four compounds were labeled as expected, if ^{13}C-methyl-methionine was fed to the cultures (Figure 4b). In the case of (methyl-d_3)-L-methionine feeding, only the mass spectra of deuterated derivatives of compounds 5, 6, and 8 could be evaluated. Their mass shift of +3 after labeling with (methyl-d_3)-L-methionine clearly showed, that no deprotonation took place at the introduced methyl group in during the reaction. The labeling data of compound 13, which makes up the highest peak area in the F205T, F205C, F205P, and F205A variant-expressing strains, is shown as well (Figure 4b) and demonstrates the preservation of the methyl group also in this substance.

Altogether, structure−function relationships described previously could be confirmed and some new insights could be drawn from the data set, although the structures of many products have not been elucidated so far. However, the product composition of the 2-MB synthase from *P. fluorescens* could be engineered in several directions after identification of F205 as an amino acid with high importance for product determination and a subsequent saturation mutagenesis approach. The microbially produced C_{11}-terpene oils produced with these variants or further engineered enzymes are highly valuable sources for the identification of, for example, novel flavor and fragrance compounds. Analysis of the available mixtures by GC-O can lead to the isolation of so far undiscovered substances with interesting olfactory characteristics. Moreover, direct comparison of the properties of pure C_{11}-terpene compounds with

https://dx.doi.org/10.1021/acssynbio.9b00432
ACS Synth. Biol. 2020, 9, 981−986

respective C_{10}-counterparts can deliver important structure−function information.

CONCLUSION

This first protein engineering report for a bacterial C_{11}-terpene synthase demonstrates the possibility of creating biosynthetic routes to a variety of noncanonical terpene structures, which might contain novel aroma compounds or pharmaceuticals. We could identify target sites for modulating product selectivity of the enzyme. Alterations of F205 were analyzed in detail and led to a range of different C_{11}-terpene product spectra and the appearance of four previously unknown C_{11}-terpenes. The deduction of a conclusion on structure−function relationships in this special type of terpene synthases is limited because there are only crystal structures of one 2-MIB-synthase and because there is a lack of complete product structure elucidation. The presented data set provides a basis for further mechanistic investigations. Furthermore, the engineered variants of the 2-MB synthase provide access to microbially produced terpene oils composed of the largely unexplored C_{11}-terpenes, for which no reasonable natural sources exist.

MATERIALS AND METHODS

Strains and Plasmids. *E. coli* MG1655 Δ*endA* Δ*recA* (DE3) (hereinafter referred to as *E. coli*)[29] was constructed in the lab of Kristala Prather (Addgene #37854) and used for production experiments.

Construction of plasmids pMK-3, pMK-5, and pMK-12 was described before.[19] pMK-5 encodes the genes of the mevalonate pathway, of an IPP isomerase and of a GPP synthase. It was used for provision of high GPP levels. pMK-6 is the respective empty control vector without the genes encoding the pathway toward GPP. pMK-12 is a pETDuet-1 vector containing the codon-optimized genes encoding the 2-MB synthase from *P. fluorescens* Pf0-1 (MBSp; DNA sequence in Figure S3; Uniprot Q3KF72) and the GPP-MTase from *S. coelicolor* A3(2) (Uniprot Q9F1Y5) in its two multiple cloning sites. pMK-3 lacks the gene for the MBSp. The MBSp variant genes were synthesized and cloned into the pMK-12 plasmid by Life Technology GmbH (Darmstadt, Germany). The DNA sequence alterations are listed in Table S2.

Protein Model Generation. The protein homology model of MBSp was built with Swiss-Model (ProMod3 version 1.1.0).[30] The crystal structure of the 2-MIB synthase of *S coelicolor* A3 (MIBS; Uniprot Q9F1Y6; PDP ID: 3v1v) was used as template. Figures of the crystal and model structure were created using UCSF Chimera.[31]

***De Novo* Terpenoid Production in *E. coli*.** Production strains harbor the pMK-5 and a pMK-12 vector with the MBSp gene or one of its mutants. A strain with the plasmids pMK-3 and pMK-5 vector was used for control experiments. Precultures in reaction tubes containing 5 mL of LB medium[32] with appropriate antibiotics were incubated overnight at 37 °C and 180 rpm. Main cultures in 15 mL 2× YT medium (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl, pH 7.0 with 2% (v/v) glycerol and appropriate antibiotics in 100 mL of baffled shake flasks were inoculated from precultures to an OD_{600} value of 0.1. After cultivation at 37 °C to an OD_{600} value of 1, gene expression was induced with isopropyl-β-D-1-thiogalactopyranoside (IPTG, 100 μM). Induced cultures had terpenoids extracted after 24 h of incubation at 30 °C and 180 rpm. For isotopic labeling experiments, unlabeled methionine, ^{13}C-methyl-methionine, or (methyl-d_3)-methionine were supplemented (c_{final} = 3 g/L) to the medium.

Stir Bar Sorptive Extraction (SBSE) GC−MS Analysis. For extraction of terpenoids, a twister (10 × 0.5 mm PDMS; Gerstel, Mühlheim an der Ruhr, Germany) was held in the liquid phase in a shake flask by an external magnet. After the production phase the twister was removed from the culture, rinsed with ddH_2O, dried with lint free wipes, and transferred to the inlet of a GC-vial, which was filled with 250 μL of *n*-pentane. After 15 min of incubation in an ultrasonic bath (Merck eurolab, Darmstadt, Germany) 5 μL of the pentane were used for GC−MS analysis using a GC−MS-QP2010 (Shimadzu) containing a DB-5 column (30 m × 0.25 mm × 0.25 μm, Agilent, Santa Clara, USA). Measurements were conducted as follows: helium as carrier gas, split ratio of 10; temperature at 250 °C, and column flow of 1.1 mL/min. The column temperature was programmed as follows: 40 °C for 1.5 min, 10 °C/min up to 250 °C followed by 20 °C/min up to 300 °C. Experiments for the determination of concentrations were performed three times, the other experiments two times, for which one representative data set is shown in Figures 3B and 4, and Figures S1 and S2 and Table S1. In the case of F205C, the SBSE-GC−MS analysis of one of the samples showed no compounds at all. Therefore, the data of this specific sample was not included. Compounds were identified via comparison of mass spectra and retention indexes (RI) to the ones of reference compounds or mass spectra of the NIST mass spectral library (v14) and RIs published by Adams. Identity was assumed if the similarity index was equal to or higher than 90 and the RI was ±10 compared to the published data. C_{11}-terpene reference compounds were synthesized by Enamine Ltd. (Riga, Latvia).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.9b00432.

TICs of SBSE-GC-MS analyses of cultures with pMK-5-containing *E. coli* additionally expressing the *P. fluorescens* 2-MB synthase or its respective alanine scan variants; comparison of SBSE-GC-MS peak areas of C11-terpenes in cultures with pMK-5-containing *E. coli* additionally expressing the *P. fluorescens* 2-MB synthase or its respective F205 variants (PDF)

AUTHOR INFORMATION

Corresponding Author

Markus Buchhaupt − *DECHEMA Research Institute, Industrial Biotechnology, Frankfurt am Main 60486, Germany;* orcid.org/0000-0003-2720-5973; Phone: +49 69 7564 629; Email: markus.buchhaupt@dechema.de

Authors

Max J. Kschowak − *DECHEMA Research Institute, Industrial Biotechnology, Frankfurt am Main 60486, Germany; Johann Wolfgang Goethe-University, Faculty of Biological Sciences, Frankfurt am Main 60323, Germany;* orcid.org/0000-0003-4378-3706

Felix Maier − *DECHEMA Research Institute, Industrial Biotechnology, Frankfurt am Main 60486, Germany; Johann Wolfgang Goethe-University, Faculty of Biological Sciences, Frankfurt am Main 60323, Germany*

https://dx.doi.org/10.1021/acssynbio.9b00432
ACS Synth. Biol. 2020, 9, 981−986

Hannah Wortmann − *DECHEMA Research Institute, Industrial Biotechnology, Frankfurt am Main 60486, Germany*

Complete contact information is available at: https://pubs.acs.org/10.1021/acssynbio.9b00432

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This research was supported by funds of the Federal Ministry of Food and Agriculture (BMEL) based on a decision of the Parliament of the Federal Republic of Germany via the Fachagentur Nachwachsende Rohstoffe e.V. (FKZ 22008713) and by the LOEWE project AROMAplus of the State of Hesse.

■ REFERENCES

(1) Cane, D. E., and Ikeda, H. (2012) Exploration and mining of the bacterial terpenome. *Acc. Chem. Res. 45*, 463−472.
(2) Dickschat, J. S. (2016) Bacterial terpene cyclases. *Nat. Prod. Rep. 33*, 87−110.
(3) Bär, P., Rabe, P., Fischer, K., Citron, C. A., Klapschinski, T. A., Groll, M., and Dickschat, J. S. (2014) Induced-fit mechanism in class I terpene cyclases. *Angew. Chem., Int. Ed. 53*, 7652−7656.
(4) Christianson, D. W. (2017) Structural and Chemical Biology of Terpenoid Cyclases. *Chem. Rev. 117*, 11570−11648.
(5) Brock, N. L., Ravella, S. R., Schulz, S., and Dickschat, J. S. (2013) A detailed view of 2-methylisoborneol biosynthesis. *Angew. Chem., Int. Ed. 52*, 2100−2104.
(6) Köksal, M., Chou, W. K., Cane, D. E., and Christianson, D. W. (2012) Structure of 2-methylisoborneol synthase from *Streptomyces coelicolor* and implications for the cyclization of a noncanonical C-methylated monoterpenoid substrate. *Biochemistry 51*, 3011−3020.
(7) Seemann, M., Zhai, G., de Kraker, J. W., Paschall, C. M., Christianson, D. W., and Cane, D. E. (2002) Pentalenene synthase. Analysis of active site residues by site-directed mutagenesis. *J. Am. Chem. Soc. 124*, 7681−7689.
(8) Driller, R., Janke, S., Fuchs, M., Warner, E., Mhashal, A. R., Major, D. T., Christmann, M., Bruck, T., and Loll, B. (2018) Towards a comprehensive understanding of the structural dynamics of a bacterial diterpene synthase during catalysis. *Nat. Commun. 9*, 3971.
(9) Vattekkatte, A., Garms, S., Brandt, W., and Boland, W. (2018) Enhanced structural diversity in terpenoid biosynthesis: enzymes, substrates and cofactors. *Org. Biomol. Chem. 16*, 348−362.
(10) Morgan, E. D. (2010) *Biosynthesis in Insects*, Royal Society of Chemistry.
(11) Ozaki, T., Shinde, S. S., Gao, L., Okuizumi, R., Liu, C., Ogasawara, Y., Lei, X., Dairi, T., Minami, A., and Oikawa, H. (2018) Enzymatic Formation of a Skipped Methyl-Substituted Octaprenyl Side Chain of Longestin (KS-505a): Involvement of Homo-IPP as a Common Extender Unit. *Angew. Chem., Int. Ed. 57*, 6629−6632.
(12) Radhika, V., Ueda, N., Tsuboi, Y., Kojima, M., Kikuchi, J., Kudo, T., and Sakakibara, H. (2015) Methylated Cytokinins from the Phytopathogen *Rhodococcus fascians* Mimic Plant Hormone Activity. *Plant Physiol. 169*, 1118−1126.
(13) Drummond, L., Kschowak, M. J., Breitenbach, J., Wolff, H., Shi, Y. M., Schrader, J., Bode, H. B., Sandmann, G., and Buchhaupt, M. (2019) Expanding the Isoprenoid Building Block Repertoire with an IPP Methyltransferase from *Streptomyces monomycini*. *ACS Synth. Biol. 8*, 1303−1313.
(14) Dickschat, J. S., Nawrath, T., Thiel, V., Kunze, B., Muller, R., and Schulz, S. (2007) Biosynthesis of the off-flavor 2-methylisoborneol by the myxobacterium *Nannocystis exedens*. *Angew. Chem., Int. Ed. 46*, 8287−8290.
(15) Komatsu, M., Tsuda, M., Omura, S., Oikawa, H., and Ikeda, H. (2008) Identification and functional analysis of genes controlling biosynthesis of 2-methylisoborneol. *Proc. Natl. Acad. Sci. U. S. A. 105*, 7422−7427.
(16) Wang, C. M., and Cane, D. E. (2008) Biochemistry and molecular genetics of the biosynthesis of the earthy odorant methylisoborneol in *Streptomyces coelicolor*. *J. Am. Chem. Soc. 130*, 8908−8909.
(17) von Reuss, S., Domik, D., Lemfack, M. C., Magnus, N., Kai, M., Weise, T., and Piechulla, B. (2018) Sodorifen Biosynthesis in the Rhizobacterium *Serratia plymuthica* Involves Methylation and Cyclization of MEP-Derived Farnesyl Pyrophosphate by a SAM-Dependent C-Methyltransferase. *J. Am. Chem. Soc. 140*, 11855−11862.
(18) Chou, W. K., Ikeda, H., and Cane, D. E. (2011) Cloning and characterization of Pfl_1841, a 2-methylenebornane synthase in *Pseudomonas fluorescens* PfO-1. *Tetrahedron 67*, 6627−6632.
(19) Kschowak, M. J., Wortmann, H., Dickschat, J. S., Schrader, J., and Buchhaupt, M. (2018) Heterologous expression of 2-methylisoborneol/2 methylenebornane biosynthesis genes in *Escherichia coli* yields novel C11-terpenes. *PLoS One 13*, No. e0196082.
(20) Rabe, P., Citron, C. A., and Dickschat, J. S. (2013) Volatile terpenes from actinomycetes: a biosynthetic study correlating chemical analyses to genome data. *ChemBioChem 14*, 2345−2354.
(21) Ignea, C., Pontini, M., Motawia, M. S., Maffei, M. E., Makris, A. M., and Kampranis, S. C. (2018) Synthesis of 11-carbon terpenoids in yeast using protein and metabolic engineering. *Nat. Chem. Biol. 14*, 1090−1098.
(22) Tomita, T., Kim, S. Y., Teramoto, K., Meguro, A., Ozaki, T., Yoshida, A., Motoyoshi, Y., Mori, N., Ishigami, K., Watanabe, H., Nishiyama, M., and Kuzuyama, T. (2017) Structural Insights into the CotB2-Catalyzed Cyclization of Geranylgeranyl Diphosphate to the Diterpene Cyclooctat-9-en-7-ol. *ACS Chem. Biol. 12*, 1621−1628.
(23) Aaron, J. A., and Christianson, D. W. (2010) Trinuclear Metal Clusters in Catalysis by Terpenoid Synthases, *Pure and applied chemistry*. *Pure Appl. Chem. 82*, 1585−1597.
(24) Köksal, M., Chou, W. K., Cane, D. E., and Christianson, D. W. (2013) Unexpected reactivity of 2-fluorolinalyl diphosphate in the active site of crystalline 2-methylisoborneol synthase. *Biochemistry 52*, 5247−5255.
(25) Starks, C. M., Back, K., Chappell, J., and Noel, J. P. (1997) Structural basis for cyclic terpene biosynthesis by tobacco 5-epi-aristolochene synthase. *Science 277*, 1815−1820.
(26) Baer, P., Rabe, P., Citron, C. A., de Oliveira Mann, C. C., Kaufmann, N., Groll, M., and Dickschat, J. S. (2014) Hedycaryol synthase in complex with nerolidol reveals terpene cyclase mechanism. *ChemBioChem 15*, 213−216.
(27) Srividya, N., Davis, E. M., Croteau, R. B., and Lange, B. M. (2015) Functional analysis of (4*S*)-limonene synthase mutants reveals determinants of catalytic outcome in a model monoterpene synthase. *Proc. Natl. Acad. Sci. U. S. A. 112*, 3332−3337.
(28) Blank, P. N., Barrow, G. H., Chou, W. K. W., Duan, L., Cane, D. E., and Christianson, D. W. (2017) Substitution of Aromatic Residues with Polar Residues in the Active Site Pocket of epi-Isozizaene Synthase Leads to the Generation of New Cyclic Sesquiterpenes. *Biochemistry 56*, 5798−5811.
(29) Tseng, H. C., Harwell, C. L., Martin, C. H., and Prather, K. L. (2010) Biosynthesis of chiral 3-hydroxyvalerate from single propionate-unrelated carbon sources in metabolically engineered *E. coli*. *Microb. Cell Fact. 9*, 96.
(30) Waterhouse, A., Bertoni, M., Bienert, S., Studer, G., Tauriello, G., Gumienny, R., Heer, F. T., de Beer, T. A. P., Rempfer, C., Bordoli, L., Lepore, R., and Schwede, T. (2018) SWISS-MODEL: homology modelling of protein structures and complexes. *Nucleic Acids Res. 46*, W296−W303.
(31) Pettersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C., and Ferrin, T. E. (2004) UCSF Chimera–a visualization system for exploratory research and analysis. *J. Comput. Chem. 25*, 1605−1612.
(32) Bertani, G. (1951) Studies on lysogenesis. I. The mode of phage liberation by lysogenic Escherichia coli. *J. Bacteriol. 62*, 293−300.