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reviews

Terpenoids: Opportunities for Biosynthesis of Natural Product Drugs Using Engineered Microorganisms

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Abstract: Terpenoids represent a diverse class of molecules that provide a wealth of opportunities to address many human health and societal issues. The expansive array of structures and functionalities that have been evolved in nature provide an excellent pool of molecules for use in human therapeutics. While this class of molecules has members with therapeutic properties including anticancer, antiparasitic, antimicrobial, antiallergenic, antispasmodic, antihyperglycemic, anti-inflammatory, and immunomodulatory properties, supply limitations prevent the large scale use of some molecules. Many of these molecules are only found in ppm levels in nature thus requiring massive harvesting to obtain sufficient amounts of the drug. Synthetic biology and metabolic engineering provide innovative approaches to increase the production of the desired molecule in the native organism, and most importantly, *transfer the biosynthetic pathways to other hosts*. Microbial systems are well studied, and genetic manipulations allow the optimization of microbial metabolisms for the production of common terpenoid precursors. Using a host of tools, unprecedented advancements in the large scale production of terpenoids have been achieved in recent years. Identification of limiting steps and pathway regulation, coupled with design strategies to minimize terpenoid byproducts wih a high flux to the desired biosynthetic pathways, have yielded greater than 100-fold improvements in the production of a range of terpenoids. This review focuses on the biodiversity of terpenoids, the biosynthetic pathways involved, and engineering efforts to maximize the production through these pathways.

Keywords: Metabolic engineering; natural products; terpenoids; microbial cells

1. Introduction

There has been remarkable recent interest in mining the immense chemical diversity in secondary metabolites for their potential in the treatment and prevention of human disease and to meet other societal needs.[1–10] As desirable molecules are found, the need to engineer the biosynthesis of these small molecules in their natural hosts or in surrogate microbes to produce the molecules in satisfactory quantities has become more apparent.[11–14] The scientific community

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has begun to re-explore the various aspects of secondary metabolite natural products as evident by the abundance of recent review articles in prominent journals.[1–32]

Natural products have undergone the evolution process for a specific purpose of interacting within a biological system, as attractants, repellents, defenses, and a host of other important ecological functions. These molecules efficiently interact with proteins, DNA, and other biological molecules in nature to produce a desired outcome, which could be exploited for the future design of natural products-derived therapeutics. Thus by sampling drug candidates from natural products, it allows the investigator to select the desired subset of chemical diversity that is known to interact with biological systems.

To better understand the impact of natural products on pharmaceuticals, one can look at our current arsenal of small molecule chemotherapeutics. Out of 155 small molecules used as chemotherapeutics, 47% are directly taken from the natural products and an additional 26% are derivatives or synthetic natural product mimics.[33] In contrast to this apparent dominance of natural products in pharmaceuticals, over the last two decades synthetic combinatorial chemistry and associated high throughput drug discovery screening yielded only one *de novo* chemical which was approved for drug use. Hence, it is not surprising that the current chemical-based drug discovery is now focusing on introducing structural and chemical diversity to natural product scaffolds to identify novel therapeutic molecules.[34]

Technological advancements made through genomics and systems biology research have put forward a new paradigm in natural products discovery and biosynthesis.[1,3,15,20,28,29] Natural products discovery can now be aided by the wealth of genomic sequence information that has become available. By combining the genomic information with the ecological mechanisms by which these natural products work, bioinformatics approaches can begin to predict a list of possible interactions of these molecules with biomolecules in humans. This information will be valuable, to predict both mechanisms of activity against disease and toxic side effects.

Supported by omics technologies, metabolic engineering and synthetic biology now allow the manipulation and

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engineering of the biosynthetic pathway of the natural product in the native host, as well as in surrogate microbial hosts.[15,20,29] Although the biopharmaceutical production using this novel engineering-driven biology is only beginning, the future promises are visible: the advent of computational systems biology to dissect and analyze the complexity of the biosynthetic pathways and associated control mechanisms, the creation of systematic enzyme classification schemes for the automated design of new pathways, *de novo* DNA synthesis for the design and construction of DNA involved in the biosynthetic pathways, the application of *de novo* evolutionary methods for generating new functional enzymes, the design of well-characterized enzymatic pathways from genetic to proteomic level and the assembly of the natural products biosynthetic pathways in easily cultured, productive hosts.

The aforemnetioned efforts provide a very powerful knowledge and technology basis for building a complete systems biology approach to (1) identify therapeutics molecules and (2) design and construct *de novo* pathways for the production of these molecules. Together, this increases our capacity to produce known biopharmaceuticals, as well as providing sufficient quantities of scaffolds for the use in diversity-oriented combinatorial chemical synthesis for new pharmaceutical molecules. Interestingly, metabolic engineering and synthetic biology driven biosynthesis efforts are well suited for three essential natural product molecules, polyketides, flavanoids, and terpenoids, which include several well-characterized and approved pharmaceutical molecules.[14–18] This review focuses on terpenoid secondary metabolites. A brief history of terpenoids and their potentials as pharmaceuticals, with more insights into current and future approaches to engineer microorganisms as terpenoid production platforms using metabolic engineering and synthetic biology for commercial scale production, are delineated.

2. A Brief Description of the Chemical and Structural Diversity of Terpenoids and Their Roles in Nature

Secondary metabolites produced by plants and microorganisms are of great interest owing to their tremendous variety of structural and functional diversity.[10] Among the various secondary metabolites, terpenes represent one of the largest and most diverse classes of secondary metabolites.[35] Terpenes exist in diverse chemical forms in an exotic array of linear functionalized hydrocarbons or chiral, carbocyclic skeletons with diverse chemical modifications such as hydroxyl, carbonyl, ketone, aldehyde and peroxide groups.[36] The functionally modified terpenes are commonly referred to as terpenoids or isoprenoids. The enormous diversity of structures is responsible for their diverse functional roles.[22,36]

More than 55,000 terpenes have been isolated, and this number has almost doubled each decade.[35,37] The diverse functional roles of some of the terpenoids are characterized as hormones (gibberellins), photosynthetic pigments (phytol, carotenoids), electron carriers (ubiquinone, plastoquinone), and mediators of polysaccharide assembly, as well as communication and defense mechanisms.[38] While the previous classes of terpenes often act within the organism, several terpenoid molecules act between organisms as toxins, repellents or attractants and play a more defensive role in the existence of the organism.[39–44] The low-molecular-weight, lipophilic nature of numerous monoterpenes and sesquiterpenes, combined with the vast structural variety and high vapor pressures at ordinary temperatures, account for its role as chemical conveyors of information. Many others may have no apparent function in the basic processes of growth and development of the producing organism; however, it has crucial ecological roles related to the existence of the organism.[45,46]

Human use of terpenes in the environment can range from environmentally benign to disastrous. A benign example is their insecticide usage, which is environmentally friendly compared to many synthetic insecticides due to its limited persistence in the environment and negligible toxicity to

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mammals and birds.[47] An impressive, albeit environmentally disastrous, demonstration of the influence of terpenoids on the ecosystem can be seen in the case of the green seaweed *Caulerpa taxifolia*, which is widely used for aquarium decoration. *C. taxifolia* contains an array of mono- and sesquiterpenes that exhibit ichthyotoxic, antibiotic,[48,49] neurotoxic[50,51] and cytotoxic[52,53] properties. *C. taxifolia* is outcompeting native algae and is spreading quickly due to its enormous growth rate and its ability to produce toxic terpenoids against predators and fouling organisms. This has subsequently affected the biodiversity by reducing the population of sea-urchins, fish, amphipods and polychaetes, in areas of the coastline of several Mediterranean countries (Croatia, France, Italy, Monaco, Spain and Tunisia) and spreading into the coasts of southern California.[54,55] The chemical and structural diversity of terpenes and their related functional role are extensively described in the literature.[56–69]

3. Terpenoids for the Prevention of Human Disease and Other Societal Needs

Though investigations into the functional biology of terpenoids have lagged far behind practical applications in medicine, agriculture, and industry, the use of terpenoids as treatment for various kinds of diseases can be seen throughout history.[35] Currently, a broad range of biological responses can be elicited in humans through various terpenoids that are applicable to human health care.[70] Different terpenoid molecules have antimicrobial, antifungal, antiparasitic, antiviral, antiallergenic, antispasmodic, antihyperglycemic, anti-inflammatory, chemotherapeutic, and immunomodulatory properties.[70–74] Terpenes are also used as skin penetration enhancers, as well as natural insecticides and can be of use as protective substances in storing agriculture products.[75,76]

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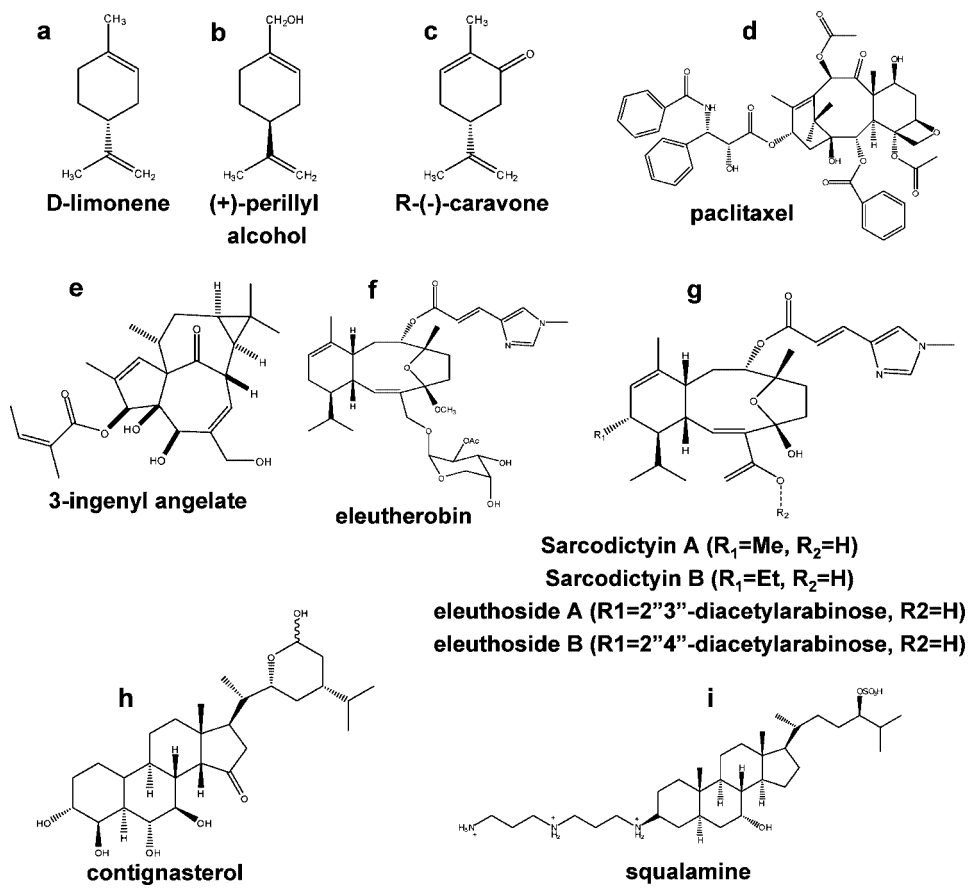

Figure 1. Examples of chemical and structural diversity in anticancer terpenoids.

Terpenoids as Anticancer Agents. Many studies have shown that several of the dietary monoterpenes are effective in the prevention and treatment of cancer.[77–80] Among these, monocyclic monoterpenes D-limonene (Figure 1 (**a**)) and perillyl alcohol (Figure 1 (**b**)) are known to inhibit the development of mammary, liver, skin, lung, colon, forestomach, prostate, and pancreatic carcinomas.[81–83] The metabolites of D-limonene such as perillic acid, dihydroperillic acid, limonene-1,2-diol, and the oxygenated molecule of D-limonene, carvone (Figure 1(**c**)), have also been shown to have anticancer activities.[80–86] The mechanism of the monoterpene antitumor effects is the inhibition of posttranslational isoprenylation of proteins regulating the growth of

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cells.[87] Terpenes, such as farnesol and geraniol, have also been shown to have chemotherapeutic activities toward human pancreatic cancers.[87,88] Moreover, monoterpenes such as carveol, uroterpenol, and sobrerol have shown activity against mammary carcinomas. Carvone (Figure 1 (**c**)) has been analyzed as an agent reducing pulmonary adenoma and fore-stomach tumor formation.[89,90] Several plant triterpenes exhibited *in vitro* antitumor activity. Betulinic acid has been shown to induce apoptosis of several human tumor cells, including melanoma and glioma, and ursolic acid and oleanolic acid reduced leukemia cell growth and inhibited the proliferation of several transplantable tumors in animals.[91]

Plant derived diterpenoids are the most effective anticancer agents approved by the FDA. Paclitaxel (Figure 1 (**d**)), a complex diterpenoid from the bark of yew, is a potent antimitotic agent with excellent activity against breast and ovarian cancers,[92] as well as AIDS-related Kaposi's sarcoma.[93] It binds tubulin heterodimers, promotes and stabilizes microtubule assembly, and stops the division of cancer cells.[94] Another recently identified, plant derived, topical chemotherapeutic for the treatment of skin cancer is 3-ingenyl angelate (Figure 1 (**e**)), a hydrophobic diterpene ester isolated from the plant *Euphorbia peplus*. 3-Ingenyl angelate causes rapid mitochondrial disruption and cell death by primary necrosis, and ultimately results in a favorable cosmetic outcome.[95]

Eleutherobin (Figure 1 (**f**)) is a glycosylated diterpene isolated from a marine soft coral, *Eleutherobia* sp., and is a potent anticancer agent that inhibits cell proliferation through promotion of tubulin polymerization into microtubules in a manner analogous to that of paclitaxel and binds to a site on microtubules that overlaps the paclitaxel binding site.[96] Like paclitaxel, eleutherobin also induces mitotic arrest, formation of multiple micronuclei, and microtubule bundling in cells.[97] Other terpenoid compounds from marine organisms such as sarcodictyin (Figure 1 (**g**)) and contignasterol (Figure 1 (**h**)) derivatives have been demonstrated to be active against cancer in preclinical or clinical studies.[98] Squalamine (Figure 1 (**i**)) is another terpenoid identified as a good candidate for drug development.[99,100] The compound has advanced into phase II clinical trials as an anticancer agent against nonsmall cell lung cancer and ovarian cancer. These terpenoid anticancer compounds have greater efficacy and safer toxicity profiles than synthetic alternatives. New molecules that are derivatives of squalamine are showing even better biological properties. Though many of these drugs have promising applications, few of these terpenoids can be obtained in large quantities from natural sources, thereby limiting their effective deployment in treating human disease.

Terpenoids with Anti-Inflammatory Activities. In addition to anticancer, there is a pool of terpenoids known for their anti-inflammatory properties.[101] There have been many monoterpenes, such as linalyl acetate, 1,8-cineole (Figure 2

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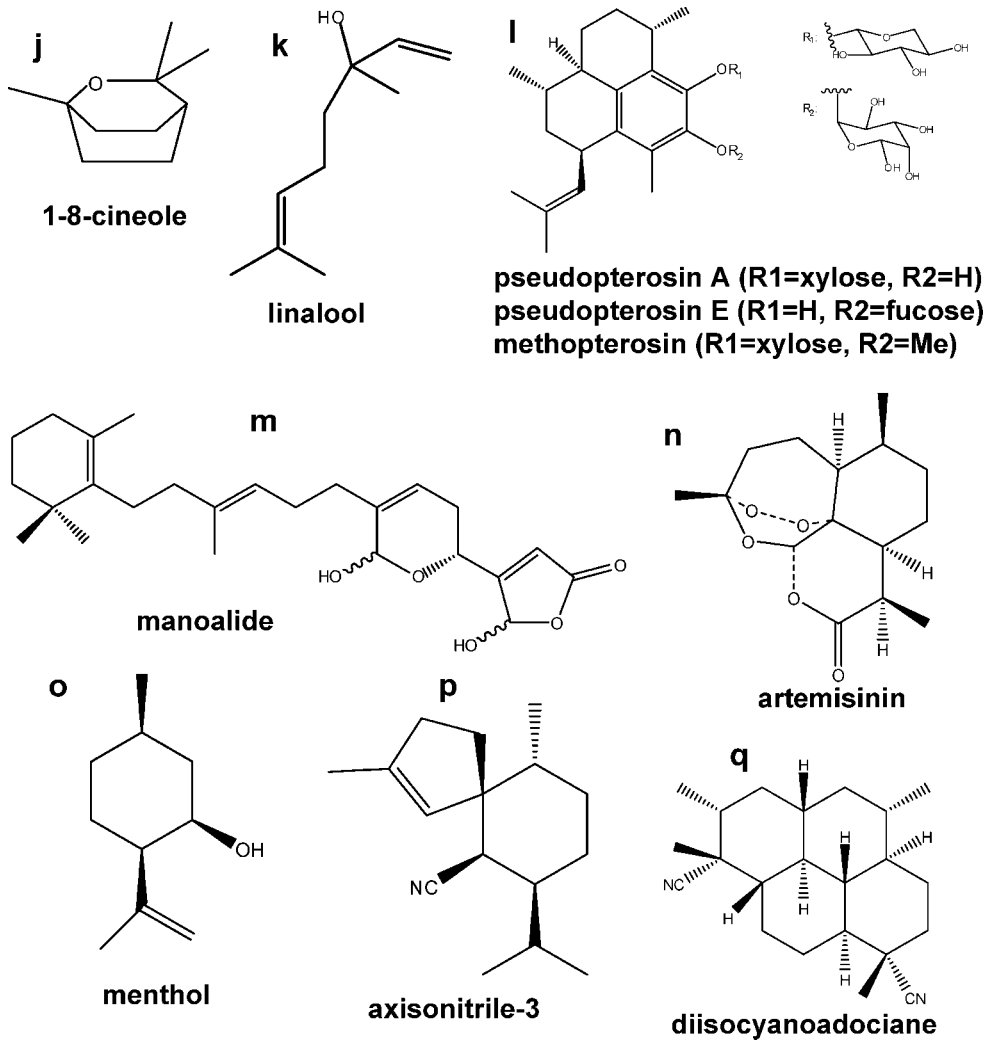

Figure 2. Examples of terpenoids with anti-inflammatory, antiparasitic, and antibacterial activities.

(**j**)), (−)-linalool (Figure 2 (**k**)), and its esters, that possess anti-inflammatory activity.[102,103] Particularly, 1,8-cineole was found to be useful in curing chronic ailments such as bronchitis sinusitis and steroid-dependent asthma, or as a preventive agent in returning respiratory infections.[104] The anti-inflammatory pseudopterosins (Figure 2 (**l**)) are diterpene glycosides with an amphilectane skeleton and were originally isolated from the gorgonian coral *Pseudopterogorgia elisabethae*.[105] Recently, several new pseudopterosin derivatives and seco-pseudopterosins were isolated from *Pseudopterogorgia* species.[106] It is interesting to note that the anti-inflammatory potential of pseudopterosins is superior to that of standard drugs such as indomethacin.[101] Contignasterol (Figure 1 (**h**)) from marine sponge, *Petrosia contignata*, and manoalide (Figure 2 (**m**)), another sponge-derived natural terpenoid, exhibit anti-inflammatory activities. Several plant derived triterpenoids, lupane, oleane, ursane, and their natural and synthetic derivatives, have also been identified as anti-

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inflammatory.[107] Another clinically proven anti-inflammatory terpenoid is the pentacyclic terpene α-amarin.[108]

Antiparasitic and Antibacterial Terpenoids. A variety of terpenoids have been described as antiparasitic agents with high efficacy and selectivity.[109,110] The most widely used parasitic drug in the world is the sesquiterpene lactone artemisinin (Figure 2 (**n**)), extracted from *Artemisia annua*, an herb, which is native to China.[111] This herb has been used for malaria therapy in China for over 1000 years. Artemisinin has a peroxide bridge to which its antimalarial properties have been attributed. It has a unique structure that lacks nitrogen containing heterocyclic rings commonly found in most antimalarial compounds. The detailed activity studies of artemisinin and its derivatives, especially in combination with synthetic antimalarial substances, have been shown to treat multidrug resistant strains of the malarial parasite.[112] Other sesquiterpene peroxides, such as yingzhaosu A and yingzhaosu C, also showed antiparasitic activity, especially against *Plasmodium berghei*.[113]

A group of monoterpenes, espintanol and piquerol A, have been found to have some antiprotozoan parasite activity. The monoterpene phenol derivative of cymene, thymol, and its structural derivatives also possess an antileishmanial potential.[114] Menthol (Figure 2 (**o**)) derivatives have also been described to possess trypanocidal activity.[115] Diterpenes and their lactones, e.g. dehydroabietinol isolated from *Hyptis suaveolens*, have been shown to have antimalarial activity.[116] Diterpenes with a nor-abietane skeleton had leishmanicidal and antiplasmodial action.[117] Betulinic acid has been described to have antimalarial activity.[118] Marine sponge *A. klethra* yielded axisonitrile-3 (Figure 2 (**p**)), an unusual and irregular terpene with an isonitrile group that was found to have antiplasmodial activity.[119] Another marine sponge diterpene, diisocyanoadociane (Figure 2 (**q**)), was found to have more pronounced antiplasmodial activity compared to axisonitrile-3. However, both of these compounds have been accompanied by some cytotoxicity.

There are many other marine sponge diterpenes such as kalihinol A that are known for their antiparasitic activity. Many terpenes have been found to be active against a variety of microorganisms.[120] Squalamine (Figure 1 (**i**)), an anticancer agent, has been reported to be a potent antibacterial, antifungal, and antiprotozoic.[99] Diterpenes extracted from *Salvia* species have exhibited antibacterial activities against a variety of organisms such as *S. aureus, S. epidermis, E. faecalis, B. subtilis, E. coli,* and *P. mirabilis*.[121] Monoterpene mixtures of terpinen-4-ol, α-terpineol, 1,8-cineole, and linalool have been shown to possess antibacterial activity against Gram-positive and Gram-negative bacteria isolated from the oral cavity, skin, and respiratory tract. The mechanism of antimicrobial action of terpenes is closely associated with their lipophilic character.[122] The order of the antibacterial activities of some of the monoterpenes against *S. aureus* is farnesol > (+)-nerolidol > plaunotol > monoterpenes (e.g., (−)-citronellol, geraniol, nerol, and linalool). The antiparasite monoterpene, phenol and thymol, demonstrated high antibacterial activity against *S. aureus*.[123] Similarly, the monoterpene, (+)-menthol, has shown anti-

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bacterial activity against *S. aureus* and *E. coli*.[123,124] Diterpenoid ferruginol and a few sesquiterpenes (including their lactones) have been reported to possess antimycobacterial activity.[125] Terpenes also display antifungal activity; one excellent example is the optical isomers of carvone, found to be active toward many kinds of human pathogenic fungi. Carvone and perillaldehyde inhibited the transformation of *Candida albicans* from the coccal to the filamentous form, which is responsible for the pathogenicity of the fungus.[86]

Terpenoids for Other Health Care and Societal Use. In addition to the aforementioned medicinal roles, terpenoids are useful as skin penetration-enhancing agents (for improving transdermal drug delivery) and as supplementary agents in topical dermal preparations, cosmetics, and toiletries, which further broadens the applications of terpenes in other areas of human health care and medicine. Terpene compounds possess several advantages, such as good penetration-enhancing abilities, low skin irritation effects, and low systemic toxicity.[125] The greatest penetration enhancement activity has been shown for monoterpenoid cyclic ethers, such as 1,8-cineol, as compared to hydrocarbon or even alcohol or ketone functionalized terpenes.[126] However, hydrocarbon terpenes, e.g. D-limonene, have already been approved as an active enhancer for steroids. Terpenes are also effective as skin permeability enhancers for the lipophilic molecule indomethacin and for hydrophilic diazepam and propranolol.[127]Moreover, monoterpenes such as linalool, carvone, and thymol were also demonstrated to enhance the permeability of model drugs such as 5-fluorouracil (5-FU) through skin and mucous membranes. The hydrophilic sesqueterpene, nerolidol, and monoterpenoid cyclic ether 1,8-cineole improved permeability 20-fold and 95-fold over D-limonene, respectively.[129] The order of skin penetration effectiveness of terpenes has been established as follows: cineole > menthol > menthone ≈ pulgeone ≈ α-terpineol > carvone > vehicle (66.6% ethanol in water) > water.[129] Generally, nonpolar terpenes such as D-limonene provide better enhancement for lipophilic agents than polar terpenes; however, terpenes containing polar groups, e.g. menthol and 1,8-cineole, enable hydrophobic permeants to traverse the human skin much more easily than terpenes without polar groups.

Finally, the use of terpenoids as flavors and fragrances in foods and cosmetics (e.g., menthol, nootkatone, linalool, and sclareol) has been known for centuries.[35] The industrial uses of monoterpenes as substitutes for ozone-depleting chlorofluorocarbons is also promising.[130] Terpenes have also been proposed as substitutes for chlorinated solvents in applications such as cleaning electronic components and cables, degreasing metal, and cleaning aircraft parts.[131]

4. A Description of the Terpenoid Biosynthetic Pathway: Converting Sugars to Pharmaceuticals

Terpenoids are present in all living organisms and are derived from the branched C5 carbon skeleton of isoprene. Each isoprenoid is constructed using a different number of repeats of isoprene, cyclization reactions, rearrangements, and further oxidation of the carbon skeletons.[132,133] The branched unsaturated diphosphate isoprene units, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), are the universal precursors in the metabolic pathway for terpenoids (Figure 3). Two biosynthetic routes to IPP and DMAPP have been characterized, the classical acetate mevalonate (MVA) pathway and the triose phosphate utilizing non-mevalonate pathway (Figure 3).[134,135] The non-mevalonate pathway is also called the 1-deoxy-D-xylulose-5-phosphate (DXP) or the 2*C*-methyl-D-erythritol-4-phosphate (MEP) pathway. Compared to the mevalonate pathway, which was completely described in 1967,[136] the non-mevalonate pathway was only characterized a few years ago.[137] The mevalonate pathway is used in eukaryotes (all mammals, the cytosol and mitochondria of plants, and fungi),

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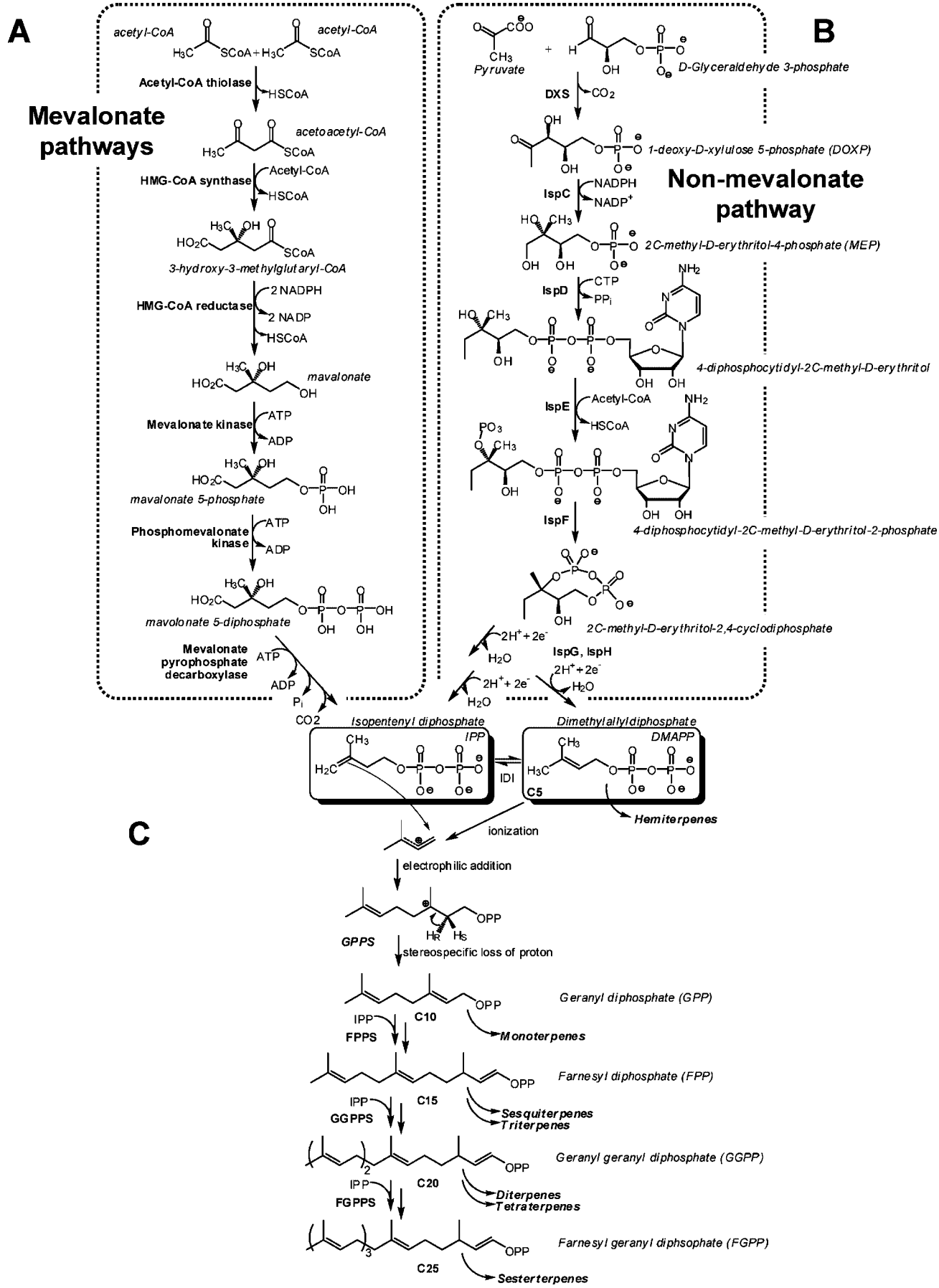

Figure 3. Schematic illustration of biosynthetic pathways to terpenoids.

archaea, and a few eubacteria.[138] The non-mevalonate pathway is present mainly in eubacteria, cyanobacteria, green algae, apicomplexan parasites, and higher plants.[135]

In higher plants, both MVA and MEP pathways are operative. The production and control mechanisms of terpenoid synthesis have been partially elucidated. It appears that the MEP pathway is plastid-related and the mevalonate pathway is localized to the cytosol.[139] Triterpenes, including the sterol precursor, cycloartenol, are known to be produced by means of the mevalonate pathway in the cytoplasmic compartment of plant cells. A large number of mono- and diterpenes are produced in the plastid compartment by means of the non-mevalonate pathway. The picture is complicated by the exchange of certain terpene precursors between the two compartments. The crosstalk between the two pathways leads to terpenes synthesized in plastids that can be derived, in part, from the cytoplasmically located mevalonate pathway intermediates and vice versa.[140–142]

The Mevalonate Pathway. The mevalonate pathway uses seven enzymatic reactions to convert the precursor acetyl-CoA to IPP and DMAPP (Figure 3). The first enzymatic bioreaction in the pathway involves Claison ester condensation between two molecules of acetyl-CoA for the production of acetoacetyl-CoA catalyzed by the acetoacetyl-CoA thiolase (AcSCoA). This is followed by an aldol condensation with another acetyl-CoA catalyzed by HMG-CoA synthase to form 3-hydroxy- 3-methylglutaryl-CoA (HMG-CoA). Next, using 2 mol of NADPH and catalyzed by the enzyme HMGA-CoA reductase, 3-hydroxy-3-methylglutaryl-CoA derivative is converted to mevalonic acid, (*R*)-MVA. Tracer studies have demonstrated that mevalonic acid can readily be fed as a precursor for terpene and sterol synthesis. However, mevalonic acid has six carbon atoms, whereas the isoprene unit has only five. Experiments using carboxylic acid labeled mevalonic acid for the production of terpenoids have shown that the carboxyl carbon is lost downstream of mevalonic acid. Mevalonic acid subsequently undergoes ATP-dependent phosphorylation to (*R*)-MVA 5-diphosphate, catalyzed by mevalonate kinase and phosphomevalonate kinase. The MVA 5-diphosphate is subsequently decarboxylated by mevalonate diphosphate decarboxylase to yield IPP, the first of the biogenic isoprene units. A stereospecific isomerization by the enzyme isopentenyl-diphosphate isomerase converts IPP to the other five carbon biogenic isoprene unit DMAPP.

The Methyl-D-erythritol-4-phosphate Pathway. The newly characterized non-mevalonate or MEP pathway consists of eight reactions catalyzed by nine enzymes (Figure 3B). Seven of the enzymes have been structurally characterized, and the probable mechanisms of action have been proposed.[134] The first reaction holds the condensation of pyruvate and glyceraldehyde 3-phosphate to produce 1-deoxy-D-xylulose-5-phosphate (DOXP), catalyzed by 1-deoxy-D-xylulose-5-phosphate synthase (DXS), using thiamine pyrophosphate as a cofactor. Next, using 1 mol of NADPH, the enzyme 1-deoxy-D-xylulose-5-phosphate reductoisomerase (IspC) converts DOXP to 2*C*-methyl-D-erythritol-4-phosphate (MEP).[143,144] The next reaction in the pathway starts with the introduction of a nucleotide as a substrate to MEP. MEP reacts with 1 molecule of CTP to produce 4-diphosphocytidyl-2*C*-methyl-D-erythritol (CDP-ME) and subsequent release of phosphate through an IspD enzyme-catalyzed reaction.[145] In the next step, the reaction is catalyzed by an ATP-dependent enzyme IspE.[146] With a cofactor, γ-phosphate, which accepts nucleophilic attack from CDP-ME, the pathway proceeds to 4-diphosphocytidyl- 2*C*-methyl-D-erythritol-2-phosphate (CDP-ME2P) and ADP. The fifth step in the pathway is catalyzed by the enzyme 2*C*-methyl-D-erythritol-2,4-cyclodiphosphate synthase (IspF), and the precursor CDP-ME2P is converted to 2*C*-methyl-D-erythritol-2,4-cyclodiphosphate (MECP) by losing 1 molecule of CMP.[147,148] The structure–function studies show that enzyme IspF requires divalent cations Zn^{2+} and Mg^{2+} for catalysis.

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The next two steps in the pathway are the least understood and catalyzed by the enzymes 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate synthase (IspG) and 4-hydroxy-3-methyl-2-(*E*)-butenyl-4-diphosphate reductase (IspH).[149,150] IspG transforms MECP to 2-methyl-2-(*E*)-butenyl diphosphate followed by the enzyme IspH, catalyzing the conversion to IPP.[151] IspH also catalyzes the formation of some DMAPP as well. The last enzyme in the universal precursor pathway is isopentenyl-diphosphate isomerase (IDI), which isomerizes the IPP carbon−carbon double bond for steriospecifc conversion to DMAPP.[152] Type I and type II IDI isomerases are characterized. IDI-I is dependent on divalent cations (Mn^{2+} or Mg^{2+}) and is widely distributed in eukaryotes and eubacteria.[153,154] The type II enzyme was recently discovered and only present in *Streptomyces* sp. strain CL190, archaea, and eubacteria.[155] It is interesting to note that the non-mevalonate pathway is not regulated in the same way as the MVA pathway.[136] To date there have been no global transcriptional regulators identified. The enzymes IspD and IspF involved in steps three and five are expressed as a bifunctional enzyme IspDF. This fusion is unusual because, unlike most bifunctional enzymes, it catalyzes nonconsecutive steps.

The Downstream Biosynthetic Pathways: Building Diversity from a Common Precursor. Starting from the universal precursors IPP and DMAPP, thousands of enzymes are involved in the biosynthetic pathways for terpenoids chain elongation, cyclization, and functionalization of hydrocarbon chains to create enormous chemical and structural diversity.[132,133,156] Among these, only a few hundred have been studied in detail. From the two basic building blocks, IPP and DMAPP, a group of enzymes called prenyltransferases catalyze the synthesis of linear prenyl diphosphates such as geranyl diphosphate (GPP), farnesyl diphosphate (FPP), geranylgeranyl diphosphate (GGPP), and farnesyl geranyl diphosphate (FGPP), up to natural rubber with a carbon chain of several million in length (Figure 3).[157] During biosynthesis, the active isoprene unit (IPP) is repeatedly added to DMAPP or a prenyl diphosphate in sequential head-to-tail condensations. The reaction starts with the elimination of a diphosphate ion from an allylic diphosphate to form an allylic cation, which is attacked by an IPP molecule with steriospecific removal of a proton to form new carbon−carbon bonds in the product molecule. Through consecutive condensations of IPP with allylic prenyl diphosphate, a prenyltransferase can synthesize a variety of molecules with fixed lengths and stereochemistry. All prenyltransferases require divalent metal ions such as Mg^{2+} or Mn^{2+} for catalysis. Prenyltransferases geranyl pyrophosphate synthase (GPS) and farnesyl pyrophosphate synthase (FPPS) catalyze the condensation of IPP and DMAPP for the formation of GPP (C10) and FPP (C15), precursors for monoterpenes and sesquiterpenes, respectively.[158,159] Geranylgeranyl pyrophosphate synthase (GGPPS) and farnesyl geranyl pyrophosphate synthase (FGPPS) catalyze the condensations to GGPP (C20) and FGPP (C25), precursors for diterpene and sesterterpene, respectively. Many functional terpenoids are synthesized from linear prenyl diphosphates through cyclization cascades, the most complex chemical reactions known in chemistry and biology.[7,133,156,160] The structural diversity in terpenoids is initiated by the differences in folding of the substrate in the active site of the terpenoid cyclase followed by the generation and propagation of carbocationic intermediates, which readily undergo dramatic structural rearrangements. Most of these reactions happen under strict sterochemical and regiochemical precision, and two-thirds of the substrate carbon atoms undergo changes in chemical bonding and/or hybridization to form a single, unique terpenoid molecule.

Terpenoids are typically classified based on the number of five carbon isoprene units in their linear precursor prenyl disphosphate as C5 hemiterpenes, C10 monoterpenes, C15

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sesquiterpenes, C20 diterpenes, C25 sesterterpenes, and C30 triterpenes. The number of different structured terpenoids with manifold functions is hard to document completely. The biosynthesis of higher isoprenoids depends on the organism, as well as vast differences in the regulation of pathways in time and location of production.[37]

5. Metabolic Engineering: Improving Terpenoid Production through Genetic Changes

The major classes of terpenoid production are (i) isolation from natural source, (ii) metabolically engineered native host, (iii) total chemical synthesis, (iv) semi-synthesis from the intermediate compounds, (v) fermentation of metabolically engineered cell culture, and (vi) fermentation of metabolically engineered bacteria or yeast.[15,20,29,30,52,161,162] Although some terpenoids are produced in relatively large quantities from natural sources (for example, essential oils, resins, and waxes), often high-value terpenoid products are found in low abundance naturally.[163,164] Thus approach (i) is severely hampered by the large requirements of natural sources that are necessary to gather in suitable quantities. This necessity for large amounts of the source is not only economically hindering, but has many ecological concerns especially for those terpenoids derived from marine organisms. Two excellent examples are the potent chemotherapeutic diterpenoids, paclitaxel and eleutherobin. Both of the compounds constitute only 0.01–0.02% extraction yield from their original sources, the bark of the plant Pacific yew (*Taxus brevifolia*) and marine coral *Eleutherobia* sp., respectively.[96,163] Excessive harvesting of the natural source limits future availability of both species, impacting future harvesting and possible environmental endangerment. At the same time, the total chemical syntheses of these and many other pharmacologically important terpenoids have been achieved; however, the multistep synthesis has extremely low yields that are not commercially viable.[161]

The development of renewable and environmentally friendly production processes for an adequate and sustainable supply of these compounds has been the major challenge addressed by modern metabolic engineering and synthetic biology research. Metabolic engineering, which integrates engineering design with systematic and quantitative analysis of metabolic pathways, provides a systematic approach to introducing and optimizing new product pathways in microbes.[11,165,166] Metabolic engineering draws on molecular biology, large scale and high throughput analytical techniques, and genomic-based bioinformatic approaches to *quantitatively* understand and modulate metabolism, with an emphasis on the global state of the cell, and not on individual reactions.[167] This represents a paradigm shift from focusing only on the product forming step, and instead understanding the pathway in the context of the entire cell.

Metabolic engineering of whole plants and plant cell cultures is an effective tool to both increase terpenoid yield and alter terpenoid distribution for desired properties such as enhanced flavor, fragrance, or color.[29,168,169] Indeed, metabolically engineered plant cell culture provides an environmentally friendly and renewable alternative to large scale production of terpenoids.[30] However, there are many limitations associated with the complexity in engineering plant cells for terpenoid production such as (i) inherent complexity in terpenoid secondary metabolite biosynthetic pathways and regulation in plant cells, (ii) lack of complete genomic sequence data of the plant system that prohibits detailed systemwide analysis on plants and plant cell cultures and associated metabolic regulatory systems, (iii) limited molecular biology tools compared to microbial systems such bacterial and yeast, and (iv) unexplained variability in terpenoid production over time and from culture to culture.[170–174]

Engineering Terpenoid Biosynthetic Pathway in Microbes. Engineering microbial cells for the sustainable production of terpenoids through the transfer of biosynthetic pathways from the native organism is a complicated task with many challenging aspects.[15,167] However, the potential

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to overcome the problem of low yield and high costs for the large scale production of these valuable compounds has motivated many to explore this alternative. The microbial system offers advantages such as (i) environmentally friendly chemistry, (ii) the use of inexpensive carbon sources, (iii) the capability of genetic manipulation to increase production yields, (iv) compatibility with large scale fermentation processes due to microbes' relative insensitivity to shear stress induced via impeller mixing in bioreactors, (v) significantly shorter doubling times in microbial systems as compared to plant cell cultures thereby significantly reducing culture times and contamination risk while increasing productivity, (vi) removal from regulation that the pathway is subject to in the native organism, and (vii) the opportunity for uncoupled growth and production phases of the fermentation, thus allowing optimal media formulations for growth and production.

For the past two decades, terpenoid metabolic engineering in microorganisms has been focused mostly on carotenoids, such as lycopene and β-carotene, precursors for approved drugs, such as artemisinin and paclitaxel, and a few other terpenoids molecules.[19,175] Among the numerous carotenoid compounds, biosynthesis of lycopene has been extensively studied in engineered bacteria. Due to its bright red color, lycopene is easily detected in bacterial colonies, a property that has allowed the design of engineering strategies for screening biodiversity and identifying improvements in isoprenoid production.[15] Since lycopene is formed from the precursor GGPP, it is possible to screen for colonies with high precursor availability and afterward express the pathways for other terpenoid molecules that use the same precursor. This introduces the idea of a "platform" strain that has been engineered to provide large quantities of a precursor. Different product pathways can be added to the "platform" strain to provide a variety of terpenoids. Two of the most utilized microorganisms for expressing heterologous enzymes for the synthesis of isoprenoids are *E. coli* and *S. cerevisiae*. A detailed account of various pathway manipulation and engineering efforts for terpenoid production in bacteria and yeast is summarized in Tables 1 and 2.

Metabolic Engineering of Terpenoid Production in *E. coli*. In the past 15 years, considerable effort has focused on cloning and expression of various heterologous genes in the downstream terpenoid biosynthetic pathways, introducing a heterologous mevalonate pathway, manipulating the upstream MEP pathways in *E. coli* for improved precursor supply through overexpression or deletion of upstream pathway genes, and altering the global metabolic network through directed changes and mutagenesis libraries. Several recent reviews cover many of these metabolic engineering approaches for the improved production of terpenoids in *E. coli*.[15,16,19]

Construction of Heterologous Biosynthetic Reactions for Terpenoids. One of the first attempts to clone and express carotenoid biosynthesis genes in *E. coli* was carried out by Misawa et al. in 1990.[176] The carotenoid biosynthetic genes *crtE*, *crtX*, *crtY*, *crtI*, *crtB*, and *crtZ* were cloned from a phytopathogenic bacterium, *Erwinia uredovora*, into *E. coli* and characterized. The genes in this pathway appear to be closer to those in higher plants than to those in other bacteria. Also, it is significant that only one gene product (CrtI) is required for the conversion of phytoene to lycopene, a conversion in which four sequential desaturations should occur via the intermediates phytofluene, zeta-carotene, and neurosporene. The obtained yields of zeaxanthin, β-carotene, and lycopene were approximately 2 mg/g dry cell weight.

The successful production of diterpene, taxadiene, the first intermediate in the biosynthetic pathway of paclitaxel, was achieved in *E. coli* by the overexpression of genes encoding isopentenyl diphosphate isomerase (*idi*) (details of modifications to the non-mevalonate pathway will be discussed below), geranylgeranyl diphosphate synthase and taxadiene synthase.[177] This demonstration supported the possibility of making other valuable terpenes in *E. coli*. However the observed yield was very low at 1.3 mg/L. The same group attempted the biosynthesis of the spearmint monoterpene ketone (−)-carvone in *E. coli*.[178] Overexpression of the pathway genes geranyl geranyl diphosphate synthase, limonene synthase, cytochrome P450 limonene hydroxylase, and carveol dehydrogenase yielded 5 mg/L intermediate (−)-limonene. Assays of pathway enzymes and intermediates indicated that flux through the initial steps catalyzed by geranyl geranyl diphosphate synthase and limonene synthase was severely limited by the availability of the isoprenoid precursors. However, by feeding the intermediate precursor (−)-limonene the functional capability of limonene-6-hydroxylase and carveol dehydrogenase to produce the end-product carvone was demonstrated. Unfortunately, inefficient uptake of (−)-limonene limited conversion efficiency. The pathways for amorphadiene and 8-hydroxycadinene have also been successfully expressed in *E. coli*.[179–181]

Modifications to the Non-Mevalonate Pathway. Many issues arise in the cloning of heterologous terpenoid pathways because of limitations in the supply of the universal precursors IPP and DMAPP. Metabolic engineering of the non-mevalonate

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Table 1. Summary of Terpenoids Produced in *E. coli* and Different Approaches for Improving the Production Yield

literature	isoprenoids produced	approach	production yield
Misawa et al. 1990[176]	zeaxanthin, β-carotene and lycopene	expression of heterologous carotenoid genes on plasmids	2,200 μg/gDW, 2,000 μg/gDW and 2,000 μg/gDW
Ruther et al. 1997[200]	zeaxanthin	expression of heterologous carotenoid genes on different plasmids and optimization	289 μg/gDW
Albrecht et al. 1997[201]	1-hydroxyneurosporene, demethylspheroidene, 1′-HO-γ-carotene and 7,8-dihydrozeaxanthin	expression of heterologous carotenoid genes on plasmids	176 μg/gDW, 46 μg/gDW, 40 μg/gDW and 122 μg/gDW
Kajiwara et al. 1997[182]	lycopene, β-carotene and phytoene	expression of IPP isomerases from *P. rhodozyma*, *H. pluvialis* and S. cerevisiae	1,029 μg/gDW, 1,310 μg/gDW and 504 μg/gDW
Wang et al. 1999[183]	astaxanthin	overexpression of native IPP isomerase and GGPP synthase from *A. fulgidus*	1,250 μg/gDW
Harker and Bramley 1999[185]	lycopene and ubiquinone (UQ-8)	expression of heterologous genes from *B. subtilis* and *Synechocystis* encoding 1-deoxy-D-xylose-5-phosphate synthase	2-fold increase and 1.5-fold increase
Matthews and Wurtzel 2000[187]	lycopene and zeaxanthin	overexpression of native gene encoding D-1-deoxyxylulose 5- phosphate synthase	1,333 μg/gDW and 592 μg/gDW
Wang et al. 2000[186]	lycopene	overexpression of the *idi* gene and the *dxs* gene, combined with the expression of a GGPP synthase from *A. fulgidus* subjected to directed evolution	45,000 μg/gDW
Huang et al. 2001[177]	taxadiene	overproduction of DXP synthase, IDI, GGPP synthase and taxadiene synthase	1,300 μg/L
Kim and Keasling 2001[189]	lycopene	expression of *dxs* and *dxr* gene with three different promoters	5,690 μg/gDW
Farmer and Liao 2000[191]	lycopene	engineering metabolic control using *glnAp2*-based artificial regulon	0.16 mg mL^{-1} h^{-1}
Farmer and Liao 2001[192]	lycopene	perturbations in the central metabolism to alter the distribution between glyceraldehydes 3-phosphate and pyruvate	25,000 μg/gDW
Carter et al. 2003[178]	limonene, carveol and carvone	inducible overexpression of heterologous genes and feeding of precursor	5,000 μg/L, 0 μg/L and 0 μg/L
Martin et. al 2003[188]	amorpha-4,11-diene	expression of the genes from *S. cerevisiae* encoding the mevalonate pathway and amorpha-4,11-synthase	~112,200 μg/L
Lee et al. 2003[202]	torulene, lycopene and β-carotene	different expression levels of mutant lycopene cyclase, optimization of growth conditions and engineering of the DXP mevalonate pathway	1,111 μg/gDW, 318 μg/gDW and 67 μg/gDW
Reiling et al. 2004[190]	lycopene, casbene, kaur-15-ene, kaur-16-ene, α-pinene, myrcene, sabinene, 3-carene, α-terpinene, limonene, β-phellandrene, α-terpinene and terpinolene	engineering of the DXP-pathway (expression of genes encoding 1-deoxy-D-xylulose-5-phosphate synthase, IPP isomerase from *H. pluvialis* and mutant FPP synthases producing either GPP or GGPP, expression of genes encoding lycopene and expression of mono- and diterpene cyclases	1,210 μg/L lycopene produced; production of other compounds detected
Alper et al. 2005[195]	lycopene	genome-wide stoichiometric flux balance analysis to discover gene knockouts that would improve lycopene production and combinatorial knockout of the identified genes	6,600 PPM
Alper et al. 2005[196]	lycopene	systematic combination of knockout targets identified by stoichiometric flux balance analysis and knockout target identified by screening of a global transposon library	18,000 PPM

Table 1. Continued

literature	isoprenoids produced	approach	production yield
Kang et al. 2005[199]	lycopene	screening of shot-gun library to identify genes that enhanced lycopene production and combinatorial expression of these genes	4,700 μg/gDW
Alper et al. 2006[197]	lycopene	characterization of knockout strains for lycopene production[196]by high cell density fermentations and optimization of growth conditions	221,600 μg/L
Yuan et al. 2006[192]	lycopene	overexpressing the chromosomal isoprenoid pathway genes, *dxs*, *idi*, *ispDF*and *ispB* using strong bacteriophage T5 promoter	6,000 μg/g DCW
Yoon et al. 2006[203]	lycopene	overexpression of genes encoding the synthesis of lycopene together with a heterologous *ipiHP1* gene; expression of genes encoding mevalonate kinase, phosphomevalonate kinase and IPP isomerase from *S. pneumonia*; supplementation of mevalonate and adding of surfactant	102,000 μg/L
Newman et al. 2006[180]	amorpha-4,11-diene	optimization of two-phase partitioning bioreactor for fermentation of amropha-4,11-diene producing strain[188]	500,000 μg/L
Yoon et al. 2007[204]	lycopene	expression of genes from the organisms *P. agglomerans* and *P. ananatis* which both encodes the production of lycopene, combined with the expression of genes encoding mevalonate kinase, phosphomevalonate kinase and IPP isomerase from *S. pneumonia*; supplementation of mevalonate	60,000 μg/L
Pitera et al. 2007[205]	mevalonate pathway optimization for amorpha-4,11-diene	balancing enzyme activity of the heterologous expressed mevalonate pathway	
Jin and Stephanopoulos 2007[198]	lycopene	combination of overexpression targets identified by screening of genomic libraries with knockout targets predicted by stoichiometric modeling	16,000 μg/gDW
Chang et al. 2007[181]	8-hydroxycadinene and artemisinic acid	expression of plant cytochrome P450s followed by optimization	105,000 μg/L and 105,000 μg/L

Table 2. Summary of Terpenoids Produced in Yeast and Different Approaches for Improving the Production Yield

literature	isoprenoid produced	organism	approach	amount produced
Yamano et al. 1994[207]	lycopene and β-carotene	*S. cerevisiae*	expression of heterologous carotenoid genes on plasmids under the control of native promoters derived from the yeast genome	113 μg/gDW and 103 μg/gDW
Miura et al. 1998[208]	lycopene	*C. utilis*	expression of heterologous carotenoid genes on plasmids under the control of native promoters derived from the yeast genome	758 μg/gDW
Miura et al. 1998[209]	lycopene, β-carotene and astaxanthin	*C. utilis*	expression of codon optimized heterologous carotenoid genes on plasmids	1,100 μg/gDW, 400 μg/gDW and 400 μg/gDW
Shimada et al. 1998[214]	lycopene	*C. utilis*	expression of heterologous carotenoid genes on plasmid combined with deletion of one ERG9 allele in diploid strain together with overexpression of truncated HMG gene	7,800 μg/gDW
Jackson et al. 2003[210]	epi-cedrol	*S. cerevisiae*	expression of heterologous gene encoding epi-cedrol synthase combined with overexpression of truncated HMG1 gene in *upc2-1* mating type **a**	370 μg/L
DeJong et al. 2005[211]	taxadiene and taxadien-5α-ol	*S. cerevisiae*	heterologous expression of five sequential paclitaxel pathway genes	taxadiene 1 mg/L and (taxadien-5α-ol) achieved at $\sim$25 μg/L
Lindahl et al. 2006[212]	amorpha-4,11-diene	*S. cerevisiae*	expression of heterologous gene encoding amorpha-4,11-diene synthase on high-copy plasmid and integration in the chromosome	600 μg/L and 100 μg/L
Ro et al. 2006[206]	amorpha-4,11-diene and artemisinic acid	*S. cerevisiae*	expression of heterologous genes combined with overexpression of tHMG gene, down regulating the ERG9 gene by substitution of ERG9 promoter for MET3 promoter, overexpression of *upc2-1*, integration of additional tHMGR, overexpression of ERG20	153,000 μg/L and 115,000 μg/L
Shiba et al. 2007[215]	amorpha-4,11-diene	*S. cerevisiae*	overexpressing native ALD6 and mutated Acs from *Salmonella enterica* in the strains from Ro et al. 2006[223]	120,000 μg/L
Asadollahi et al. 2007[213]	valencene, cubebol and patchoulol	*S. cerevisaie*	downregulating the ERG9 gene by substitution of ERG9 promoter for MET3 promoter and expression of heterologous genes	3,000 μg/L, 1,600 μg/L and 11,500 μg/L (nonspecific synthases results in a mix of isoprenoids)

pathway initially focused on the overexpression of the genes *dxs* and *idi*, which significantly improved the intracellular pool of precursors for isoprenoid biosynthesis and subsequent carotenoid yield up to a 5–10-fold improvement.[177,182–190] The more dramatic increase was found when *dxs* and *idi* were overexpressed together, showing that multiple limitations must be overcome to ensure a large capacity to produce isoprenoids.[189] Wang et al.[183] reported that simultaneous amplification of *idi* and GGPP synthase (*gps*) in astaxanthin-producing *E. coli* strains revealed that the conversion from FPP to GGPP is the first bottleneck, followed by IPP isomerization and FPP synthesis. The engineered *E. coli* strain showed 50-fold improvement in astaxanthin compared to the control strain. Further directed evolution of the rate limiting *gps* enzyme resulted in a 100% increase in lycopene yield.[186] Thus modulating both transcriptional levels and specific activity is important for optimizing the metabolic flux distribution. The same research group further investigated the metabolic flux using *glnAp2*-based artificial regulon, which allows the expression of the rate controlling enzymes (Idi and Pps) for lycopene biosynthesis.[191] The lycopene-producing strain (*dxs*, *gps*, *crtBI*) constructed with an artificial regulon controlling *idi* and *pps* (*glnAp2-idi* + *glnAp2- pps*) increased 3-fold in lycopene productivity (0.16 mg mL^{-1} h^{-1}). In another study, overexpression of *dxs, idi,* and *ispA* in *E. coli* was carried out for the production of monoterpenes and diterpenes.[190] The engineered *E. coli* produced the diterpenes casbene and ent-kaurene and the monoterpene δ-3-carene. The same strain yielded 6-fold more lycopene than the control strain. In order to minimize the metabolic burden associated with plasmid overexpression of the rate controlling genes in the MEP pathway, Yuan et al.[192] recently reported a newly engineered strain by replacing the native promoters of the chromosomal isoprenoid pathway genes *dxs*, *idi*, *ispDF*, and *ispB* with the strong bacteriophage T5 promoter (PT5). The modified strain yielded 6 mg/g DCW of β-carotene.

Altering the Global Metabolic Network To Improve Isoprenoid Flux. Several approaches were undertaken to improve the availability of central carbon metabolism precursors toward the non-mevalonate pathway. Farmer et al. overexpressed or inactivated several enzymes that leads to alterations in central metabolism that redirect flux from pyruvate to G3P resulted in higher lycopene production in *E. coli*.[193] It suggests that G3P is a limiting factor in lycopene production and modifications that achieve a more equitable distribution between the two precursors are able to increase the lycopene yield in metabolically engineered *E. coli*. Another recent attempt to alter central metabolism by the inactivation of various competing pathways at the nodes of acetyl-CoA and pyruvate divert more carbon flux to IPP, increasing lycopene production by 45% over the parent strain.[194] Using this background strain, a heterologous

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mevalonate pathway was incorporated that increased lycopene production 2-fold (the heterologous mevalonate pathway will be discussed below).

Model-based rational manipulations and library screening were used to increase the supplies of G3P, PEP, reducing equivalents, and energy carriers (ATP, GTP, CTP, etc.). A computational search using a stoichiometric, genomewide bioreaction model of *E. coli* metabolism was used to identify gene knockouts which are predicted to increase the flux to lycopene by eliminate competing reactions or providing more energy or reducing equivalents for the pathway.[195] By using a sequential approach, the model was able to predict single and multiple gene knockout, including a triple knockout construct deleting the genes of *gdhA/aceE/fdhF*. This predicted strain was created in the background of the *dxs*, *idi*, and *ispDF* overexpression strain and was experimentally validated to produce the highest yield, 6,600 ppm, 37% above that of the parental strain. The stoichiometric model-based method can account only for stoichiometric effects on lycopene production; as such it cannot predict important kinetic and regulatory effects that may affect lycopene production.

Different combinatorial approaches (commonly called inverse metabolic engineering) have been used to identify genetic changes that are not predicted by current understanding of terpenoid metabolism. A transposon mutagenesis library, which has a transposon sequence randomly inserted in the genome of each mutant, thereby inactivating the gene it lands in, was used to search for additional knockout targets that affect the lycopene phenotype via regulatory, kinetic, or other unknown mechanisms.[196] The screening of the transposon library identified three gene targets, *rssB* (hnr), *yjfP*, and *yjiD*, that correlated with lycopene overproduction. In the case of *yjiD*, the transposon was inserted between the identified promoter region and the gene for *yjiD* (notated as Δp*yjiD*).

Further attempts were made to combine the rationally identified gene targets with combinatorial gene targets. Using the two *distinct sets* of (1) stoichiometric (discovered by the computational model) and (2) combinatorial gene targets (identified from the transposon library) identified in the above study, a global maximal strain with triple knockout Δ*gdhA*Δ*aceE*Δp*yjiD* was generated.[196] It is interesting to note that this global optimum strain contained two targets from the stoichiometric model and one from the combinatorial library. This optimum would not have been found without using these two approaches in combination. Under optimized conditions, this lycopene producing strain accumulated lycopene up to 18,000 ppm in shake flask and 220 mg/L from a 27 g DCW/L, high-cell-density fed-batch fermentations.[197]

Another combinatorial approach, involving the overexpression of random genes in the *E. coli* genome, was carried out in the computational model-based *gdhA/aceE/fdhF* strain. This search strategy screened genomic libraries of *E. coli* and identified two hypothetical regulatory genes, *yjiD* and *ycgW*, that improved lycopene production.[198] The final lycopene engineered strain included four overexpressions and three knockouts, (T5P-*dxs*, T5P-*idi*, rrnBP-*yjiD*-*ycgW*, Δ*gdh*Δ*aceE*Δ*fdhF*, pACLYC) which might exhibit three different mechanisms for improving lycopene production: push, pull, and global regulation favoring lycopene production. First, the strain contained three knockouts of metabolic enzymes in the central carbon metabolism, which might push precursors into the non-mevalonate pathway. Second, the strain had overexpressions of two isoprenoid pathway genes which pull precursor metabolites from a central metabolic pathway into the non-mevalonate pathway. Finally, the strain also overexpresses two hypothetical proteins, which presumably affect lycopene production through a regulatory manner. In another library overexpression approach, Kang et al.[199] identified another set of genes enhancing lycopene production in *E. coli*. They identified three regulatory genes such as *crl*, *rpoS*, and *appY*, which could act as enhancers of lycopene production when expressed alone or coexpressed with the rate-limiting gene of *dxs* in the MEP pathway. The combination of *dxs* with *crl*, *rpoS*, and *appY* produced 4.7 mg/g dry cell weight of lycopene, which is significantly higher than that of overexpressing *dxs* alone.

Engineering Heterologous Mevalonate Pathway To Provide the Universal Precursors IPP/DMAPP. To address the precursor limitations, an *E. coli* strain was engineered with the heterologous mevalonate pathway to yield large amounts of the terpenoid precursors, resulting in a high-level, *in vivo* production of amorphadiene, the sesquiterpene olefin precursor to the potent antimalarial drug artemisinin.[179] By engineering the expression of a synthetic amorpha-4,11-diene synthase gene and introducing the mevalonate pathway from *Saccharomyces cerevisiae* into the

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strain, amorphadiene production reached a titer of 112.2 mg/L. By employing an optimized two-phase partitioning bioreactor, this same strain yielded 500 mg/L amorphadiene.[180] Later, the same MVA pathway engineered strain was used for the additional heterologous expression of plant P450s derived from their native biosynthetic pathway for the production of sesquiterpenes 8-hydroxycadinene and artemisinic acid at titers of 60 ± 2 mg/L and 105 ± 10 mg/L respectively.[181]

Metabolic Engineering of Terpenoid Production in Yeast. To date, efforts have focused primarily on *E. coli*; however, there are many advantages to using yeast as a host for terpenoid production. As with *E. coli,* overexpressions, deletions, or modifications to central metabolic pathways, as well as introduction of heterologous pathways, have been employed to improve precursor supply for yeast. Some of these modifications have been adopted from prior bacterial engineering efforts in carotenoid production. The engineering efforts have resulted in yeast strains capable of producing high titers of up to 153 mg/L terpenoids (Table 2).[206]

Construction of Non-Native Downstream Terpenoid Pathways for New Products. Prior to the year 2000, attempts to produce terpenoids in yeast can be summarized as biosynthesis of (1) lycopene and β-carotene in *S. cerevisiae*;[207] (2) lycopene, β-carotene and astaxanthin in *Candida utilis*.[208,209] The first attempt by Yamano et al.[207] to produce terpenoids in *S. cerevisiae* by cloning *CrtE*, *CrtB*, *CrtI*, and *CrtY* genes for lycopene and β-carotene used a Yep-13 derived vector and individual promoters and terminators with each gene. Lycopene and β-carotene accumulated together with their intermediates in the yeast cells. This was the first attempt to express a multigene metabolic pathway in a eukaryotic system. Though this attempt did not produce high titers, however, it demonstrated the potential for redirecting carbon flux toward an engineered carotenoid pathway away from the native downstream sterol synthesis. After this, Miura et al.[208,209] engineered another yeast strain, *C. utilis,* by expressing *crtE*, *crtB*, *crtI*, *crtY*, *crtZ*, and *crtW* to produce carotenoids. The *C. utilis* strain was chosen because of its ability to accumulate high amounts of ergosterol, which implied a high flux or precursor availability in the MVA pathway.[208] Here, the production was improved by using codon biased heterologous genes encoding the production of lycopene, β-carotene, and anstaxanthin.

Since the year 2000, the focus on engineering terpenoid production in yeast has shifted from carotenoids to high value therapeutic sesquiterpenes and diterpenes. Some of these recent attempts are production of epi-cedrol,[210] taxadiene-5α-acetoxy-10β-ol, a precursor of paclitaxel,[211] amorpha-4,11-diene[213] and artemisinic acid precursors for artemisinin[206] and valencene, cubebol, and patchoulol in engineered *S. cerevisiae*.[213]

Modifications to the Mevalonate Pathway To Increase the Precursor Supply. Most of the successful metabolic engineering approaches to yeast have been very similar and have focused on increasing flux through the mevalonate pathway by (1) increasing the flux from acetoacetyl-CoA to HMG-CoA by overexpressing the tHMG gene, (2) reducing the flux toward erosterol by targeting ERG9, and (3) overexpressing ERG20. Among these approaches, the down-regulation of ERG9 seems to be the most effective at increasing the production of terpenoids. Since ergosterol is crucial for cell growth, there will always be a trade-off between production of isoprenoids and growth rate.

To improve the production of terpenoids, Shimada et al.[214] attempted to increase the flux through the mevalonate pathway by overexpressing a truncated version of the native HMG reductase gene and deleting one allele of the ERG9 gene in the diploid *C. utilis*. Neither modification improved terpenoid production individually; however, the combined modification improved lycopene accumulation 4-fold. The hypothesized mechanism for this improvement is that HMG-

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CoA synthase is negatively regulated by ergosterol. By reducing the ERG9 gene dose, the pool of ergosterol is lowered, activating HMG-CoA synthase. However, even with HMG-CoA synthase active, the pathway is still limited by HMG reductase. Upon overexpression of HMG reductase, a high flux through the MVA pathway was observed. Part of this flux was used in the ergosterol pathway to allow for cell growth, and the excess flux was diverted to carotenoid synthesis.

Jackson *et al.*[210] identified three different genetic loci in *S. cerevisiae* to improve the sesquiterpene epi-cedrol production by (1) overexpressing a truncated 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, (2) overexpressing the *upc2-1* allele, and (3) using a mating type **a** *S. cerevisiae* background. Points (2) and (3) will be discussed in global regulation of terpenoid production. In addition, they observed that the overexpression of ERG20 gene encoding FPP synthase in combination with the other two genetic modifications did not result in a significant improvement in epi-cedrol production. Recently, Ro et al.[206] has attempted a similar modification by downregulating ERG9 transcription by substituting the native ERG9 promoter with a MET3 promoter. This modification resulted in a 5-fold improvement in amorpha-4,11-diene production due to a reduced flux to ergosterol. This manipulation, in combination with an overexpression of tHMG, resulted in a 2-fold increase in amorpha-4,11-diene production. Further increases were found by overexpressing the *upc2-1* allele (a global transcription factor) and chromosomally integrating another copy of the tHMG gene. In this set of experiments, it was again observed that overexpression of ERG20 did not result in significant increase in production yield. The final, genetically optimized strain produced the highest titer to date of 153 mg/L terpenoids. In yet another approach, Asadollahi et al.[213] showed that the ERG9 downregulation by MET3 promoter substitution without any other modification was very efficient in increasing the production of three different sesquiterpenes.

The MET3 downregulation has a dual effect of reducing the flux of carbon to ergosterol, a competing pathway for terpenoids, and increasing flux through the MVA pathway by increasing HMG-CoA synthase specific activity through lowering of the ergosterol concentration, an inhibitor of the HMG-CoA synthase.[136] While this increased flux to MVA can reduce growth by diverting carbon that would have otherwise been used for biomass, this trade-off is avoided when using the MET3 promoter, which is used because this promoter can be repressed when the fermentation is about to enter stationary phase and shift the flux toward isoprenoid production. The regulation of the mevalonate pathway has recently been reviewed by Maury et al.[20]

Altering Central Carbon Metabolism to Increase Flux to MVA Pathway. An example of increasing the flux toward the mevalonate pathway was published by Shiba et al.[215] for producing amorpha-4,11-diene. Overexpression of acetaldehyde dehydrogenase (ALD6) and introduction of acetyl-CoA synthetase (ACS) from *Salmonella enterica* increased the supply of acetyl-CoA to the MVA pathway in *S. cerevisiae*, resulting in an increased amorphadiene production. The overexpression of ALD6 alone reduced amorphadiene production, due to decreased cell mass, presumably because of a measured increase in the byproduct, acetate. To overexpress acetyl-CoA synthetase activity, ACS1 was selected over other isoenzymes due to its three times higher V_{max} and thirty times lower K_m. The overexpression of ACS1 showed an 8–23% increase in production, without affecting the growth. However, the co-overexpression of ACS1 and ALD6 did not show any improvement in productivity, possibly due to high acetate accumulation resulting from an imbalance in enzyme activities around acetyl-CoA. A mutated ACS from *Salmonella enterica* was overexpressed in the background of an ALD6 overexpression strain and showed an increase in acetyl-CoA synthetase activity that resulted in high amounts of amorphadiene accumulation. The mutated ACS from *Salmonella enterica* is not inhibited by acetyl-CoA levels due to a proline to leucine substitution at position 641 preventing acetylation of *Salmonella* acetyl-CoA synthetase. In general the mevalonate pathway in yeast is well-characterized, offering great possibilities to engineer and produce different isoprenoids.

Global Regulation of Terpenoid Production. Mating type **a** and the *upc2-1* allele were identified by Jackson et al.[210] as global effects that would improve terpenoid production. The mating type, **a,** has been observed to have higher sesquiterpene accumulation compared to the mating type **α**. The observed higher production with the mating type **a** may be due to its ability to biosynthesize more FPP, a precursor for mating factor prenylation or less control on the diversion of FPP to sterol biosynthesis. Further overexpression of the *upc2-1* allele, encoding a mutated global transcription factor, and tHMG1 gene in a mating type **a** yeast strain yielded the highest amount of epi-cedrol.

6. Challenges in Engineering Complex Multistep Metabolic Pathways in a Microorganism: Paclitaxel as a Case Study for Sustainable Biosynthesis

Though a partial success was realized in transferring the biosynthetic pathway and engineering *E. coli* and yeast for sustainable production of terpenoids, the metabolic pathways leading to some terpenoids are too complex, such as that leading to paclitaxel, which required 19 enzymes to convert IPP/DMAPP to the final product (Figure 4).[216] Recent research efforts using induced taxoid production in *Taxus* cell cultures has shown considerable progress in understanding the pathway, enzymology, and molecular genetics of

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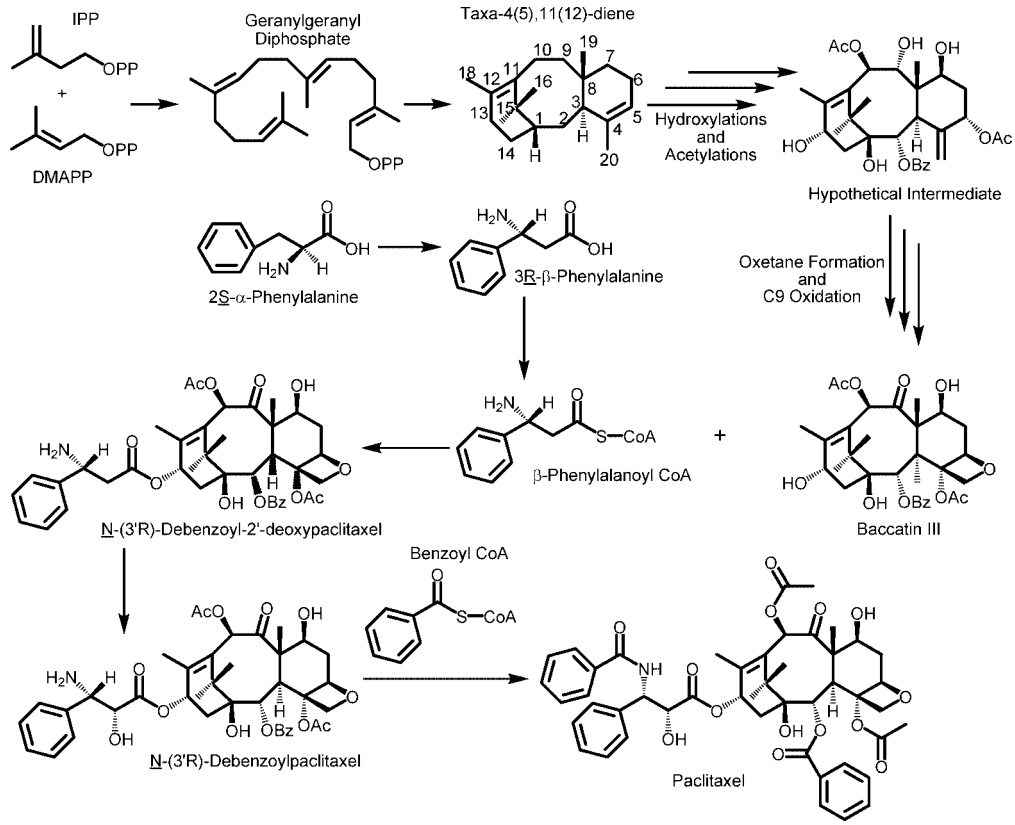

Figure 4. The paclitaxel biosynthetic pathway and associated key metabolite intermediates. The genes involved in this biosynthetic pathways are GGPP synthase, taxadiene synthase, taxoid 5α-hydroxylase, taxoid-5α-*O*-acetyltransferase, taxoid 13α-hydroxylase, taxoid 10β-hydroxylase, taxoid 2α-hydroxylase, taxoid 2-*O*-benzoyltransferase, taxoid 7β-hydroxylase, taxoid 10-*O*-acetyltransferase, taxoid 1β-hydroxylase,* taxoid 9α-hydroxylase, taxoid 9-keto-oxidase,* taxoid C4,C20- β- epoxidase,* phenylalanine aminomutase, side chain CoA-ligase,* taxoid 13 *O*-phenylpropanoyl-transferase, taxoid 2′-hydroxylase,* taxoid 3′-*N*-benzoyltransferase.[216,219] Genes marked with an asterisk are yet to be identified or characterized.

paclitaxel biosynthesis.[216–219] The pathway includes eight cytochrome P450 (CYP450) hydroxylases, acyl, aroyl CoA-dependent transferases, keto-oxidase, and epoxidase, and expression of redox partners for P450 that are necessary for an active pathway. However, still there are gaps that exist in defining several pathway steps and establishing the sequences of this extended series of reactions with an additional five genes encoding presumable biosynthetic enzymes yet to be identified.

Paclitaxel Biosynthetic Pathway. The first committed enzyme of the paclitaxel pathway is taxadiene synthase, which cyclizes the common precursor geranylgeranyl diphosphate to taxadiene. This taxadiene core structure is then functionalized by seven cytochrome P450 oxygenases and decorated with two acetate groups and a benzoate group by acyl and aroyl CoA-dependent transferases. Though the order of oxygenation beyond the initial C5α-hydroxylation is uncertain, the suggested progression of oxygenation from C5 to C10, C2, C9, C13, C7, and finally C1, to the level of an acylated heptaol of uncertain identity.[216] Interestingly previous studies and sequence analysis indicated that the taxoid hydroxylases derived from a common progenitor by gene duplication and differentiation to evolve alternative substrate selectivities and new regio- and stereochemistries of the hydroxylation reaction on the core taxadiene structure. Similar to P450 taxoid oxygenases, the family of taxoid acyl and aroyl transferases might be also derived from a common ancestral gene by duplication and differentiation to evolve alternative acyl/aroyl CoA substrate selectivities and new regiochemistries for ester synthesis at the various hydroxylated positions on the taxane core and amidation of the C13-phenylisoserinoyl side chain. Previous studies of acylation patterns of existing taxoid metabolites, feeding studies, and evaluation of the apparent selectivity of the recombinant acyl/aroyl transferases have suggested that the initial acetylation at C5 of the taxane core is followed by benzoylation at

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C2, acetylation at C10, the addition of the side chain at C13, and *N*-benzoylation of the C13 side chain.[216]

Partial Pathway Reconstruction in Microbial Systems. Several drawbacks are associated with the early attempted production of paclitaxel intermediates through *E. coli* and yeast. In the case of *E. coli*, the taxadiene synthase gene was heterologously expressed and the first intermediate, taxadiene, was produced at levels of 1.3 mg/L.[177] One major limitation with the further assembly of the pathway is that the prokaryotic *E. coli* cannot functionally express the P450 enzymes that widely participate in the paclitaxel biosynthetic pathway. However, recent success in the functional expression of engineered plant P450 enzymes, responsible for the biosynthesis of flavanoids and other valuable terpenoids, put forward future possibilities of paclitaxel pathway assembly.[181,220]

Unlike bacteria that require extensive protein engineering, eukaryotic microbial hosts such as yeast could readily express functional P450 monooxygenases and other complex enzymes in the paclitaxel bioysnthesis. In *S. cerevisiae*, five sequential pathway steps leading from primary isoprenoid metabolism to the intermediate taxadien-5α-acetoxy-10*β*-ol were functionally expressed.[211] The *S. cerevisiae* host expressed the genes from the *Taxus cuspidate* including geranylgeranyl disphosphate synthase (GGPPS), taxadiene synthase (TS), cytochrome P450 taxadiene 5α-hydroxylase (TYH5a), taxadienol 5α-O-acetyl transferase (TAT), and taxoid 10*β*-hydroxylase (THY10b). The production of taxadiene was recorded at 1 mg/L, and the second intermediate (taxadien-5α-ol) was achieved at ∼25 *μ*g/L. However the very limited flux from the taxadiene and the 5a-hydroxylation steps prevents the *in vivo* production of other intermediates. Thus by using metabolic engineering approaches that differentiate this field from genetic engineering with its unique focus on the pathway *in its entirety* as opposed to the properties of single genes or enzymes, the future paclitaxel pathway assembly and optimization in microbial hosts are promising. This is in recognition of the fact that the microbial production of this multistep biosynthesis is a *systemic property of the pathway* instead of any individual enzyme. Although the metabolic engineering of paclitaxel biosynthesis in *E. coli* and yeast has only begun, the application of new protein engineering techniques combined with synthetic biology approaches to improve the rate-controlling enzymes appears promising.

In summary, the functional expression of these enzymes and supporting complexes in the paclitaxel pathway represent a large challenge that will require scalable methods for protein expression and optimization *in vivo*. To date, no technologies can be deployed to engineer the pathway in its entirety, and further step-by-step optimization will be required to complete the paclitaxel pathway.

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7. Future Perspective for the Commercial Scale Production of Terpenoids

There are many challenges that lie ahead in the commercial production of terpenoids from either plants or microbes. In plants, there are limitations with both the understanding of terpenoid metabolism and the use of genetic tools to modify the metabolism for higher yields of terpenoids. Basic research into understanding the mechanisms by which the terpenoids are used in nature will be a way into understanding the regulatory switches that are involved in producing given terpenoids at the time and place that is best for the organism. Only by understanding this regulation can environmental signals or genetic modifications be used to improve terpenoid yield from a genetic regulation standpoint. As well, terpenoids that will be marketed under governmental regulation will face challenges recovering the product from the cultivation site in ways that will meet regulatory demands. In many cases, the native organism may be very slow growing or difficult to cultivate plants, making the native organism impractical for production.

In order to avoid challenges with recovering terpenoids in the field, microbial based approaches become very attractive. In these model microbial systems, a much better understanding of metabolic regulation and a wealth of tools are available to modify the system. Here, the challenges can be separated into the (1) precursor supply problem and (2) pathway optimization for a given terpenoid product. Much of the work to date has focused on improving the precursor supply for relatively simple terpenoids. Current yields of 500 mg/L of product are adequate for very high value compounds, but will not be adequate for lower value consumer additives or therapeutics that will be deployed in the developing world. An order of magnitude increase to 5–20 g/L yields appears to be possible, although the challenges will be in (a) further optimization of flux through MEP or MVA pathways (or perhaps a clever combination of the two), (b) engineering microbial tolerance to the very high levels of terpenoids, and (c) developing culturing methods to remove volatile terpenoids during production.

One strain will unlikely be appropriate for producing all terpenoids due to the differing toxicities expected by different terpenoids to different microbes, as well as the varying abilities to express eukaryotic enzymes. For a given strain that produces IPP and DMAPP in adequate supply, the different pathways from IPP/DMAPP to the many possible products has to efficiently convert the precursors to the desired product. Challenges here include (a) the identification of pathway enzymes in the native organism or the directed evolution of existing organisms to give *de novo* activities desired for the pathway, (b) the functional expression of foreign enzymes in the microbial host, and (c) balancing enzymes in the pathway to avoid the accumulation of toxic intermediates. While the enzymes in some pathways are known, many other steps are still missing to produce many of the most desirable terpenoids. Functional genomics and bioinformatics ap-

proaches will be important to identify candidate enzymes for the pathway.[221] As genome sequencing becomes nearly routine, a terpenoid producer can be quickly sequenced and annotated, and loci with predicted functions similar to the expected pathway can be identified. Beyond this, efforts are underway to evolve enzymes to have new activities that are desirable.[222] Enzymes from the native organism must be functionally expressed in the surrogate host. Codon optimization and gene synthesis have significantly improved the translation of many plant enzymes in microbes, but there are many challenges specifically in expressing P450s correctly in microbes. Membrane-bound proteins have to be correctly modified to be soluble in the surrogate hosts, and toxic side reactions must be identified and minimized. Identifying important folding chaperones may also be important to produce the desired enzyme. Finally, a given pathway must be balanced to maintain a homeostasis and avoid the accumulation of intermediates that may have detrimental effects on the cell. Promoter engineering based approaches[223] or operon intergenic region engineering[224] can provide ways to regulate the relative transcriptional and translational levels of enzymes in an operon.

8. Conclusion

Natural products-based therapeutics have seen a resurgence in interest and discovery of interesting compounds. However, unlike the large scale efforts to screen natural products for therapeutic activity that took place in the 1970s, this postgenomics approach is not limited to compounds that can only be isolated from the native host. Enabling technologies have allowed the engineering of plant cells for increased terpenoid flux, the transfer of biosynthetic pathways to microorganisms, and redirection of flux in these microorganisms to the upstream MEP or MVA pathways through various overexpressions, deletions or downregulation of genes identified in experimental and computational studies. The microbial biosynthetic route has several advantages compared to extraction from natural sources, metabolically engineered plant cells, or conventional chemical synthesis. Crucial issues in microbial biosynthesis are the expansion of existing approaches to identify optimal kinetic and regulatory effects in central metabolic pathways for redirecting carbon flux to terpenoid pathways, and the identification of new microbial hosts which could make higher titer production possible. Despite the obvious advantages of engineering microbial cells for terpenoid supply, either some plant terpenoid metabolic pathways are too complex for efficient transfer to a microbe or parts of the pathway are partially undefined. These obstacles prohibit complete pathways from being reconstituted in microbial systems. Additionally, cytochrome P450 (CYP450) hydroxylases and their redox partners involved in the biosynthetic pathways are difficult to functionally express, but this difficulty must be overcome to enable the efficient synthesis of terpenoids in microbial systems. Development of innovative engineering approaches and technologies to address these needs will be central to completing the shift to an economically feasible, microbial-based production scheme for terpenoids.

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