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Isoprenoid Drugs, Biofuels, and Chemicals—Artemisinin, Farnesene, and Beyond

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Abstract Isoprenoids have been identified and used as natural pharmaceuticals, fragrances, solvents, and, more recently, advanced biofuels. Although isoprenoids are most commonly found in plants, researchers have successfully engineered both the eukaryotic and prokaryotic isoprenoid biosynthetic pathways to produce these valuable chemicals in microorganisms at high yields. The microbial synthesis of the precursor to artemisinin—an important antimalarial drug produced from the sweet wormwood *Artemisia annua*—serves as perhaps the most successful example of this approach. Through advances in synthetic biology and metabolic engineering, microbial-derived semisynthetic artemisinin may soon replace plant-derived artemisinin as the primary source of this valuable pharmaceutical. The richness and diversity of isoprenoid structures also make them ideal candidates for advanced biofuels that may act as "drop-in" replacements for gasoline, diesel, and jet fuel. Indeed, the sesquiterpenes farnesene and bisabolene, monoterpenes pinene and limonene, and hemiterpenes isopentenol and isopentanol have been evaluated as fuels or fuel precursors. As in the artemisinin project, these isoprenoids have been produced microbially through synthetic biology and metabolic engineering efforts. Here, we provide a brief review of the numerous isoprenoid compounds that have found use as pharmaceuticals, flavors, commodity chemicals, and, most importantly, advanced biofuels. In each case, we highlight the metabolic engineering strategies that were used to produce these compounds successfully in microbial

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hosts. In addition, we present a current outlook on microbial isoprenoid production, with an eye towards the many challenges that must be addressed to achieve higher yields and industrial-scale production.

Keywords Isoprenoids · Antimalarial · Artemisinin · Biofuel · Sesquiterpene · Monoterpene · Isopentenol

Contents

1 Introduction

Isoprenoids are the largest and most diverse group of natural products, composed of over 50,000 compounds including primary metabolites such as sterols, carotenoids, and quinines, and secondary metabolites that are often used for medical purposes [1–3]. Chemists have long marveled at the structural diversity of terpenes in natural products and have engineered their biosynthetic pathways to develop numerous isoprenoid-derived commercial drugs. Recently, advanced biofuels have garnered attention as global climate change has driven the development of carbon-neutral energy sources. The chemical structure of isoprenoids confers several beneficial

aspects as fuel compounds. For example, methyl branching is a common structural feature of isoprenoids that lowers the freezing point significantly. Also, cyclic structures, which are frequently observed in isoprenoids, increase energy density and are generally considered valuable features for jet fuels. In recent years, some isoprenoids have been tested and produced as potential gasoline, diesel, and jet fuels due to their favorable energy content, cold weather properties, and high octane/cetane numbers (Table 1) [4–11].

Isoprenoids are usually classified into groups according to the number of carbons: hemiterpenoids (C5), monoterpenoids (C10), sesquiterpenoids (C15), diterpenoids (C20), and triterpenoids (C30). Isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) are the two universal C5 building blocks to synthesize isoprenoids. These starting precursors can be synthesized by two routes: the 1-deoxy-D-xylulose-5-phosphate (DXP) or 2-methyl-d-erythrito-4-phosphate (MEP) pathway and the mevalonate (MVA) pathway (Fig. 1).

Often found in bacteria and plant plastids, the MEP or non-mevalonate pathway forms IPP and DMAPP from 2-methyl-D-erythrito-4-phosphate (MEP) via the condensation of pyruvate and glyceraldehyde 3-phosphate (G3P). The mevalonate (MVA) pathway is present in archaea, fungi, plant cytoplasm, and other eukaryotes including mammalian cells where IPP and DMAPP are formed from the condensation of three molecules of acetyl-CoA to mevalonate. For decades, the mevalonate pathway has been studied to understand the production of isoprenoids inasmuch as it is responsible for cholesterol biosynthesis in humans and other mammals. In plants, these two isoprenoid biosynthesis pathways exist in parallel for primary and secondary metabolism, which could be useful for environmental adaptation and more efficient carbon utilization [12].

The first step in the MEP pathway is the formation of 1-deoxy-D-xylulose 5-phosphate (DXP) by the condensation of pyruvate and D-glyceraldehyde 3-phosphate, catalyzed by DXP synthase (DXS) encoded by the *dxs* gene (Fig. 1). This step is crucial and known as the rate-limiting step of the entire pathway. The second step is catalyzed by DXP reductoisomerase (encoded by the *dxr* gene) to convert DXP to MEP. MEP is then converted to 4-(cytidine-5′-diphospho)-2-methyl-D-erythritol (CDP-ME), 2-phospho-CDP-ME (CDP-ME2P), 2-methyl-D-erythritol 2,4-cyclodiphosphate (cMEPP), and IPP and DMAPP via the series of enzymatic reactions.

The mevalonate pathway initiates with the condensation of two acetyl-CoAs by thiolase to extend the carbon backbone to produce acetoacetyl-CoA. Subsequently, another acetyl-CoA is condensed with acetoacetyl-CoA to produce 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), which is physiologically almost irreversible. Then, mevalonic acid, a stable precursor of isoprenoid/sterol biosynthesis, is produced by the HMG-CoA reductase (HMGR) from HMG-CoA using primarily NADPH as a cofactor. Two kinases, mevalonate kinase (MK) and phosphomevalonate kinase (PMK), catalyze the phosphorylation of mevalonate to produce mevalonate 5-phosphate and mevalonate 5-diphosphate, respectively. The final step of the mevalonate pathway to produce IPP is the ATP-driven decarboxylative elimination reaction catalyzed by mevalonate diphosphate decarboxylase (PMD). Following the production of IPP, isomerization by Idi leads to DMAPP formation

Table 1 Properties of conventional fuels and isoprenoid-based biofuels

Fuel Name	Density (range) (kg/m^3)	Flash point (°C) (min)	Freezing point (°C) (max)	Net heat of combustion (MJ/kg) (min)	Cetane number (min 40)	References
Amorphane						
Jet-A (ASTM)	775–840	38	−40	42.8	N.D.	[9]
Jet-A	811	43	−47	43.42	N.D.	
20 % Amorphane[a]/JetA	818	49	−48	43.1	N.D.	
50 % Amorphane[a]/JetA	846	60	−53	42.97	N.D.	
100 % Amorphane[a]	880	113	<−52	42.79	N.D.	
Farnesene						
#2 Diesel	864.6	73	N.D.	42.4	41.6	[7]
5 % AMD-200/#2 Diesel	859.5	75	N.D.	N.D.	41.7	
20 % AMD-200/#2 Diesel	845.4	78	N.D.	42.8	45.2	
50 % AMD-200/#2 Diesel	820.4	86	N.D.	43.2	50.7	
100 % AMD-200 (Farnesane)	773.7	109	N.D.	44.2	58.6	
Monoterpene dimers						
Alpha-Pinene_Dimer	935	N.D.		42.047	N.D.	[136]
Beta-Pinene_Dimer	938	N.D.		42.118	N.D.	
Limonene_Dimer	914	N.D.		41.906	N.D.	
JP-5	820	N.D.	<−46.15	42.4	N.D.	
JP-10	940	N.D.	<−79.15	42.1	N.D.	
RJ-5	1,080	N.D.	>−18.15	41.6	N.D.	
Gasoline	740	N.D.	<−100.15	43.6	N.D.	
Diesel no. 2	850	N.D.	−12.15 to −6.15	42.4	N.D.	
Biodiesel	880	N.D.	>−10.15	37.4	N.D.	
β-Pinene	860	N.D.	−61.15	42.9	N.D.	

(continued)

Table 1 (continued)

Fuel Name	Density (range) (kg/m^3)	Flash point (°C) (min)	Freezing point (°C) (max)	Net heat of combustion (MJ/kg) (min)	Cetane number (min 40)	References
α-Pinene	860	N.D.	−64.15	42.9	N.D.	
Bicyclic monoterpenes						
Jet-A ASTM	775–840	38	−40	42.8	N.D.	[8]
Jet-A	811	43	−47	43.4192	N.D.	
20 % AMJ-400[b]/Jet-A	820.4	43	−51	43.0534	N.D.	
50 % AMJ-400[b]/Jet-A	834.9	44	−56.5	42.9881	N.D.	
100 % AMJ-400[b] (pinane)	860.3	43	<−70	42.8011	N.D.	
Hydrogenated limonene and myrcene						
Diesel		58.3	N.D.	N.D.	45.6	[10]
Hydrogenated limonene (limonane)						
10 % in diesel	N.D.	58.9	N.D.	N.D.	42.8	
Hydrogenated myrcene						
2 % in diesel	N.D.	63.9	N.D.	N.D.	44.9	
5 % in diesel	N.D.	61.7	N.D.	N.D.	44.3	
10 % in diesel	N.D.	60	N.D.	N.D.	44.7	
Bisabolane						
D2 diesel	850	60–80	N.D.	N.D.	40–55	[6]
Biodiesel	880	100–170	N.D.	N.D.	48–65	
Bisabolane	820	111	N.D.	N.D.	41.9	

[a] Partially mixed with nonhydrogenated amorphadiene

N.D. Not determined

[b] 98.7 % of pinane

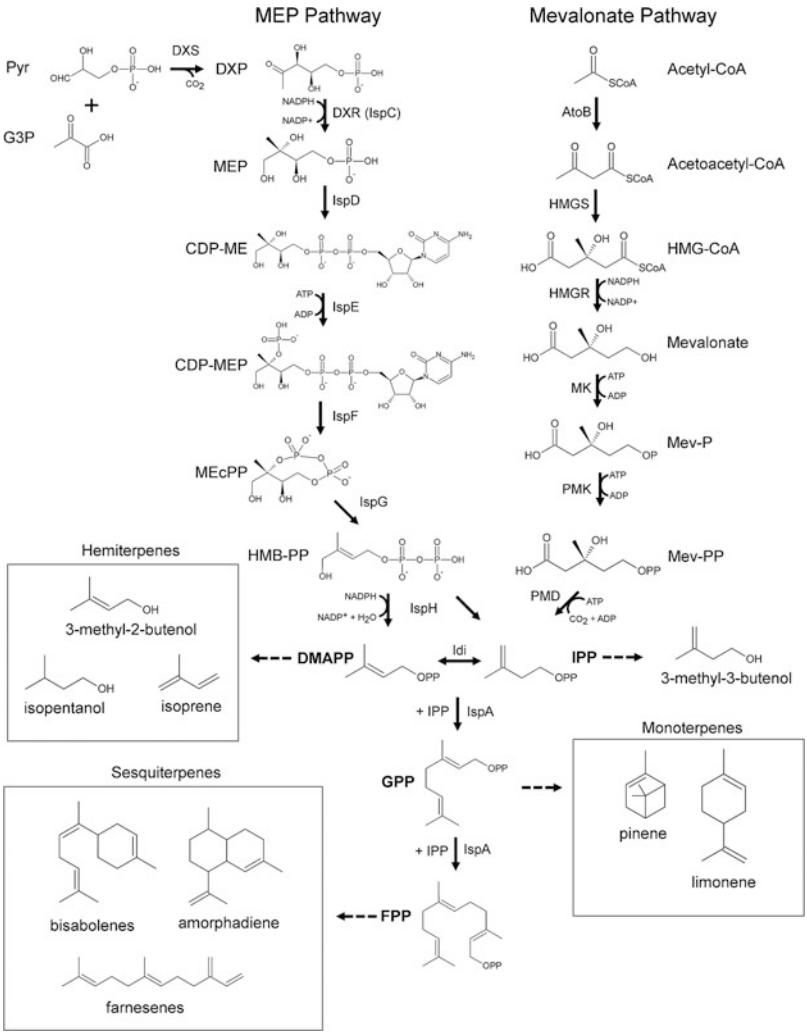

Fig. 1 Isoprenoid biosynthetic pathways. The MEP pathway (or DXP pathway) and mevalonate (*MVA*) pathway are two major isoprenoid biosynthetic pathways. Both pathways produce IPP and DMAPP as five-carbon building blocks for isoprenoid biosynthesis. The MEP pathway initiates with the condensation of pyruvate and glyceraldehyde 3-phopshate by DXS and an additional six steps transform DXP to IPP and DMAPP. In the mevalonate pathway, three molecules of acetyl-CoA condense to form HMG-CoA and an additional four steps transform HMG-CoA to IPP, which is isomerized to DMAPP by the isomerase (Idi). The condensation of DMAPP with one or two molecules of IPP leads to monoterpene or sesquiterpene production, respectively

and the initiation of isoprenoid precursor chain elongation (Fig. 1). The first successful engineering of a complete mevalonate (MVA) pathway in a heterologous host was accomplished about a decade ago for the biosynthesis of pharmaceutical

isoprenoids [13]. The sesquiterpene precursor of the antimalarial drug artemisinin was produced at high titers using a heterologous mevalonate pathway in *E. coli*, and this work is extensively reviewed in Sect. 2.

In this review, we present current efforts and future outlooks towards the biosynthesis of various isoprenoid drugs, biofuels, and chemicals. In Sect. 2, we briefly review the general engineering strategy for the construction and optimization of a heterologous mevalonate pathway for isoprenoid biosynthesis. In particular, we focus on the biosynthesis of the antimalarial drug artemisinin, which is perhaps the most successful example of isoprenoid biosynthetic pathway engineering. We then further review the advances in this platform technology for biofuel and chemical production. Sesquiterpene biofuel precursors including farnesene and bisabolene are reviewed in Sect. 3, and monoterpene biofuel precursors such as limonene and pinene are discussed in Sect. 4. Other important sesquiterpenoids and monoterpenoids are also discussed. In Sect. 5, hemiterpenoid fuels and chemicals such as isopentenol are briefly reviewed along with the issue of IPP toxicity, which is a crucial engineering factor in high titer isoprenoid production.

2 Mevalonate Pathway Assembly and Artemisinin Production

2.1 Efforts Towards Microbial Production of Isoprenoids

As the most numerous and structurally diverse family of natural products, isoprenoids have a variety of commercial uses as flavors, fragrances, and pharmaceuticals [2]. Due to the small quantities of isoprenoids commonly produced by plants and naturally occurring microorganisms, there has been considerable interest in the development of engineered microbial platforms for isoprenoid production in large-scale fermentations [14]. Research in this area has focused primarily on the production of pharmaceutically important compounds including carotenoids, sterols, the anticancer drug Taxol, and the antimalarial artemisinin [15–18]. Rather than review the vast numbers of studies in each of these areas, we focus this section on efforts leading to the development of a microbial platform for semisynthetic artemisinin production (Fig. 2). This decade-long project covers the initial assembly of a high-flux isoprenoid pathway in both *E. coli* and *S. cerevisiae* and details numerous pathway optimizations. The advances in metabolic engineering and synthetic biology achieved during this project have ultimately influenced current efforts to produce diverse terpenoid compounds including sesquiterpene (Sect. 3), monoterpene (Sect. 4), and hemiterpene (Sect. 5) biofuels.

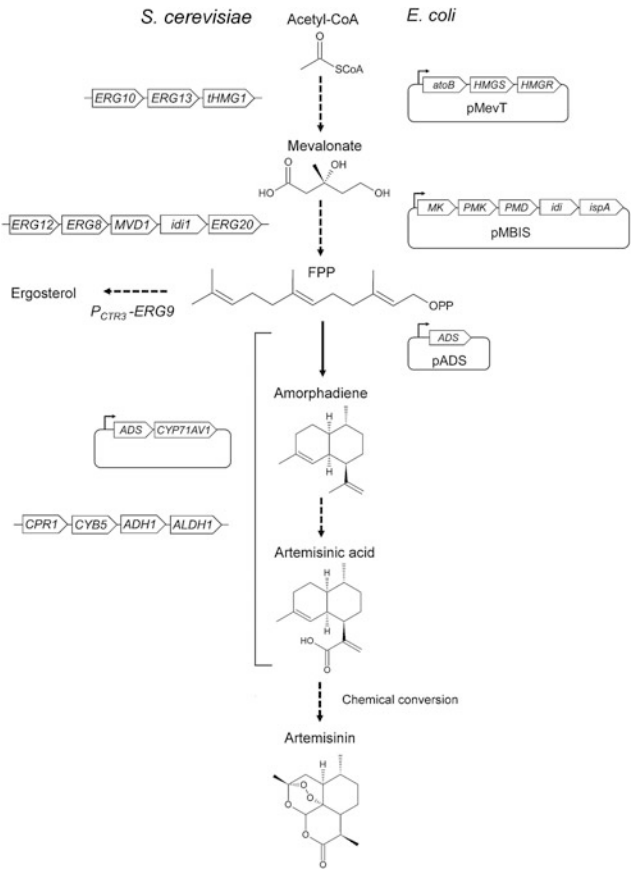

Fig. 2 Artemisinin strain engineering. An overview of important engineering breakthroughs in *S. cerevisiae* (*left*) and *E. coli* (*right*) for the production of amorphadiene, artemisinic acid, and semisynthetic artemisinin. Genes that were overexpressed on plasmids (*circular*) or on the chromosome (*straight line*) are highlighted. In the case of *S. cerevisiae*, ergosterol biosynthesis was also downregulated

2.2 Assembly and Optimization of Mevalonate Pathway for Large-Scale Production of Semisynthetic Artemisinin

Artemisinin is a potent antimalarial drug naturally produced by the sweet wormwood *Artemisia annua*, a plant long recognized for its medicinal properties [19]. Artemisinin and its derivatives have been designated as first-line antimalarial drugs [20] and are currently key components of antimalarial combination therapies (ACTs). Unfortunately, artemisinin availability and price has fluctuated over the past decade, largely due to the 18-month lag between planting, harvesting, and eventual supply. Although chemical synthesis could provide a stable supply of

artemisinin, this is not a feasible or cost-effective option [21]. Chemically, artemisinin is an isoprenoid containing 15 carbon atoms (sesquiterpene) derived from farnesyl diphosphate (FPP). The first committed step in artemisinin biosynthesis is the conversion of FPP to amorphadiene, a step catalyzed by amorphadiene synthase (ADS). Because isoprenoids are readily produced in nature, the biosynthesis of amorphadiene and, eventually, artemisinic acid, served as an attractive alternative to chemical synthesis.

In nature, the two common building blocks of isoprenoids, isopentenyl diphosphate and dimethylallyl diphosphate, are produced either from the mevalonate pathway or the methylerythritol phosphate pathway [22]. The MVA pathway is generally present in eukaryotes and archaea, whereas the MEP is active in most bacteria including *E. coli*. Though the MEP pathway has recently been used to produce high levels of taxadiene, an isoprenoid precursor to the anticancer drug Taxol (paclitaxel; [15]), early efforts to engineer a high-flux MEP pathway in *E. coli* were met with limited success. The endogenous regulation of the MEP pathway was suspected to be a reason for this intractability and to bypass this suspected limitation, expression of the *S. cerevisiae* mevalonate pathway was engineered in *E. coli* [13]. This approach provided a high-flux route to produce IPP and DMAPP and thus the longer chain terpene FPP, the precursor to amorphadiene in an *E. coli* host.

The heterologous mevalonate pathway was initially divided into a three-enzyme "top" portion (MevT) responsible for the conversion of acetyl-CoA to mevalonate and a five-enzyme "bottom" portion (MBIS) that transformed mevalonate into FPP. The MevT operon was made up of acetoacetyl-CoA thiolase from *E. coli* (*atoB*), along with HMG-CoA synthase (*HMGS*) and reductase (*HMGR*) from *S. cerevisiae*. The MBIS operon consisted of *S. cerevisiae*-derived *MK*, *PMK*, and *PMK,* along *idi* and FPP synthase (*ispA*) from *E. coli.* Enzymes in these two operons were expressed under an IPTG-inducible *lac* promoter in two plasmids. To make amorphadiene from FPP, an *E. coli* codon-optimized amorphadiene synthase gene (*ADS*) from *A. annua* was synthesized and expressed under a *trc* promoter in a high copy plasmid (pTrc99A). Coexpression of MevT and MBIS in *E. coli* DH10B complemented an MEP pathway mutant even in the absence of mevalonate, confirming the functional expression of both operons. By feeding mevalonate to a strain expressing only MBIS and ADS, the authors showed that flux from the MBIS operon did not limit amorphadiene production at the highest mevalonate concentration used (40 mM). If MBIS was induced without ADS, growth inhibition increased with the amount of added mevalonate, suggesting that FPP is toxic. This effect was more extreme in a truncated bottom pathway expressing only MK, PMK, and PMK (pMevB), suggesting that IPP accumulation is even more deleterious [13]. With a complete mevalonate pathway (pMevT + pMBIS) and ADS, 3.1 μg caryophyllene equivalent/mL/OD_{600} of amorphadiene was produced in a shake-flask culture in LB medium after 9 h, a 36-fold improvement over the native MEP pathway. A glycerol-amended culture reached higher biomass yields and prolonged amorphadiene production into the stationary phase. When accounting for loss of amorphadiene to the headspace, a total production of 112 mg/L was calculated from

the LB + 0.8 % glycerol culture. The heterologous mevalonate pathway assembled in this work proved far superior to earlier efforts to engineer the MEP pathway and laid the groundwork for assembly of a highly efficient platform for isoprenoid production [13].

A number of follow-up studies resulted in greatly improved amorphadiene yields in *E. coli.* Due to volatility, amorphadiene titers were severely underestimated in earlier work [13]. Use of a hydrophobic dodecane overlay relieved this issue and resulted in a significantly improved titer of 281.4 mg/L [23]. Other improvements included the use of TB medium and the "pulsing" of glycerol and other carbon sources during the stationary phase. With a dodecane overlay and increased carbon and complex nutrients, amorphadiene titers reached 480 mg/L, a 20-fold improvement over the original process.

The precursor pathway was improved through a variety of metabolic engineering techniques. The accumulation of HMG-CoA, later shown to inhibit fatty acid biosynthesis [24], was identified as a metabolic bottleneck in the MevT operon that limited mevalonate production [25]. Through growth analysis and LC-MS quantification of pathway intermediates, the authors demonstrated that improved expression of tHMGR, *N*-terminally truncated HMG-CoA reductase, relieved growth inhibition, reduced HMG-CoA accumulation, and improved mevalonate production threefold. This requirement for a balanced metabolic pathway was further emphasized in a related study [26]. In this work, the authors used a standardized vector system to alter gene dosage and identify MK and ADS as rate-limiting enzymes. Through modulation of additional parameters such as codon usage, promoter strength, and plasmid copy number, the authors assembled a pathway in *E. coli* DH1 that was sevenfold more efficient than the original strain [13].

Gene variants of *HMGS* and *HMGR* derived from *Staphylococcus aureus* (*mvaS* and *mvaA*, respectively) were used to more than double amorphadiene production [27]. This work also developed an effective process for high-density fermentation. A defined medium was used instead of TB, and parameters including carbon feed rate and nitrogen concentration were systematically changed to reduce acetate formation, curtail carbon flow towards protein synthesis, and improve amorphadiene yield. With a carefully balanced dual restriction of glucose and nitrogen, DH1 harboring the optimized pathway produced 27.4 g/L of amorphadiene, the highest-reported titer in *E. coli* to date.

In order to produce artemisinin from the sesquiterpene precursor, amorphadiene must first be biologically converted to artemisinic acid which can be easily processed to the final product via chemical synthesis. To accomplish this conversion in vivo, the *A. annua* native cytochrome P450 *CYP71AVI* was expressed in *E. coli* coexpressing the heterologous MVA pathway and ADS [28]. By changing expression vectors and the host to DH1 instead of DH10B, fully oxidized artemisinic acid was produced at a titer of 105 mg/L. This work served as the first demonstration of in vivo production of functionalized terpenoids with native plant P450s in *E. coli.*

Despite the high yields and titers of amorphadiene attained in *E. coli*, the artemisinin project was ultimately carried out using a refactored mevalonate pathway in

S. cerveisiae due primarily to difficulties in achieving efficient artemisinic acid production in the *E. coli* host [29]. Prior to the switch, Ro and coauthors initially devised a *S. cerevisiae* strain capable of high flux to artemisinic acid through a variety of host modifications [18]. To increase FPP production in *S. cerevisiae, tHMGR* was overexpressed along with the transcriptional regulator UPC2, which is involved in the biosynthesis of sterols. In addition, *ERG9*, which encodes squalene synthase, was downregulated to prevent carbon loss from FPP. By combining these modifications with the expression of ADS, 153 mg/L of amorphadiene was produced. Next, the authors sought to produce artemisinic acid from amorphadiene. To accomplish this goal, the genes responsible for this conversion in *A. annua* [cytochrome P450 *CYP71AV1* and NADPH:cytochrome P450 oxidoreductase (*CPR*)] were isolated and overexpressed in *S. cerevisiae* (Fig. 2). This work also developed an efficient purification technique for synthesized artemisinic acid. Artemisinic acid was efficiently transported out of the cell and remained bound to the cell surface while protonated. Thus, treatment with an alkaline buffer removed >96 % of artemisinic acid from the cell pellet. Using this feature, the authors developed a one-step purification method that routinely yielded >95 % pure artemisinic acid. Altogether, artemisinic acid was produced at a titer of 115 mg/L.

Westfall and coauthors focused on further improving amorphadiene and artemisinic acid productivity, achieving >40 g/L of amorphadiene in a fermentation process [30]. A variety of changes in the host, pathway, and fermentation process facilitated these improvements. The endogenous consumption of galactose, commonly used as an inducer, was eliminated through a *GAL1* deletion. This modification allowed for the production of amorphadiene and artemisinic acid using glucose as a sole carbon source and galactose as an inducer. In the pathway, heterologous genes derived from *A. annua* were codon-optimized, and the orientation of the *GAL1* promoters was altered to prevent recombination. These modifications were reconstructed in *S. cerevisiae* CEN.PK2, a better-characterized strain, rather than S288C. In the final production strain, every enzyme of the mevalonate pathway up to *ERG20* was transcribed from galactose-regulated, divergent *GAL1/GAL10* promoters, and three copies of *tHMG1* were integrated in the chromosome. *GAL80*, encoding the negative regulator of the galactose regulon, was also deleted to obviate the use of galactose entirely. With these optimizations, 41 g/L of amorphadiene were produced after 116 h at a yield of 16.98 Cmol %. Because yields of biosynthetic artemisinic acid were considerably less than amorphadiene, a three-step chemical conversion was also developed in this report that achieved a 48.4 % yield of artemisinic acid from amorphadiene.

Most recently, a highly efficient, complete biosynthetic process to artemisinic acid was constructed and allowed for the high-level, semisynthetic synthesis of artemisinin [31]. The combination of multiple components including an engineered host for an improved precursor pathway, artemisinic acid biosynthesis pathway, fermentation process engineering, and chemical conversion to artemisinin was required to accomplish this extraordinary feat. A copper-regulated *CTR3* promoter was used to downregulate *ERG9*, allowing for less-expensive $CuSO_4$ to be used as a repressor instead of methionine. To increase cell viability, expression of *CPR1*, the

cognate reductase of *A. annua* amorphadiene oxidase *CYP71AV1,* was reduced. Cytochrome *b5* from *A. annua* (*CYB5*) was integrated into the yeast chromosome, yielding higher production of artemisinic acid and artemisinic aldehyde. *A. annua* artemisinic aldehyde dehydrogenase (*ALDH1*) was expressed, further increasing artemisinic acid production. To complete the artemisinic acid pathway and reduce artemisinic alcohol production, a putative *A. annua* alcohol dehydrogenase (*ADH1*) was expressed in conjunction with *CYP71AV1*, *CPR1*, *CYB5*, and *ALDH1*. With the complete biosynthetic pathway, artemisinic acid was produced as a crystalline extracellular precipitate that complicated sampling procedures. To overcome this complication, the authors used extractive fermentation with isopropyl myristate (IPM) oil and developed a method to extract artemisinic acid from IPM at high purity. With all of these optimizations, 25 g/L of artemisinic acid were produced, a >10-fold improvement over earlier efforts. Finally, an inexpensive chemical process based on singlet oxygen was used to convert artemisinic acid into semisynthetic artemisinin at a high yield. Remarkably, the strains and processes described in this work are currently being used in an industrial process for artemisinin production and distribution [29].

2.3 Tangential Development of Tools for Metabolic Engineering

A number of promising metabolic engineering tools were constructed in association with the artemisinin project. Many of these tools were developed in *E. coli*, where the heterologous mevalonate pathway served as a flexible model system. Some of the most promising tools served the purpose of balancing the expression of multiple genes, a reoccurring theme in the artemisinin project. A method for randomly tuning the expression of multiple genes within an operon by altering the sequences of intergenic regions was described and applied to the MevT operon (*atoB*, *HMGS*, *HMGR*; [32]). Tunable intergenic regions (TIGRs) containing posttranscriptional control elements including hairpins and RNase E sites were combinatorially generated and inserted in the MevT operon. Unexpectedly, reduced expression of HMGS and HMGR was shown to yield a sevenfold increase in mevalonate concentration. This observation highlights the strength of this methodology, as an intentional reduction in HMGS and HMGR expression would not have been a priority in rational pathway design.

MevT also served as a testbed for the development of synthetic protein scaffolds, another approach to tune expression and provide modular control over metabolic flux [33]. To test this system, AtoB, HMGS, and HMGR in the MevT operon were tagged with the metazoan protein–protein interaction ligands GBD, SH3, and PDZ. A synthetic scaffold containing the cognate binding domains for these tags was then coexpressed in this same system, allowing the tagged proteins to colocalize on the scaffold. By altering the number and/or the order of binding sites, enzyme

stoichiometry could be predictably altered. Using this scaffold, a 77-fold increase in mevalonate yield was observed [33].

The application of targeted proteomics to metabolic pathway optimization provided a third method for tuning pathway expression [34]. A selected reaction monitoring (SRM) proteomics method was applied to the entire mevalonate pathway for amorphadiene production and used to measure concentrations of all nine pathway proteins. Protein levels of HMGR, MK, and PMK were shown to be low and were thus hypothesized to limit pathway flux. Codon-optimization of these genes and the addition of a supplemental promoter upstream of MK improved protein expression and facilitated a threefold increase in amorphadiene titer.

The toxicity of prenyl diphosphates such as IPP and FPP [13] provided a means to develop novel screening methods. Because IPP and FPP cause growth inhibition, overexpression of a terminal enzyme (such as a terpene synthase) that consumes these toxic precursors restores growth. For example, using this concept, a library of 19,000 clones harboring fragments of *B. subtilis* genomic DNA was screened for hemiterpenoid production in an IPP-accumulating *E. coli* strain. Two genes, identified as *yhfR* and *nudF*, overcame IPP toxicity and restored growth. Further analysis of *nudF* showed that it was capable of converting IPP into isopentenol, a potential biofuel (see Sect. 4.2). The toxicity of prenyl diphosphates was further exploited to program dynamic pathway expression using stress–response promoters [35]. In this work, a microarray was performed on *E. coli* harboring a truncated mevalonate pathway ending in the terminal production of FPP. A subset of promoters from genes that were differentially expressed under FPP accumulation was subsequently used to drive the mevalonate pathway. Specifically, the promoter for *gadE*, a gene downregulated during FPP accumulation, was used to drive expression of mevalonate pathway enzymes (MevT–MBIS operon) up to FPP synthase, whereas the promoter from *rstA*, a gene upregulated during FPP accumulation, was used to drive terpene synthase *ADS* that consumes FPP. In this manner, the cell would dynamically adjust expression to maintain FPP levels below a toxic threshold: high FPP concentrations would simultaneously slow its production (downregulate MevT–MBIS) and increase its consumption (upregulate ADS). Compared to the inducible pathway, this dynamic pathway produced twofold more amorphadiene ($\sim$1.5 g/L), accumulated less acetate, and reached higher ODs. Since this strategy does not require the development of biosensors or synthetic transcription factors, it should be broadly applicable to other metabolic pathways.

2.4 Future Work

The artemisinin project has served as a compelling example of the potential of metabolic engineering and synthetic biology to deliver real-world results. Semi-synthetic artemisinin is functionally identical to the plant-derived drug [31], has been approved by the World Health Organization (WHO), and is currently being produced on an industrial scale by the multinational pharmaceutical company

Sanofi [29]. Given the remarkable diversity of isoprenoid compounds, it is likely that a similar engineering approach can yield a variety of medicinally relevant molecules such as paclitaxel, prostratin, and lovastatins [29]. The remainder of this review focuses primarily on an additional application: isoprenoid-derived advanced biofuels.

3 Sesquiterpenoid Biofuels and Chemicals

3.1 Sesquiterpenoids Properties and Chemical Diversity

Sesquiterpenoids are one of the largest groups (>7,000 compounds) of isoprenoid natural products and perform important physiological functions in a wide range of organisms including plants, insects, and fungi [36]. This chemical diversity is derived from the flexibility of terpene synthases, enzymes that catalyze the conversion of FPP into a large variety of sesquiterpene skeletons that can be further modified into more complex molecules with increased functionality [36, 37]. Functionally, sesquiterpenes have a wide range of activities from antimicrobial agents (such as phytoalexins capsidiol [38]) to alarm pheromones (such as farnesene [39]). Structurally, sesquiterpenes can be acyclic, monocyclic, bi- or even tricyclic structures depending on the identity of the terpene synthase (Fig. 3).

Acyclic sesquiterpenoids can be found in essential oils and insect pheromones. They include farnesene and isomeric alcohols such as nerolidol and farnesol, and often act as important compounds for the fragrance, flavoring, and pharmaceutical industries [40–43]. In addition to these uses, acyclic sesquiterpenoids such as farnesane have been proposed as potential diesel and jet-fuel alternatives [7, 42, 44, 45].

Monocyclic sesquiterpenoids also include compounds for the pharmaceutical and perfumery industries. For example, humulene has antiallergic and anti-inflammatory properties [46, 47]. Elemol, zingiberene, and bisabolene occur in many essential oils and fragrances [40] and recently the hydrogenation product of bisabolene (i.e., bisabolane) has been proposed as a promising diesel replacement [6].

Bi- or tricyclic sesquiterpenoids are often valuable chemicals. Nootkatone, for example, found in low concentrations in diverse plant essential oils, may be used in foods, cosmetics, and pharmaceuticals due to its distinct grapefruit-like odor or as an insect repellent or insecticide [40, 48, 49]. Due to its low natural abundance, nootkatone has been produced by sequential oxidations of valencene, a cheaper sesquiterpene produced from citrus fruit, and also from engineered microbes [50, 51].

There are many sesquiterpenoid lactones (SLs) with chemical and structural properties favorable for selective activity towards tumor and cancer stem cells. These compounds function by targeting specific signaling pathways and characteristics present in cancerous cells and are thus lead compounds in cancer clinical trials.

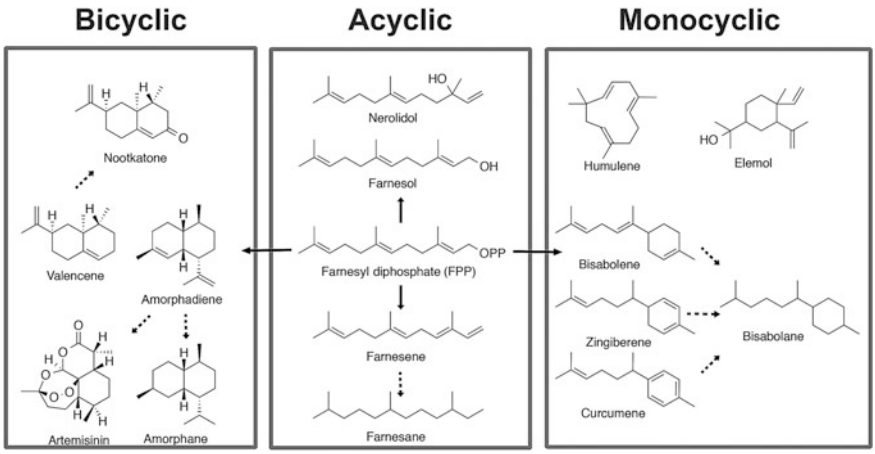

Fig. 3 Examples of sesquiterpenoids. All sesquiterpenoids are produced from the C15 precursor FPP (farnesyl diphosphate). According to the nature of terpene synthase, they can be either acyclic, monocyclic, or bi- or tricyclic compounds. The solid arrows show enzymatic processes, and the *dotted arrows* represent downstream conversion by either chemical or enzymatic processes

Artemisinin, thapsigargin, parthenolide, and many of their synthetic derivatives have been identified and tested for anticancer activities (Ghantous et al. 2010; [52–54]).

As mentioned above, farnesane and bisabolane have been identified and tested as diesel fuel alternatives. D2 Diesel, the fossil fuel for compression ignition engines, is a mixture of linear, branched, and cyclic alkanes with an average carbon length of 16. Sesquiterpenes are hydrocarbons of 15 carbons, close to the average length of diesel (C16), but with a branched—rather than a straight-chain structure. From a fuel performance point of view, the branching degree of the isoprenoid translates into greater molecular stability under high pressure, reduced premature ignition, increased octane number, and lowered freezing points through reduced molecule stacking [55]. However, having a slightly branched hydrocarbon rather than a straight-chain alkane also lowers the quality of combustion (i.e., reduced cetane number) in diesel engines. In the next section, we review these two biodiesel alternatives and the efforts to produce them in microbial hosts.

3.2 Farnesene and Bisabolene

The C15 isoprenoids farnesane and bisabolane have cetane numbers of 58 and 52, respectively, putting them within the expected range for diesel fuels (40–60; [55]). In addition, they display better cold properties, with cloud points of −78 and −25 °C compared with D2 diesel's cloud point of −3 °C. The ring portion of bisabolane increases the density of the fuel (0.88 g/mL), which will increase the energy density per volume of fuel [6]. Farnesane has a lower density (0.77 g/mL)

than bisabolane, however, it has a better cetane number and is the closest to commercialization [7].

Although plants are the natural source of bisabolene and farnesene, which are sesquiterpene precursors of bisabolane and farnesane, respectively, engineered microbial platforms may be the most convenient and cost-effective means to produce these compounds [6, 56, 57]. Microbial production of bisabolene and farnesene has been explored, but an efficient biological route for the hydrogenation of these sesquiterpenes to produce the corresponding biofuels has not been established despite promising initial work [58]. As a result, isoprenoid biofuels can be produced through a hybrid process, using a microbial platform for sesquiterpene overproduction and then a chemical route to produce the fully reduced fuel.

Previously, the mevalonate pathway was engineered in both *E. coli* and *S. cerevisiae* to overproduce FPP and, potentially, any sesquiterpene for which the corresponding terpene synthase is known. The highest reported titers of any isoprenoid are those of the sesquiterpene amorphadiene: ~25 and ~40 g/L amorphadiene have been obtained by overexpression of the MVA pathway in *E. coli* and *S. cerevisiae,* respectively [27, 31]. The flexibility of the *E. coli* and *S. cerevisiae* FPP-overproducing platforms allowed scientists to switch rapidly from the production of amorphadiene to the production of bisabolene [6] and farnesene [7].

Farnesene is the generic name for a series of sesquiterpene isomers that in nature act as chemical signaling molecules with diverse functions in numerous organisms [16], playing roles as attractants in pollination [59] and predation response [60] in plants or as alarm pheromones in insects [61].

In heterologous hosts such as *E. coli* and *S. cerevisiae*, farnesene has been produced for use as a precursor for renewable fuels and chemicals from FPP via heterologous expression of farnesene synthase [7, 16, 62]. Farnesene synthases have been isolated from different sources including *Mentha piperita* [39], *A. annua* [63], *Picea abies* [64], *Zea mays* [60], or *Citrus junos* [65].

The molecular structure of *trans-β*-farnesene (commercialized under the name Biofene® by the biotech company, Amyris, based in Emeryville, California) makes it attractive as a scaffold for specialty chemical applications such as solvents, emollients, and vitamins (Amyris website at www.amyris.com). The fully reduced form of farnesene (farnesane) is being pursued as an alternative biosynthetic diesel and is the closest of the isoprenoid-based biofuels to commercialization [57].

Using the previously described gene expression systems of the MVA pathway [13, 66] with farnesene synthase from *A. annua* and *P. abies,* Amyris described the bioproduction of farnesene using both *E. coli* and yeast (up to 1.1 g/L farnesene in *E. coli* expressing the MVA pathway after 120 h, and 728 mg/ L in yeast after 72 h) and methods to hydrogenate the biologically produced sesquiterpene into farnesane and other derivatives in a two-step semisynthetic process [7]. Farnesene is currently produced by Amyris from sugarcane using laboratory-evolved strains of the industrial yeast *S. cerevisiae* PE-2. By iterations of random mutagenesis, analysis, and selection, evolved strains have produced farnesene at >50 % of theoretical mass yield [16]. According to the last public report of Amyris at the end of 2010, total titers reached 104.3 g/L of farnesene with a productivity of 16.9 g/L/d and a

recovery of 95 % (source: http://www.biomassboard.gov/pdfs/biomass_tac_todd_pray_09_29_2010.pdf). Current titers are probably higher, but the company has not made the numbers public. Amyris began industrial manufacturing of farnesene in Brazil in December 2012. The plant is located at the Paraíso Bioenergia mill, where microbes convert sugarcane into Biofene that is then processed into specific renewable products. Amyris, currently selling renewable diesel in metropolitan areas in Brazil, has plans for additional production facilities to meet growing demand for its renewable products (www.amyris.com). Very recently, Amyris partnered with TOTAL (the French oil company) and the Brazilian airline GOL to fly the first commercial flight with farnesane and attained industry approval for this renewable jet fuel. Also, Amyris has patented amorphane (the hydrogenated form of the artemisinin precursor amorphadiene) as a component in a jet-fuel replacement [9, 27].

To produce the biofuel precursor bisabolene in *E. coli*, bisabolene synthases from various plant sources were coupled with the heterologous mevalonate pathway [6]. A bisabolene synthase from *Abies grandis* was the most promising, where an *E. coli* codon-optimized variant produced ~400 mg/L of bisabolene. Metabolic engineering of the precursor pathway to improve flux to FPP led to final bisabolene titers of ~900 mg/L in both *E. coli* and *S. cervisiae* [6]. Using carotenoid production as a visual phenotype, genes that affected isoprenoid synthesis in yeast were identified and knocked-out to increase terpene production. Combinations of these deletions and other pathway modifications improved titers of bisabolene more than 20-fold to 800 mg/L in flask and 5.2 g/L in a fermentation process [67]. The discovery of bisabolene synthase as the limiting factor of bisabolene synthesis in these studies prompted the identification of the crystal structure of the most efficient bisabolene synthase (from *A. grandis*) to aid in its engineering for increased microbial bisabolene production [68].

3.3 Farnesol

Farnesol ($C_{15}H_{26}O$) is an acyclic sesquiterpenoid alcohol derived from FPP. It is found in plant essential oils and is commercially important in the flavor and fragrance industries. In addition, farnesol is pharmaceutically relevant as an antimicrobial [69], anticancer drug precursor [43, 70], and is useful in agriculture as a biopesticide [45, 71]. Furthermore, this branched chain alcohol has also been considered as a diesel or jet-fuel substitute due to its low water solubility, high-energy content, and relatively low volatility [45, 57].

Farnesol has been microbially produced using some naturally occurring microbes such as *Candida albicans*, which uses farnesol as a quorum sensing molecule [72]. However, the best production of farnesol has been achieved by the dephosphorylation of FPP in engineered *S. cerevisiae* and *E. coli* overexpressing MVA pathway genes [16, 45]. Efforts have been directed towards increasing the FPP pool size through pathway engineering and redirecting FPP flux to farnesol formation by downregulating competing pathways [45]. A titer of 135 mg/L was

reported in *E. coli* following heterologous expression of the MVA pathway and augmentation of IspA (FPP synthase; [42]). In developed strains of *S. cerevisiae,* titers up to ~5 g/L farnesol have been achieved after 215 h in 1-L fed-batch fermentations [73]. This farnesol production strain contained a mutation in the ERG9 gene that presumably led to an increased FPP pool and therefore increased farnesol production by nonspecific endogenous phosphatases, such as LPP1 and DPP1 [74, 75]. Further modifications included higher expression of HMG-CoA reductase (HMGR), found to be a key element in improved farnesol production [76].

FPP dephosphorylation is carried out by some endogenous promiscuous phosphatases, but can also be done by more specific sesquiterpene synthases [74, 75, 77]. However, the promiscuous phosphatases have high Km and low kcat values towards FPP due to their broad substrate specificity, and the farnesol synthases (usually of plant origin [77]) have poor expression in the microbial production host which limit the use of these enzymes. Protein engineering to improve the specificity of dephosphorylation and the soluble expression of farnesol synthases is likely needed to improve microbial farnesol production further [42].

3.4 Future Work

Microbial production of sesquiterpenes appears to be an attractive alternative to extraction from plants or chemical synthesis from petroleum-derived materials. However, the biological production of sesquiterpenes is still far from optimum because overexpression of the isoprenoid production pathway in the microbial host leads to toxicity and metabolic stress. Even though most sesquiterpenes are not toxic to the producing host, the toxicity of the intermediates leads to the reduced carbon flux to the final product and eventually leads to final yields quite lower than the theoretical maximum. Many studies have already identified many different pathway bottlenecks such as MK expression [34], HMG-CoA accumulation [25], and low activity of the terpene synthase [6, 67, 78] in microbial sesquiterpene production. Although partially addressed [25, 34, 79, 80], the unbalanced expression of the pathway is still a primary cause of low isoprenoid yields [81]. Therefore, further efforts to balance enzyme expression and enzyme screening/evolution seem a good strategy to follow in order to achieve higher yield of the final product.

4 Monoterpene Fuels and Chemicals

4.1 Monoterpene Properties and Chemical Diversity

Monoterpenes are C10 compounds built from two C5 isoprenoid units (one IPP and one DMAPP). Monoterpenes are generally synthesized in the glandular structures of plants and are thus common components of essential oils. As with sesquiterpenes,

advances in metabolic engineering now allow for the microbial production of diverse, monoterpene-derived oils, flavors, and pharmaceuticals [57]. Moreover, recent success in the microbial production of limonene and pinene have expanded the microbial platform to include jet biofuels [10, 78, 82].

Monoterpenes can be divided into three major subgroups based on structural features (Fig. 4): acyclic, monocyclic, and bicyclic monoterpenes. Acyclic monoterpenes include compounds such as myrcene and ocimene, which are hydrocarbons very similar to classical petrochemicals. Both are valuable compounds for the perfume industry, and myrcene is an especially versatile starting material for flavors, fragrances, cosmetics, vitamins, and pharmaceuticals due to its reactive diene structure [83]. The hydrogenated forms of these acyclic monoterpenoids (i.e., 2,6-dimethyloctane) have been proposed as alternative biofuels [10]. Hydroxylated acyclic monoterpenoids such as linalool and geraniol are also potential biofuels in addition to being important components in fragrances and pesticides [37, 84–86].

Cyclic monoterpenoids include a huge variety of molecules, most of which are derived from the α-terpinyl cation. As a result, many important flavor and medicinal compounds have the same carbon skeleton as limonene. Most notable derivatives of monocyclic monoterpenes are oxygenated compounds, such as α-terpineol, perillyl alcohol, carveol, carvone, and menthol [87]. The fully hydrogenated form of limonene, limonane, is considered a promising jet-fuel replacement [10], and recently limonene itself has been evaluated as a jet-fuel additive [88]. Among bicyclic monoterpenoids, there are pinenes, which are the main components of turpentine produced from tapping trees [10, 78, 89]. Many valuable compounds that are used in the fragrance, flavor, and drug industries are derived from pinenes. Pinene-derived verbenone, for instance, is used in perfumery, as a cough suppressant, and in insect control [84, 90, 91], and camphor is used as a flavor compound, plasticizer, cosmetic, and pharmaceutical [92]. Other bicyclic monoterpenoids include sabinene and related compounds such as thujone, thujene, and umbellulone which are valuable as flavoring agents, perfume components, antimicrobials, and potential biofuels [41, 93, 94], however, toxicity of these compounds might limit their use [95–97].

Although isoprenoid alternatives to diesel fuel have been identified and microbially produced, metabolic engineering for the production of high-energy density tactical fuels (jet and missile fuels, e.g.) has lagged behind. Existing biosynthetic jet fuels derived from natural oils have been used to power aircraft in 50:50 blends with Jet-A fuel [98, 99], but lack the specifications required to replace jet fuels such as JP-10 [78].

4.2 Pinene and Limonene

α, β-Pinenes and limonene are widely used in fragrances, drugs, and as commodity chemicals. Recently, the demand for these compounds has risen due to their suitability as renewable, high-density jet fuels [78, 98]. Attaining the volumetric energy

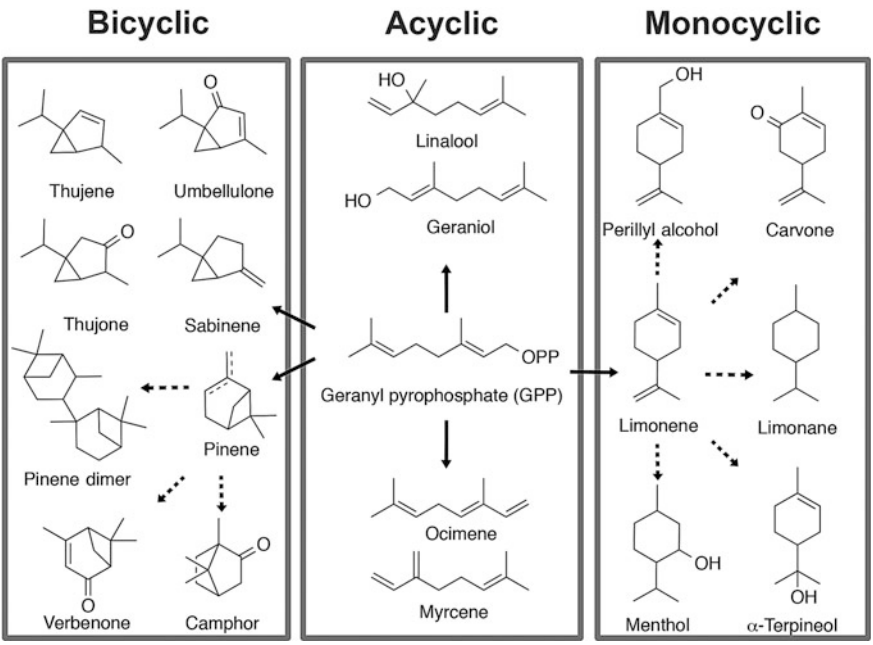

Fig. 4 Examples of monoterpenoids. All monoterpenoids are produced from the C10 precursor GPP (geranyl diphosphate). Depending on the terpene synthase, they can be acyclic, mono-, or bicyclic compounds. The *solid arrows* represent enzymatic processes, and the *dotted arrows* show downstream conversion by either chemical or enzymatic processes

content necessary for jet fuels requires mimicking the strained ring systems found in JP-10 [78]. Hydrogenated pinene dimers, synthesized via chemical dimerization of the bicyclic terpenes (pinenes), have been shown to contain high-volumetric energy similar to that found in JP-10 [4]. Similarly, the hydrogenated form of limonene has been reported to have favorable properties for next-generation jet biofuels and fuel additives that enhance cold-weather performance [10].

Currently, α-pinene and (+)-D-limonene are mainly obtained from the plant biomass of tapping trees (turpentine) or as a byproduct of orange juice production, respectively. However, fluctuations in their production from natural producers (i.e., plants) and subsequent cost limit their use as biofuels and chemical feedstock even though the demand for these monoterpenes is increasing [57, 78, 82, 87, 89]. Therefore, it is necessary to seek sustainable technologies for monoterpene production.

Although microbes can produce various isoprenoids through either MEP or MVA pathways to supply the essential metabolites DMAPP and IPP, they are usually unable to produce monoterpenes due to the lack of efficient geranyl diphosphate synthases (GPPS) and adequate monoterpene synthases. With the growing interest in these compounds, many metabolic engineers have explored biosynthetic methods for monoterpene production. A decade ago, the production of

the monoterpenes 3-carene and limonene was demonstrated through heterologous expression of carene synthase and limonene synthase (LS) of plant origin in *E. coli* [100, 101]. However, yields were low: production levels of 3 μg/L/OD600 and 5 mg/L were achieved for 3-carene and limonene, respectively. In these early works, the authors hypothesized that low precursor availability (i.e., IPP and DMAPP) was the primary cause of low titers [13, 100, 101]. Although the endogenous MEP pathway in *E. coli* may not be sufficient for high flux supply of IPP and DMAPP, the heterologous expression of the MVA pathway in *E. coli* considerably improves flux to isoprenoid precursors [13].

Two recent publications managed to produce pinene in *E. coli* at reasonably high titers by using a heterologous MVA pathway. In the first study [89], *α*-pinene production was significantly enhanced in *E. coli* by assembling a heterologous MVA pathway, codon-optimized GPPS2 from *A. grandis*, and codon-optimized pinene synthase (PS) Pt30 from *Pinus taeda*. The final pinene-producing strain, YJM28, accumulated α-pinene up to 5.44 mg/L in a shake-flask and 0.97 g/L under the fed-batch fermentation conditions. In more recent work, the authors used a previously engineered *E. coli* strain for the overproduction of IPP and DMAPP and combinatorially screened PS and GPPS enzymes to improve flux through the last two steps of the pathway [78, 102]. By combining expression of three PS and three GPPS from conifers, they achieved about 28 mg/L of pinene using GPPS and PS from *A. grandis*. Furthermore, they designed GPPS-PS protein fusions to reduce GPP product inhibition and toxicity by substrate channeling, producing 32.4 mg/L of pinene [78].

A platform for high titer production of limonene was also recently described [82]. As explained above for pinene production, a heterologous MVA pathway was used to provide IPP and DMAPP precursors and combined with GPPS from *A. grandis* and LS from *Mentha spicata*. In this study, a series of engineering steps yielded much higher titers of limonene than previously reported: the limonene titer was over 450 mg/L, comparable to those achieved for sesquiterpenes (500–1,000 mg/L). Despite using a similar platform, this titer is also considerably higher than that of pinene ($\sim$32 mg/L), probably because the LS enzyme expresses better and/or has higher efficiency than any of the PS enzymes tested.

Compared to sesquiterpenes, one of the main differences and concerns in the microbial overproduction of monoterpenes is the toxicity of monoterpene products [103, 104]. In fact, efforts to detect and overexpress efflux pumps to counteract monoterpene toxicity has proven useful in the improvement of yields [105].

4.3 Acyclic Monoterpenes

Efforts to produce acyclic monoterpenes microbially have focused primarily on geraniol due to its value in the fragrance, agrochemical, and pharmaceutical industries [43, 86, 106]. As a biofuel compound, geraniol ($C_{10}H_{18}O$) has similar properties to farnesol, a potential sesquiterpenoid biofuel alcohol [57]. Geraniol is

produced by dephosphorylation of GPP and has recently been produced in an engineered *E. coli* strain [86]. As practiced in other successful efforts, the authors engineered *E. coli* to have an exogenous MVA pathway for improved precursor production. They used an engineered GPPS mutated from native *E. coli* FPP synthase for the biosynthesis of GPP, and a truncated geraniol synthase from *O. basilicum* for efficient conversion of GPP to geraniol. After eliminating competing pathways, they achieved a final production titer of 182.5 mg/L of geraniol.

The hydrogenated products of acyclic monoterpenes such as myrcene and ocimene are also considered good biofuel replacements [10]. Microbial production of these acyclic monoterpenes, which originally are found in plants, has been reported in the product mixture of an engineered *E. coli* strain that produced various terpenes [101]. To our knowledge, however, specific microbial metabolic engineering efforts to overproduce these acyclic monoterpenes have not been reported. Because terpene synthases that synthesize these acyclic monoterpenes have been described [107], it is likely that microbial production with high product specificity will be achieved soon.

4.4 Future Work

In order to be considered to be economically competitive alternatives to petroleum-based fuels, near-theoretical yields would be required for biofuel production. To produce the monoterpene limonene, for example, it is known that the required microbial yields and titers significantly exceed current microbial limonene toxicity limits [105]. Clearly it will be necessary to remove the product continuously from the producing culture broth and/or engineer more tolerant strains.

There are various ways to overcome the product toxicity, but one of the most promising approaches for monoterpene production was the use of efflux pumps [105]. Efflux pumps are membrane transporters that recognize and export toxic compounds from the cell using the proton motive force. The best-studied solvent tolerance pumps are those from the resistance–nodulation–division (RND) family in gram-negative bacteria, but many other organisms also harbor efflux pumps [108]. Recent experiments have demonstrated that heterologously expressed RND efflux pumps can improve tolerance to biofuels [105]. Expression of efflux pumps is a promising engineering strategy to engineer tolerance for many isoprenoid biofuels, but there are several avenues for future research in this area. Directed evolution may help produce designer pumps that are specific to a particular biofuel, and fine-tuned efflux pump expression is also necessary inasmuch as it is well known that overexpression of membrane proteins can be detrimental [109, 110].

Though targeted efflux pump expression has been proven successful as a tolerance mechanism, traditional strain improvement methods such as chemostat-mediated adaptation, chemical mutagenesis, directed evolutionary engineering, genome shuffling [111], or targeted recombineering methodologies [112] constitute powerful tools for rapid evolution of tolerance mechanisms [105, 113]. It is also

important to recognize that the tolerance engineering strategies are not universal and what works for one biofuel in one host strain may not work for others [104]. Another alternative to withstand toxicity would be to engineer the pathway in alternative hosts such as *Pseudomonas* that are known to have better mechanisms to respond to stress [104, 114, 115].

5 Hemiterpenoid Fuels and Chemicals

5.1 Hemiterpenoid Properties and Chemical Diversity

Hemiterpenes and hemiterpenoids are C5 compounds derived from a single isoprene subunit. Efforts to produce hemiterpenoids in microbial hosts have primarily targeted isoprene, a volatile monomer of significance to the rubber industry. Recently, there has been interest in the biosynthetic production of C3–C5 alcohols as advanced biofuels [57, 116]. One successful approach for microbial C3–C5 alcohol production utilizes refactored amino acid biosynthesis pathways [117]. Broadly speaking, this strategy relies on the overexpression of a promiscuous 2-keto-acid decarboxylase (KDCs) and alcohol dehydrogenase (ADHs) to produce a diverse range of alcohols from amino acid precursors. The leucine biosynthesis pathway, for example, was used to produce 3-methyl-1-butanol or isopentanol at high titers (Fig. 5a; [118]). Isoprenoid biosynthesis pathways provide additional routes to C5 alcohol production, namely isopentenols (3-methyl-3-butenol and 3-methyl-2-butenol) and isopentanol (3-methyl-1-butanol; Fig. 5b). It was recently determined that these alcohols have energy content and octane numbers that make them potential gasoline replacements [119]. In addition to favorable energy content, the alcohols 3-methyl-3- and 3-methyl-2-butenol were recently shown to function as ideal antiknock additives in spark ignition engines [5].

5.2 Isopentenols

Isopentenols are derived from the dephosphorylation of IPP and DMAPP, which form 3-methyl-3- and 3-methyl-2-butenol, respectively (Fig. 5b). Although there have been numerous studies concerning the engineering of both the MEP and MVA pathways for the production of C10, C15, and longer-chained terpenes, work on isoprenoid-derived short-chain alcohols was comparatively minimal until their characterization as biofuel candidates.

Isopentenol was first detected in *E. coli* cultures following the overexpression of the native MEP pathway and was suspected to result from IPP dephosphorylation [120]. More recent work has identified genes capable of catalyzing this dephosphorylation reaction [121]. Using a heterologous MVA pathway and a screening

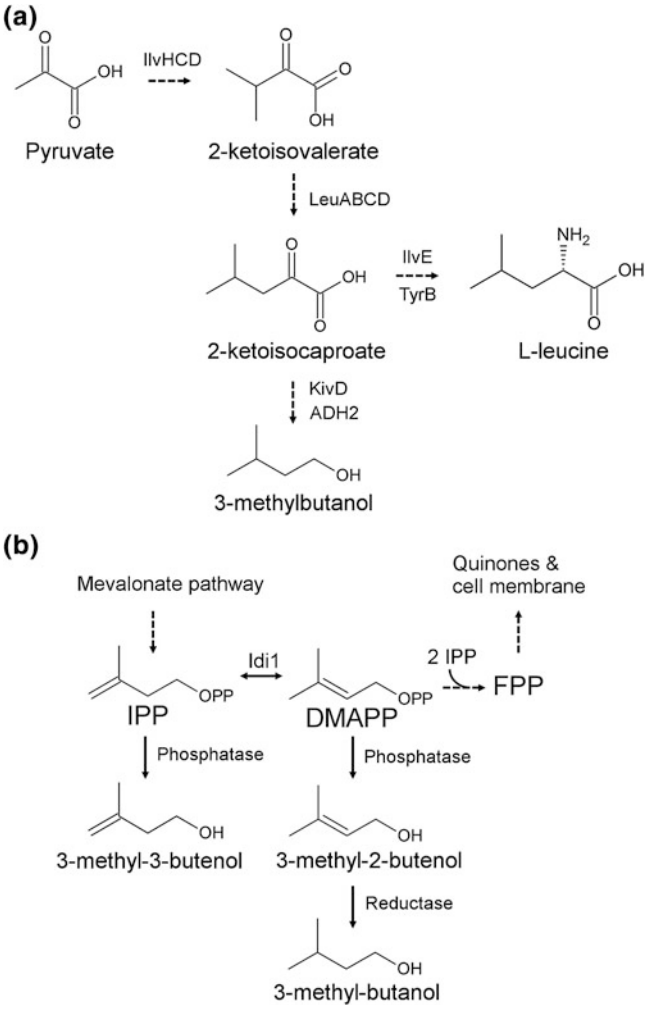

Fig. 5 Biosynthesis of five carbon (C5) alcohols. **a** Amino acid biosynthesis (or keto-acid) route to 3-methyl-butanol. **b** Isoprenoid biosynthesis route to three C5 alcohols

method based on prenyl diphosphate toxicity (see Sect. 2.3), Withers et al. demonstrated that expression of the *B. subtilis* gene *nudF* resulted in the production of isopentenol at relatively high concentrations ($\sim$110 mg/L). In a later study, NudF was compared with alternative phosphatases in *E. coli* in an effort to assemble a synthetic pathway for C5 alcohol production [122]. In this work, efficient IPP phosphatase activity was discovered by screening HAD [123] and Nudix [124] superfamilies of enzymes. Out of 23 HAD-like phosphatases and 13 Nudix hydrolases in *E. coli*, the Nudix hydrolase NudB was found to be the most efficient enzyme. By expressing NudB on a high-copy vector with pMevT and pMevB [13],

3-methyl-3-butenol was produced at 8.3 % of theoretical yield. Using a fusion protein of Idi1 and NudB, IPP-derived 3-methyl-3-butenol and DMAPP-derived 3-methyl-2-butenol were produced. The total C5 alcohol content was reduced from the strain expressing only NudB, however, and was hypothesized to result from competition with FPP biosynthesis. By expressing a promiscuous *E. coli* reductase (NemA) with the fusion protein, 3-methyl-2-butenol could be reduced to 3-methyl-1-butanol, resulting in the production of three C5 alcohols (Fig. 5b).

Optimizations to the heterologous mevalonate pathway have resulted in significantly higher yields of isopentenol in *E. coli.* Replacement of *HMGS* and *HMGR* from *S. cerevisiae* with versions of *Enterococcus faecalis* improved mevalonate production 45-fold [125]. Expression of this upper pathway with *MK*, *PMK*, *PMD*, and *nudF* from *B. subtilis* resulted in the production of 1.3 g/L of 3-methyl-3-butenol at a yield of 12 % [125]. When *idi* was also expressed, a mixture of 3-methyl-3- and 3-methyl-2-butenol was produced, albeit at a reduced total C5 alcohol yield as observed previously [122]. With NudF from *E. coli*, 3-methyl-2-butenol was produced with higher specificity, but at a significantly lower yield [125].

More recently, 3-methyl-3-butenol was produced at a yield of 46 % following extensive pathway engineering [81]. Using a systematic method, rationally constructed variants of the precursor pathway were assayed for pathway protein levels, glucose, acetate, and isopentenol. According to a correlation analysis, variations in the level of HMGS and MK explained the majority of pathway behavior. Using this information, a conceptual model of isopentenol pathway function was developed and used to guide engineering efforts. With the expression of *E. coli*-derived NudB, the most efficient pathway produced 1.5 g/L of 3-methyl-3-butenol at 46 % yield, consumed all available glucose, and produced low amounts of the waste-product acetate.

5.3 IPP Toxicity

Inasmuch as IPP is the immediate precursor of isopentenol, preventing or overcoming IPP toxicity is an important issue to consider when engineering isopentenol pathways. IPP toxicity was first identified a decade ago, when a high-flux mevalonate pathway was assembled in *E. coli* [13]. The kinetics of NudB, the protein used to produce the highest reported titer of isopentenol [81], show that this enzyme is quite slow, with a turnover rate significantly less than other mevalonate pathway enzymes [122]. When this enzyme was paired with a high-flux precursor pathway, IPP accumulation and toxicity were observed [81]. Specifically, cell growth and glucose consumption were attenuated when IPP concentrations were high. Expression of an additional copy of *nudB* alleviated this toxicity, albeit at a significant increase to metabolic burden. A similar reduction in OD was also observed using NudF from *B. subtilis*, though IPP measurements were not taken to confirm its accumulation [125]. Due to this toxicity, the development of more efficient

hydrolases to catalyze isopentenol production is needed to maximize productivity. In addition, the mechanism of IPP toxicity should be studied to aid in tolerance engineering.

5.4 Future Work

The initial progress in the microbial production of hemiterpenes is encouraging: in shake-flask experiments, yields of hemiterpene alcohols approach or exceed the reported yields of monoterpene and sesquiterpene biofuels. In addition, the prospect of creating hemiterpene derivatives with attractive properties appears relatively straightforward and has yet to be explored. Through expression of alcohol acetyl transferases, for example, esters of C5 alcohols with promising fuel properties such as isoamyl acetate [126–128] can be readily produced.

Additional work is needed to increase the specificity of alcohol production. Fully reduced isopentanol, the most attractive target from a biofuel perspective, can currently only be produced as a component of a three-alcohol mixture at very low yields. Mitigation of carbon loss to FPP through a genetic knockdown of *ispA* may improve yields, and further engineering of the previously described Idi1 ~ NudB fusion [122] may improve flux to 3-methyl-2-butenol and subsequently isopentanol. Altering the substrate preference of NudB or NudF, both of which have native substrates that differ significantly from IPP and DMAPP [124], could also increase yields by providing improved product specificity. Finally, an improved reductase may be capable of increasing the conversion of 3-methyl-2-butenol to isopentanol.

6 Outlook

In this review chapter, we have summarized some of the engineering efforts towards the microbial synthesis of isoprenoid-based drugs, chemicals, and fuel compounds. The incredible diversity of isoprenoid compounds provides nearly countless target molecules for biological engineers. Though many challenges exist, even complex medicinal isoprenoids such as artemisinin and Taxol may eventually be produced entirely in vivo. The complete biosynthesis of these medicinal isoprenoids frequently requires several oxidations of the original terpene backbone, mostly by cytochrome P450 enzymes as shown for artemisinin and Taxol [15, 18]. Because it is important to carry out these P450-based oxidations in the engineered host, further study of P450 enzymes is critical to build a complete biological route for these compounds. P450s are frequently found as membrane-bound enzymes and require colocalized CPR (cytochrome P450 reductase) as a reductase partner. Although *E. coli* has been useful as a high-titer platform for terpene biosynthesis, achieving efficient P450 activity in this host has been challenging. The complex folding of P450 proteins, coupled with *E. coli*'s requirement for membrane-unbound, soluble

P450s, has severely limited progress. However, recent progress on the directed evolution of bacterial P450 enzymes has yielded P450 candidates that are functional in *E. coli* and have specific activity towards terpene targets.

Considerable progress has been made in the microbial production of isoprenoid-based advanced biofuels. With a few exceptions, however, most compounds are far from commercialization. There are several risks in commercialization such as capital risk, technology risk, market risk/volatility, and operational risk. To overcome these risks and prove economic feasibility are the most important considerations in commercialization. The technoeconomic analysis on feedstock to fermentable sugar and to biofuel suggests that there are many variables to consider such as feedstock prices, biomass depolymerization costs, the yield of the microbial process for biofuel production, and the scalability [129]. For example, when we assume a break-even price of sugar at the mill to be close to $0.10/lb, which is close to the long-term nominal price of the commodity, a rough calculation of the theoretical price of microbial sesquiterpene biofuel gives about $6/gal of biofuel based on data for ethanol production when we assume near 100 % theoretical yield for sesquiterpene production [6]. Due to these challenging economics, high product yields (generally at least 85 % of the theoretical maximum yield) are absolutely essential if biofuels are to be competitive with nonrenewable, petroleum-based fuels [57].

Optimization of the isoprenoid pathway itself is perhaps the most direct way to improve titer, yield, and productivity. A common theme that has emerged in isoprenoid pathway optimization is the requirement for balanced enzyme expression. In recent years, analytical tools such as targeted proteomics [34, 81] and metabolomics have paired with genetic standardization and modern cloning techniques to make this task far easier. In most cases, pathway balancing has led directly to improvements in titer [15, 25, 34, 81]. For C10 and C15 terpenoids, the slow kinetics of most terpene synthases poses another significant barrier to economical production. The construction of more efficient terpene synthases through protein engineering or directed evolution will undoubtedly provide further improvements in yield and productivity. Though pathway optimization is crucial, interactions with the microbial host must also be considered: competing pathways must be eliminated and perturbations to cellular central metabolism, redox balance, and energetics must be minimized. Systems biology and metabolic modeling hold particular promise for addressing these challenges.

Another important aspect to address is the scalability of microbial production inasmuch as it has a huge impact on the overall economics and feasibility of isoprenoid biofuel production. Production data from high-volume, fed-batch fermentations are necessary to assess the feasibility of large-scale production. At large scale, the addition of exogenous antibiotics or inducers becomes cost-prohibitive. Recently developed technologies that alleviate the requirement for both antibiotics [130] and inducers [35] should thus be used to improve pathway stability and lower production costs. Because successful scale-up is critical—and because the conditions for optimal production may change when fermentation takes place in a bioreactor—dynamic promoters and expression systems that respond to environmental queues are particularly desirable. Ideally, the cell should be able to adjust the

pathway activity according to the metabolic status of the host or the concentrations of key pathway intermediates [131]. Although a successful example was recently reported for sesquiterpenoid production [35], further applications of dynamic regulation could potentially eliminate the accumulation of toxic intermediates, increase strain stability, and improve pathway efficiency.

The high cost of carbon sources such as purified glucose is detrimental to biofuel cost and must be reduced to achieve economical production. Cheaper, more abundant carbon sources are clearly required. Lignocellulosic biomass is perhaps the most promising due to its availability, and work is underway to construct microbes that efficiently metabolize plant-derived sugars. Sucrose serves as another promising alternative to purified glucose. In countries such as Brazil and Australia, sucrose is currently used as a major carbon source in the biobased chemical and fuel industry. Most native *E. coli* hosts, however, cannot metabolize sucrose, and engineering this capability is necessary. More recently, photosynthetic autotrophic microbes have been studied for isoprene production, and methanotrophic bacteria are being investigated as shale gas is increasingly available as a cheap and abundant carbon source.

Although we have focused primarily on simple isoprenoids in this review, there are more complex natural terpenoids that have important medicinal value and are potential targets for biological and metabolic engineers. Indeed, it may soon be possible to produce valuable new classes of complex isoprenoid derivatives biosynthetically due to recent advances in genomics and related disciplines. Meroterpenoids, oxygenated medicinal terpenoids biosynthesized from polyketide and terpenoid precursors [132], serve as attractive examples. As genomic and metagenomic sequencing data become more abundant and broadly applicable to natural products discovery, there have been efforts to understand and engineer meroterpenoid biosynthesis in bacteria and fungi [133, 134]. Though less genetically tractable, fungal systems are particularly promising for meroterpenoid production as they usually have a larger range of functional P450 enzymes and contain endogenous polyketide synthases and isoprenoid pathways [135]. With proper engineering, these fungal hosts will provide a promising route to improved terpene and medicinal meroterpenoid production in non-*E. coli*, nonyeast hosts.

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