



Review in Advance first posted online  
on September 21, 2015. (Changes may  
still occur before final publication  
online and in print.)

# Engineering of Secondary Metabolism

Sarah E. O'Connor

The John Innes Centre, Norwich Research Park, Norwich NR4 7UH, United Kingdom;  
email: sarah.oconnor@jic.ac.uk

Annu. Rev. Genet. 2015. 49:5.1–5.24

The *Annual Review of Genetics* is online at  
[genet.annualreviews.org](http://genet.annualreviews.org)

This article's doi:  
10.1146/annurev-genet-120213-092053

Copyright © 2015 by Annual Reviews.  
All rights reserved

## Keywords

natural product, secondary metabolite, synthetic biology, combinatorial biosynthesis, compartmentalization

## Abstract

Secondary (specialized) metabolites, produced by bacteria, fungi, plants, and other organisms, exhibit enormous structural variation, and consequently display a wide range of biological activities. Secondary metabolism improves and modulates the phenotype of the host producer. Furthermore, these biological activities have resulted in the use of secondary metabolites in a variety of industrial and pharmaceutical applications. Metabolic engineering presents a powerful strategy to improve access to these valuable molecules. A critical overview of engineering approaches in secondary metabolism is presented, both in heterologous and native hosts. The recognition of the increasing role of compartmentalization in metabolic engineering is highlighted. Engineering approaches to modify the structure of key secondary metabolite classes are also critically evaluated.

## INTRODUCTION

Secondary or specialized metabolites are small molecules that are not essential for life, although these compounds likely confer evolutionary advantage to the producer organism (62, 96). Although primary metabolic processes (e.g., glycolysis, amino acid biosynthesis) are found in many or all species of life, the pathways that lead to secondary metabolites are specialized, with particular pathways found in a taxonomically restricted group of organisms. Secondary metabolites exhibit enormous structural variation and consequently display a wide range of biological activities.

### Classes of Secondary Metabolites

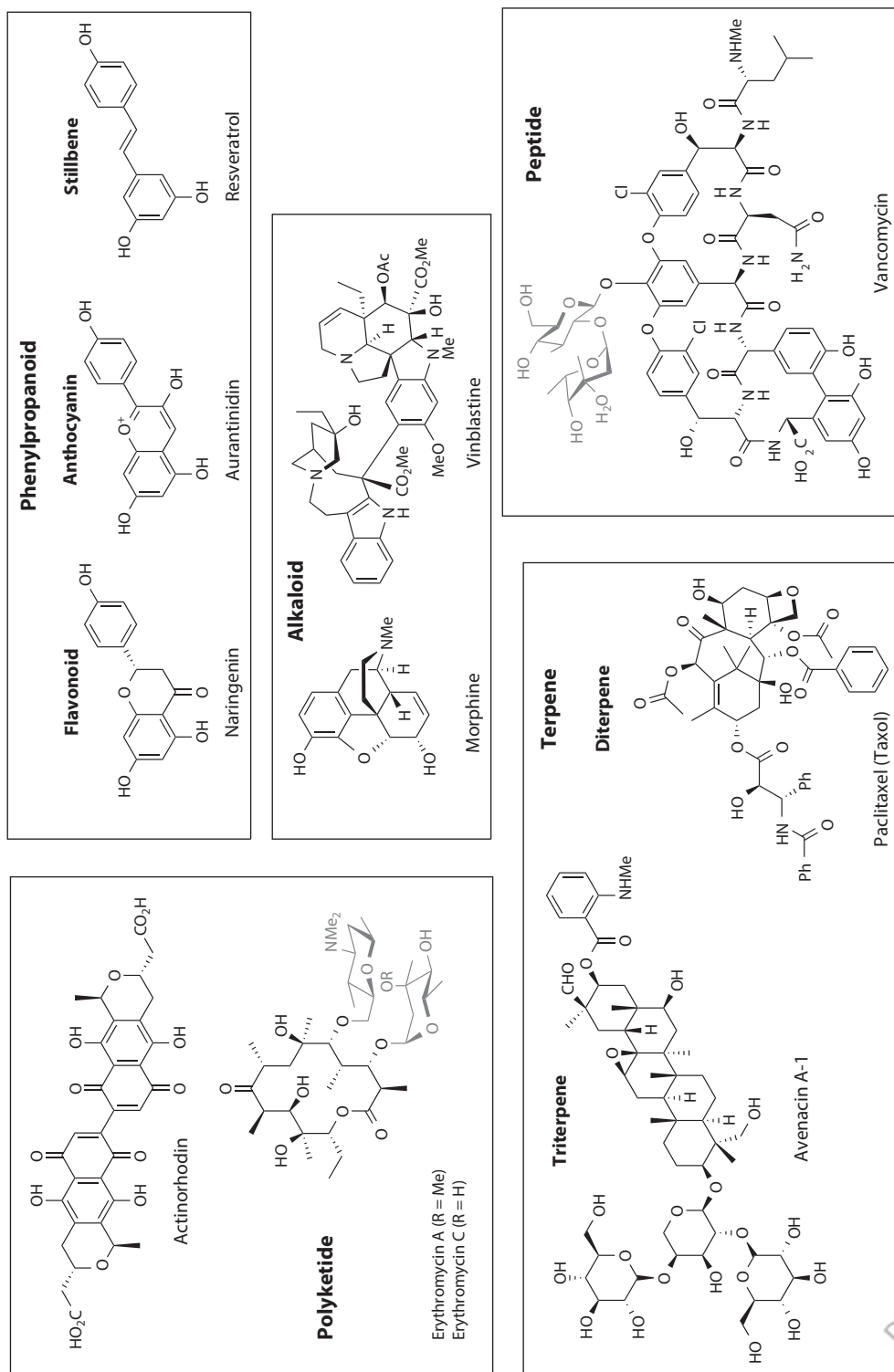
Secondary metabolites are classified according to biosynthetic origin (**Figure 1**). Terpenes are derived from precursors [dimethylallylpyrophosphate (DMAPP) and isopentenylpyrophosphate (IPP)] containing five carbons (73) and are further classified by size. Polyketides are synthesized from acetate units and are also subdivided into several classes (95). In plants, polyketide biosynthesis intersects with aromatic amino acid biosynthesis to generate phenylpropanoid structures, such as flavonoids, flavonones, stilbenes, and anthocyanins (143). Some carbohydrates can also be classified as secondary metabolites (93), and saccharides that are unique to secondary metabolism often decorate the core structure of many secondary metabolites. Peptide metabolites are built from amino acids using either the ribosome (118) or dedicated enzymes (nonribosomal peptide synthetases) (146). Alkaloids encompass a broad range of metabolites with the sole commonality of a basic nitrogen atom (5, 34, 152, 163).

To date, secondary metabolites have been isolated from all domains of life. Those from bacteria were among the first to be discovered and were subject to the first engineering efforts. From the eukaryotic kingdom, fungi and plants are known to be prolific sources of secondary metabolites. Notably, animals do not produce many such compounds, most likely because these organisms have other ways of interacting with the environment (e.g., movement). Secondary metabolites have also been isolated from archaea, although the number of examples reported is relatively low; this may be in part due to challenges associated with culturing many species of this domain.

### Metabolic Pathway Elucidation

The biosynthetic genes and/or regulatory elements of a metabolite must be characterized prior to metabolic engineering. The study of bacterial-derived secondary metabolites was hugely accelerated by Hopwood and coworkers' discovery that the biosynthetic genes for the polyketide actinorhodin are clustered on the genome of *Streptomyces* (125). This phenomenon of clustering is now known to be generally true for all bacterial secondary metabolic pathways. Bacterial genomes can be sequenced and subjected to genome mining to rapidly identify new metabolic pathway clusters for functional characterization (13). Over the past decade, it has become clear that the vast majority of fungal secondary metabolite pathways are also clustered on the genome (15, 20, 25).

Although an ever-increasing number of metabolic pathway clusters are being identified in plant genomes (113), not all plant pathways are clustered; the extent to which clustering prevails will be clarified as more plant genomes become available. Because genes of a plant metabolic pathway are frequently coordinately regulated, a known biosynthetic gene can be used as bait to search for undiscovered genes that share similar expression profiles using transcriptomic (RNA-seq) data (132). Additionally, transcriptome-metabolome comparisons can point to potential biosynthetic genes by correlating gene expression with the presence of the secondary metabolite of interest (156).



**Figure 1** Representative members of the major classes of secondary metabolites. Carbohydrates unique to secondary metabolism decorate the structures of many of these compounds (*gray*).

## Principles of Metabolic Engineering

Secondary metabolites have been used for pharmaceutical, cosmetic, and agrochemical purposes for thousands of years. Even today, secondary metabolites account for more than one-third of all therapeutic compounds (110). Although the pharmaceutical industry has largely turned to relatively inexpensive synthetic compounds as a source of new drug leads, in the past decade there has been renewed interest in secondary metabolites (83). This is likely due to the increasing recognition that secondary metabolites are evolutionarily preselected for biological activity; secondary metabolites are termed privileged scaffolds. Unfortunately, many secondary metabolites are produced in small quantities by the producer organism, and these organisms can be slow growing or impossible to culture under laboratory conditions. Metabolic engineering of biological systems has the potential to be a scalable, selective, and cost-effective way to access these high-value molecules with good yields and levels of purity. Furthermore, modifications to the structures of secondary metabolites can often improve or change the biological activity of the compound (26). Pathway engineering can be used to modify the structure of the secondary metabolite, or to make completely novel molecules, with new or improved biological properties. Finally, engineering of secondary metabolism can improve the function or fitness of the host organism.

The choice of host is an essential first consideration in metabolic engineering. Manipulation of the native producer to enhance yields has a long history in industry. Nonengineering strategies, such as random mutation followed by screening or selective breeding, have led to enhancements in secondary metabolite levels, indicating that the yields in the native host can be substantially improved. If the native producer can be genetically manipulated, then engineering the native producer is a viable option. Additionally, if the metabolite is being used to confer a beneficial property, such as enhanced defense or nutritional benefit, to the host, then engineering must take place in the native producer. However, metabolic pathways are tightly regulated, and overexpressing a single biosynthetic enzyme usually has a limited impact on yield. Recent advances have demonstrated that the expression of transcription factors can be used to achieve more global upregulation of secondary metabolism (11). Additionally, altering the site of biosynthesis also has the potential to override the endogenous regulatory controls (151).

Transfer of entire metabolic pathways into a heterologous host is an increasingly recognized alternative to using the native producer. Although heterologous hosts for secondary metabolite production were reported as early as 1985, the development of modern molecular biology tools has made heterologous expression much more accessible. Genes can now be synthesized relatively inexpensively, allowing the codon usage of foreign genes to be optimized for the host organism; this typically results in improved protein expression. Techniques to incorporate genes into dedicated locations in the chromosome ensure stable and controllable gene expression. Isolation and characterization of a wide range of promoters (constitutive and inducible) allow tuning of protein expression levels; it is important to note that high expression levels do not always lead to the highest product yields, and substantial tuning of gene expression plays a crucial role in many successful metabolic engineering efforts. Finally, the ability to assemble large blocks of DNA has made heterologous reconstitution of lengthy and complex pathways possible.

Four heterologous hosts are assessed in this review: *Streptomyces*, *Escherichia coli*, *Saccharomyces cerevisiae*, and *Nicotiana benthamiana*. *Streptomyces*, a soil bacterium that is a prodigious producer of secondary metabolites, was the first genus used for modern metabolic engineering efforts (47). Because *Streptomyces* produces many secondary metabolites, minimal upstream engineering is required to ensure that the appropriate building blocks for the foreign metabolite are available. *Streptomyces* is typically used as a host for pathways from other related bacteria, and there are usually minimal issues with gene expression.

Although *Streptomyces* is still used widely as a host for many bacterial pathways, compared with *E. coli* *Streptomyces* is relatively slow growing, and far fewer genetic tools are available (23, 61). However, *E. coli* is not a major producer of secondary metabolites, so reconstitution in this host typically requires substantial engineering of the native cellular metabolism to ensure that a sufficient amount of precursors are available to build the desired secondary metabolite. Early examples demonstrated that *E. coli* could be used to reconstitute bacterial products, although more recent work has demonstrated that plant metabolites can also be reconstituted successfully in this host. However, many plant enzymes, such as cytochrome P450s, are membrane bound, making expression in a bacterial host problematic (21). Additionally, many plant and some fungal pathways use compartmentalization of biosynthetic enzymes to control biosynthesis (53), and compartmentalization cannot be easily replicated in a bacterial host.

*S. cerevisiae* (baker's yeast), although slower growing than *E. coli*, can be transformed readily; efficient homologous recombination enables stable integration of genes into the chromosome, and a host of genetic tools are available. Numerous metabolic engineering approaches have been developed for *S. cerevisiae* (76, 135). Yeast as a heterologous host has been particularly important for plant-derived pathways such as complex terpenes (136); because plant cytochrome P450s can be challenging to express in *E. coli*, yeast remains the more common choice of host for plant-derived pathways. Additionally, proteins can be targeted to different cellular compartments in a yeast host, which can be an important aspect of a metabolic engineering strategy.

*N. benthamiana*, a relative of tobacco, is emerging as a practical expression host for certain secondary metabolite pathways. This species is relatively fast growing and can be transformed efficiently, and coexpression of large numbers of genes (gene-stacking), which is required for reconstitution of lengthy metabolic pathways, has been successfully demonstrated. Both stable and transient expression of pathway enzymes are possible, with exceptionally high levels of protein expression achieved in some transient expression systems (130). Despite this, microbial expression hosts are still more convenient for many laboratories, and *N. benthamiana* has so far been used primarily for expression of secondary metabolites derived from plants.

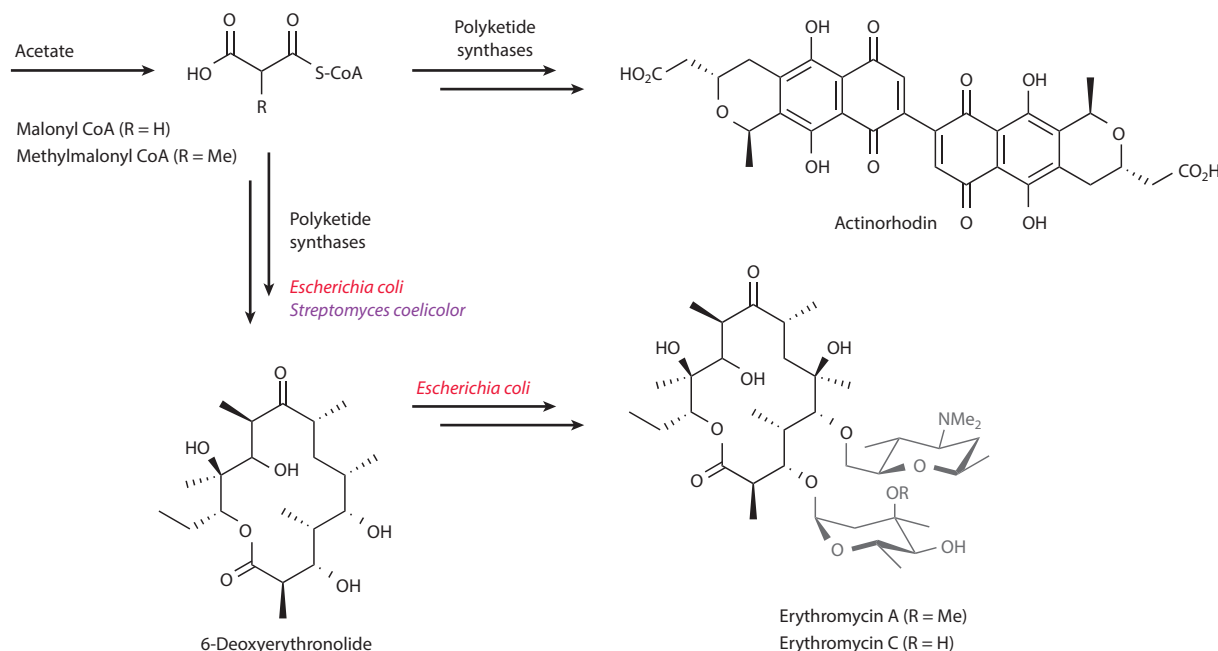
Assessment of yield must be taken on a case-by-case basis. An acceptable yield depends on how much of the metabolite is required, how much is produced by the native producer, and the value of the biological activity of the compound. Generally, in microbial hosts, titers over 1 g/L are considered outstanding.

A critical survey of engineering approaches in secondary metabolism is presented here. Specific examples have been provided to illustrate key developments in the field; unfortunately, many excellent examples have not been included because of space constraints. Additionally, several rigorous reviews in engineering secondary metabolism have been published recently (45, 121, 148, 154).

## PATHWAY RECONSTITUTION IN HETEROLOGOUS HOSTS

An important strategy in secondary metabolic engineering is reconstitution of an entire metabolic pathway into a new host. Although many natural producer organisms are difficult to culture or transform, heterologous expression allows the use of a convenient host that can be genetically manipulated and is safe and cheap to culture. Below, the major heterologous hosts that are used in metabolic engineering are assessed, along with examples of important secondary metabolites that have been produced using these hosts. Other pathway hosts are being developed, including the gram-positive bacterium *Bacillus subtilis* (141), the filamentous fungus *Aspergillus nidulans* (88, 129, 155), the microalga *Chlamydomonas reinhardtii* (44), the moss *Physcomitrella patens* (7), and the liverwort *Marchantia polymorpha* (59).





**Figure 2**

Polyketides have been the workhorse of engineering efforts in secondary metabolism in both *Streptomyces* and *Escherichia coli*. Actinorhodin and erythromycin biosynthetic pathways are shown.

### *Streptomyces*

*Streptomyces* is a genus of soil bacteria that is a rich producer of secondary metabolites, particularly polyketide and peptide-type antibiotics (57). Secondary metabolites derived from *Streptomyces* were among the earliest targets of metabolic engineering; nearly 30 years ago, Hopwood and coworkers reported the reconstitution of actinorhodin (**Figure 2**), an aromatic polyketide secondary metabolite from *Streptomyces coelicolor*, into the heterologous host *Streptomyces parvus* (91). Since then, a range of genetic tools has been developed for *Streptomyces*, most notably *S. coelicolor*, including promoters for gene expression and vectors to integrate large inserts into the chromosome (48). Because *S. coelicolor* produces polyketide- and peptide-derived secondary metabolites, this host requires minimum upstream engineering to heterologously produce these classes of secondary metabolites.

The power of *S. coelicolor* as a heterologous host was further demonstrated in 1994 when Khosla and coworkers reported the first reconstitution of 6-deoxyerythronolide B, a precursor to the polyketide antibiotic erythromycin (**Figure 2**), in titers of 40 mg/L (68). Many other secondary metabolites have subsequently been expressed in this host (48, 89). An optimized *S. coelicolor* strain, in which the gene clusters of the four major metabolites have been deleted (47), has recently been developed, which greatly simplifies isolation of heterologous metabolites when used as a host.

### *Escherichia coli*

*E. coli* is a well-characterized prokaryotic host with a wealth of genetic tools and a fast doubling period. As such, this organism has served as the host for numerous metabolic engineering efforts.

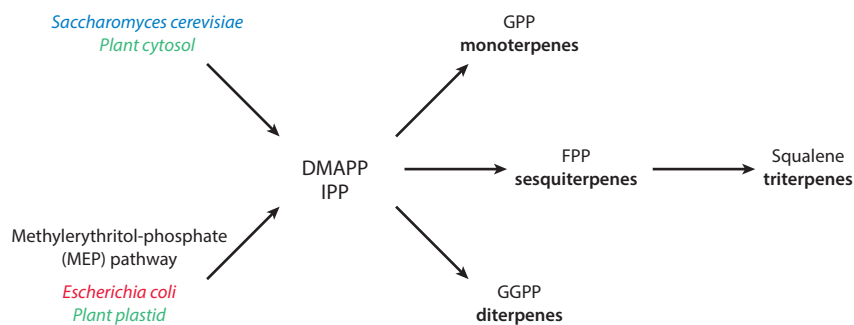
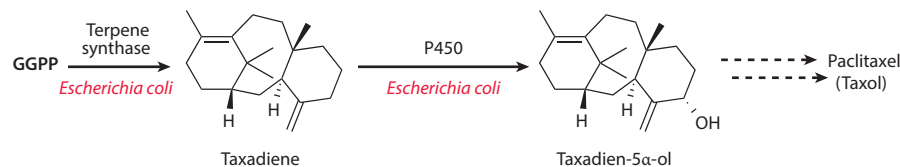
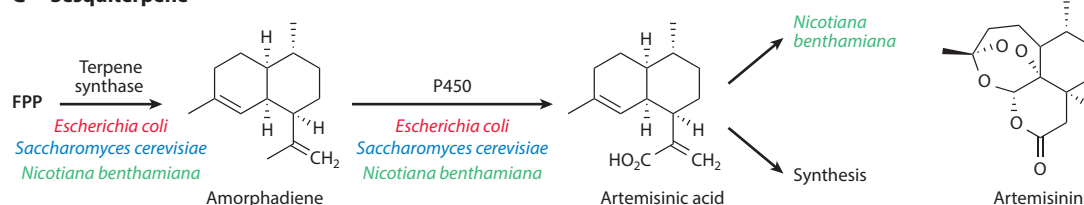


**Erythromycin: a bacteria-derived polyketide.** The reconstitution of a complex polyketide secondary metabolite into *E. coli* was first reported in 2001 (120). The metabolite 6-deoxyerythronolide B, the precursor for the polyketide erythromycin, was produced in *E. coli* at titers comparable to those of an industrially relevant native producer strain. In addition to expressing the biosynthetic enzymes (polyketide synthases) on plasmids, a biosynthetic pathway for the precursor methylmalonyl-CoA was introduced, along with the enzyme required for post-translational modification of the polyketide synthase. Erythromycin has been used as an exemplar for metabolic engineering, and numerous improvements of this initial system have been reported (64, 65, 106, 145). Most notably, reconstitution of the more highly derivatized end product of 6-deoxyerythronolide B, erythromycin C, was reported in 2005 at titers of <1 mg/L (119), and erythromycin A was reported in 2010 in titers of approximately 10 mg/L (159). The production of erythromycins from 6-deoxyerythronolide B requires the addition of 17 additional genes responsible for deoxysugar biosynthesis and macrolide tailoring. Many other bacterially derived natural products have been produced in *E. coli*, indicating that this is a robust choice of host for secondary metabolites from bacteria secondary metabolism (23).

**Paclitaxel (Taxol): a plant-derived diterpene.** Paclitaxel is a diterpene produced by yew (*Taxus brevifolia*, *Taxus baccata*) that has anticancer activity. The terpene precursors, DMAPP and IPP, can be produced from either the methylerythritol-phosphate (MEP) or mevalonic acid (MVA) primary metabolic pathways (Figure 3a). *E. coli* uses the MEP pathway exclusively. A precursor to paclitaxel, taxadiene, was produced at titers of 1 g/L in *E. coli* (2) by overexpressing all of the downstream taxadiene biosynthetic enzymes, along with four rate-controlling MEP genes to ensure adequate amounts of terpene precursors (Figure 3b). Importantly, the MEP genes and the downstream genes were constructed as two separate modules and the levels of the modules were optimized together, emphasizing the importance of balancing the two parts of the pathway together, as opposed to considering pathway enzymes in isolation (154). The next step of the pathway, catalyzed by a cytochrome P450 that hydroxylates taxadiene, was then introduced. It is challenging to express plant P450s into *E. coli* because these enzymes are membrane bound and often require engineering of the N terminus for functional expression in *E. coli* (2). Although yields dropped after introduction of this P450, nevertheless approximately 100 mg/L of hydroxylated taxadiene intermediate was observed. Whether plant-derived pathways containing numerous P450s can be efficiently expressed in *E. coli* remains to be seen (66). The precursors for diterpene biosynthesis have also been improved by combinatorial mutagenesis of some of the upstream biosynthetic enzymes in *E. coli* (82).

**Artemisinin: a plant-derived sesquiterpene.** Artemisinin is a sesquiterpene isolated from sweet wormwood (*Artemisia annua*) that has potent antimalarial activity (Figure 3c). Production of amorphadiene, a biosynthetic intermediate of artemisinin, has been engineered in *E. coli* (92). In this case, the MVA precursor pathway, which is not naturally present in *E. coli*, was engineered into the host and used as the source of the IPP/DMAPP precursor supply (92). A completely heterologous pathway may not be subject to the regulatory controls of the native host and could therefore be more useful for overproduction efforts. This system was subsequently improved by identifying two rate-controlling steps of amorphadiene biosynthesis and then tuning the expression levels of these enzymes, resulting in a titer of 300 mg/L (4). After substantial optimization of the cytochrome P450 that acts on amorphadiene, titers of artemisinic acid of 100 mg/L could be achieved (21). The merits of using the MEP or the MVA pathway for metabolic engineering of terpene biosynthesis are currently being debated (102).



**a Mevalonic acid (MVA) pathway****b Diterpene****c Sesquiterpene****Figure 3**

Terpene secondary metabolism. (a) All terpenes are derived from either the mevalonic acid (MVA) or methyl-erythritol phosphate (MEP) pathways, depending on the organism or location of biosynthesis. (b) Intermediates for Paclitaxel (diterpene) can be reconstituted in *Escherichia coli*. (c) The biosynthesis of artemisinin (sesquiterpene) and corresponding biosynthetic intermediates is described for *E. coli*, *Saccharomyces cerevisiae*, and *Nicotiana benthamiana*.

**Reticuline: a plant-derived benzyloisoquinoline alkaloid.** Reticuline is the precursor for all benzyloisoquinoline alkaloids, which are produced in a variety of plants, such as the opium poppy (*Papaver somniferum*), and include the opiates morphine, codeine, and noscapine. Engineering a five-gene pathway for reticuline in *E. coli* from an exogenously supplied dopamine precursor was first reported in yields of >10 mg/L (100). *E. coli* does not have the intrinsic metabolic capacity to produce alkaloids, and this system was subsequently improved in 2011 when *E. coli* was engineered to produce dopamine de novo using pathway enzymes from a variety of organisms (bacterial and mammalian). In this system, reticuline could be produced from glycerol at yields of 6 mg/L (108).

**Plant-derived phenylpropanoids.** Phenylpropanoids from plants, which are composed of stilbenes, flavonoids, flavonols, flavonones, and anthocyanins, have beneficial health properties (Figure 1) (122, 140). Production of flavonones in *E. coli* by transformation of a plasmid harboring three heterologous genes was first reported in 2003 in yields up to 11 µg/L, when the precursor



aromatic amino acid (phenylalanine or tyrosine) was added exogenously (56). These yields have been substantially improved over the past decade; by testing enzymes from numerous plant sources and tuning expression levels, titers of 84 mg/L of the common flavonoid naringenin have been achieved from glucose (131). Resveratrol, a stilbene found in red wine, has been produced in excellent titers (2.3 g/L) when the intracellular malonyl-coenzyme A pool was enhanced (84).

### *Saccharomyces cerevisiae*

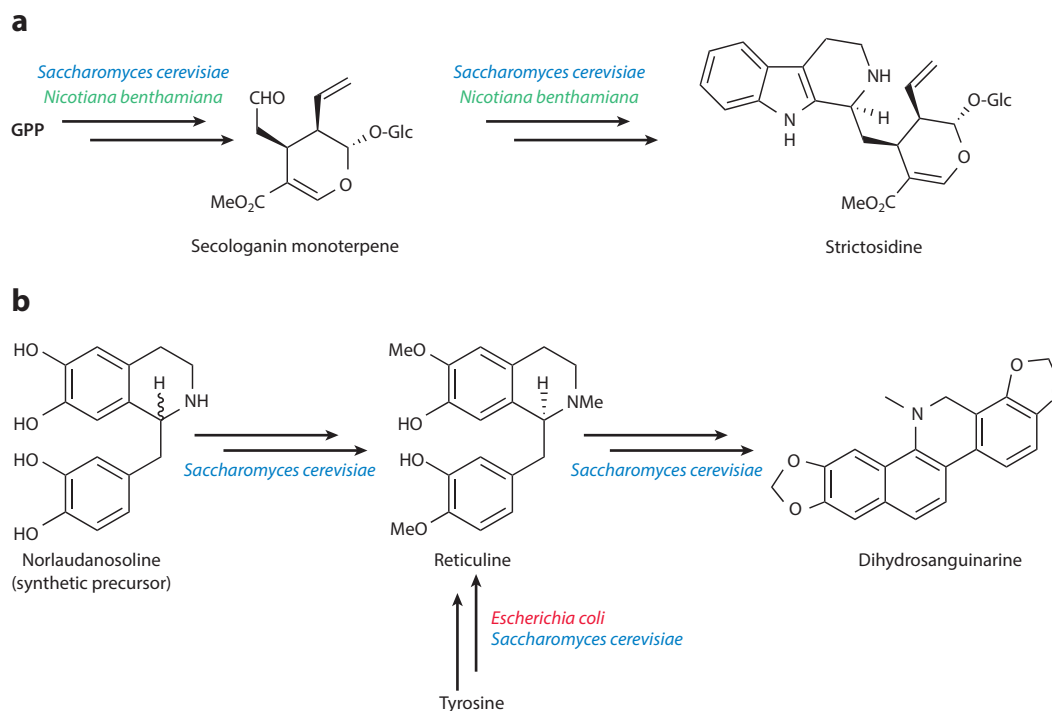
*S. cerevisiae* (baker's yeast) is a common eukaryotic host for metabolic engineering. Efficient homologous recombination enables stable integration of genes into the chromosome, and many genetic tools are available for this organism. Yeast also contains different cellular compartments, which can be exploited in metabolic engineering strategies.

**Artemisinin: a plant-derived sesquiterpene.** A prominent case study of metabolic engineering in yeast is production of artemisinic acid (123), an advanced biosynthetic precursor of artemisinin (Figure 3c) (116). In this case, the MVA pathway—the pathway used naturally by yeast for terpene precursor production—was optimized by overexpressing rate-controlling genes and by deleting genes responsible for precursor diversion into sterol biosynthesis. Additionally, a novel cytochrome P450 involved in artemisinic acid formation was identified. Overall titers in this strain were 100 mg/L. This process was improved by the discovery of two additional enzymes that assisted with the formation of a carboxylic acid present on artemisinic acid, allowing titers of at least 25 g/L (117, 147), which are sufficiently high to enable commercialization. In a related approach, an engineered cytochrome p450 was introduced into the system to oxidize amorphaadiene into the alternative semisynthetic substrate dihydroartemisinic acid (32).

**Strictosidine: a plant-derived monoterpene indole alkaloid.** Strictosidine is a key intermediate in the biosynthesis of the monoterpene indole alkaloids, which includes the anticancer agents vinblastine and vincristine from *Catharanthus roseus* (Figure 4a). Strictosidine is derived from a monoterpene, a class of terpene that yeast does not naturally produce. However, yeast can be engineered, by expressing key enzymes of the upstream MVA pathway and dedicated monoterpene synthases, to direct flux into monoterpene synthesis (58). For strictosidine biosynthesis, a total of 21 genes were introduced into yeast to increase levels of monoterpene precursors and to convert the precursor to the final product. This is one of the longest plant pathways to be reconstituted into yeast, and titers of strictosidine at 0.5 mg/L were reported (16).

**Plant-derived isoquinoline alkaloids.** A number of benzyloisoquinoline alkaloid pathways have been partially reconstituted into *S. cerevisiae* (Figure 4b). Although the upstream pathway leading from tyrosine to the biosynthetic intermediate reticuline can be expressed in *E. coli*, these upstream enzymes have not been expressed in an active form in yeast until recently (31). Production of reticuline from glucose in yeast was achieved by developing an enzyme-coupled biosensor to identify a tyrosine hydroxylase that was active when expressed in yeast. Prior to this recent development, all isoquinoline alkaloids expressed in yeast have been produced from an exogenously supplied substrate, the commercially available compound norlaudanosoline. Hawkins et al. (52) reported production of the key intermediate reticuline from norlaudanosoline, and the downstream alkaloids scoulerine, tetrahydrocolumbamine, salutaridine, and tetrahydroberberine, which are derived from reticuline. Enzyme expression levels could be tuned and correlated with product titers (52). A subsequently reported system could produce the highly derivatized alkaloids codeine,





**Figure 4**

Alkaloid metabolism. (a) The biosynthesis of the alkaloid strictosidine has been reconstituted in both *Saccharomyces cerevisiae* and *Nicotiana benthamiana*. (b) Alkaloids such as dihydrosanguinarine can be produced in *S. cerevisiae* from the synthetic intermediate norlaudanosoline. The native biosynthetic intermediate reticuline can be produced in *Escherichia coli*.

morphine, hydromorphone, hydrocodone, and oxycodone from the advanced intermediate thebaine (137). Dihydrosanguinarine has also been produced in yeast from norlaudanosoline by introduction of a 10-gene pathway into *S. cerevisiae*. Yields were 1.5% from the norlaudanosoline precursor (40).

**Plant-derived phenylpropanoids.** The stilbene resveratrol was first reconstituted in yeast from p-coumaric acid at titers of 1  $\mu\text{g/L}$  by expressing two genes required for resveratrol biosynthesis on plasmids (10), and subsequently improved to approximately 300  $\mu\text{g/L}$  (139). Naringenin, the central precursor of all flavonoids, was reconstituted by introducing three genes from the phenylpropanoid pathway into *S. cerevisiae* under an inducible promoter to yield titers of approximately 7 mg/L of naringenin (63). More complex derivatives of naringenin have been reconstituted from phenylalanine in metabolically engineered yeast strains harboring plasmids with heterologous biosynthetic genes in yields up to 7 mg/L (139). After extensive optimization of expression levels, de novo production of the flavonoid naringenin from glucose has been achieved (75). Notably, the yields for phenylpropanoids are consistently higher in an *E. coli* host, which is surprising because yeast is phylogenetically closer to the native plant producer.

**Fungal-derived polyketides.** Fungi are increasingly recognized as a rich source of secondary metabolites. A number of fungal-derived polyketide pathways have been successfully

reconstituted in *S. cerevisiae*. In a breakthrough study, a large, multifunctional polyketide synthase from *Aspergillus terreus* responsible for lovastatin biosynthesis was expressed in *S. cerevisiae* (90). Additional pathways have since been reconstituted in product titers of approximately 1–15 mg/L (e.g., 126, 161). Filamentous fungi produce a host of other secondary metabolite classes, some of which have also been successfully expressed in yeast at good titers (60).

### *Nicotiana benthamiana*

*N. benthamiana*, a relative of tobacco, has been used as a practical expression host. This plant is relatively fast growing and can be transformed efficiently, and multiple genes can be expressed (stacked) using both stable and transient expression.

**Plant-derived glucosinolates.** The amino acid–derived glucosinolate metabolites benzylglucosinolate and glucoraphanin, which have health-giving properties for humans and insect deterrence properties for the host plant, were reconstituted in *N. benthamiana* using a transient expression system (43, 99). These examples were one of the first proof-of-concept studies demonstrating that a large number of genes (14) could be stacked in a plant host to successfully produce a desired metabolite at reasonable yields (e.g., 0.4  $\mu\text{mol}$  glucoraphanin/g dry weight of plant tissue). Expression cassettes containing multiple genes could be expressed under the control of a single promoter by using a viral 2A autoprocessing sequence. This pathway was also the basis for a study involving engineering of transport.

**Strictosidine: a plant-derived monoterpene indole alkaloid.** The first reconstitution of strictosidine, the common precursor for all monoterpene indole alkaloids, was performed in the *N. benthamiana* host (98). Ten genes encoding the strictosidine pathway, along with two genes to boost terpene precursor formation, were transiently expressed on individual plasmids. From these experiments, an advanced precursor of strictosidine was isolated. Co-infiltration of early intermediates of the pathway along with the plasmids improved yields of strictosidine. Upstream biosynthetic intermediates appeared to be derivatized by endogenous *N. benthamiana* enzymes (54), thereby reducing the flux into end-product synthesis. Unfortunately, although the rich metabolism of *N. benthamiana* ensures an ample supply of precursors for secondary metabolite biosynthesis, these native enzymes can derivatize some foreign biosynthetic intermediates, diverting them into dead-end products. Successful genome editing has recently been demonstrated in *N. benthamiana* (109), and it may be possible to use this technology to create *N. benthamiana* hosts that are more optimized for metabolite production (e.g., by eliminating certain endogenous secondary metabolite enzymes).

**Artemisinin: a plant-derived sesquiterpene.** Biosynthesis of artemisinin—not an advanced intermediate—was achieved in a stable transgenic tobacco line in 2011 (37). Transgenic plants expressed five plant- and yeast-derived genes from the mevalonate and artemisinin pathways. These five genes were cloned into a single vector, targeted to mitochondria, which appeared to enhance yields. Overexpression of one of the rate-limiting MVA genes was also important in achieving good titers. Artemisinin was produced at 7  $\mu\text{g/g}$  dry weight plant tissue, which is lower than the native plant producer *Artemisia annua*. However, the transgenic plant has not yet been subjected to optimization efforts. Transient expression of these same enzymes in *N. benthamiana* does not lead to artemisinin, but yields of artemisinic acid reached 4  $\mu\text{g/kg}$  of fresh weight plant tissue (138).



**Plant-derived triterpenes.** A three-gene module from a triterpene gene cluster from oat was transiently expressed in *N. benthamiana* as three individual genes to produce a glycosylated, acetylated triterpene product in yields of 40 nmol/mg fresh weight of plant tissue (104). Substantial improvements in triterpene production levels in tobacco have also been achieved by changing the location of biosynthesis.

## PATHWAY ENGINEERING IN NATIVE HOSTS

Titers of secondary metabolites have been improved by random mutation or selective breeding of the native host, but genetic manipulations of the native producer—if the host is amenable to genetic manipulation—often result in modest changes to the product profile. Secondary metabolism is tightly and redundantly regulated, requiring innovative strategies to alter titer levels in the native host.

### Turning on Cryptic Clusters in Native Microbial Producers

The genomes of many bacteria and fungi encode gene clusters for secondary metabolites that are not expressed under laboratory conditions. Although heterologous expression of these clusters is one way to access the product encoded by these molecules, engineering the native host to activate these clusters is an attractive alternative (8). Provided that the native host can be genetically manipulated, the awakening of silent clusters can allow rapid functional characterization of the corresponding product.

In the bacteria *Streptomyces*, several approaches have been used to awaken secondary metabolite production. Constitutive expression or deletion of a regulatory element, usually present within the gene cluster, often stimulates metabolite production (18, 49, 81). Insertion of a heterologous promoter in front of selected cluster genes has also proven successful (114). Certain point mutations to the ribosome have been linked to overproduction of silent clusters, although this method is not widely used for discovery of new secondary metabolite pathways (8). Finally, random mutagenesis has been coupled to a promoter-reporter system that selects for mutants in which transcription from the targeted gene cluster is activated (51). *Aspergillus*, a genus of fungi that produces a diverse range of secondary metabolites, is also highly amenable to gene cluster activation (24, 85). One early study fused a heterologous promoter to a transcription factor associated with the cluster, leading to expression of the product (11). Given that many fungal gene clusters contain a dedicated transcription factor, this is a widely applicable approach. Chromatin modifications have also been linked to regulation of secondary metabolites, and silencing of biosynthetic gene clusters has been reversed in *A. nidulans* by removal of genes involved in histone methylation, suggesting that expression of secondary metabolite gene clusters is mediated by chromatin configuration (14).

The complexity of plant metabolism and plant genome biology makes identification of cryptic gene clusters in plants more challenging. However, as more plant genomes become available, analogous strategies to identify and mine cryptic or unexpressed pathway in plants may become an important tool in plant secondary metabolite discovery.

### Enhancing and Introducing Pathways in Native Plant Producers

Introduction of desirable traits into plants can be achieved by manipulation of secondary metabolism. Engineering the metabolism of native, nonmodel plant producers poses substantial challenges, but an enormous range of tools for gene overexpression, knockdown, and regulation has been developed in the past several years (157) since the earliest demonstrations of secondary



metabolite engineering in plants (e.g., 97). Both silencing and overexpression of transcription factors and key pathway enzymes have been used to modify secondary metabolite profiles in plants. The push strategy refers to using early pathway enzyme genes to push the flux downstream, whereas the pull strategy refers to expression of downstream genes to draw the substrates toward the final product (157). Although in a few cases these engineering efforts are used for the overproduction and isolation of a specific product (e.g., 160), in many cases the goal is to create plants with improved taste, scent, color, or defensive properties. However, the legislation concerning genetically modified plants may limit widespread application of these new plants; for discussion of one high profile case study on Golden Rice, see Reference 33.

**Enzyme overexpression.** Tomatoes are a principal dietary source of flavonoids and anthocyanins, secondary metabolites that are highly beneficial for human health. Flavonol biosynthesis was upregulated in tomato by transforming tomato with a flavonoid biosynthetic gene from petunia, leading to an increase of up to 78-fold in flavonols in the peel (105). The taste of tomatoes was improved by overexpressing a monoterpene synthase from basil under the control of a ripening-specific promoter. Notably, the increased monoterpene resulted in a decrease in lycopene, the red pigment of tomato, also derived from the terpene pathway (28).

Overexpression of certain secondary metabolite enzymes can enhance the plant's defense response. Maize was transformed with a terpene synthase gene from oregano that yields a sesquiterpene that attracts beneficial nematodes. The resulting maize plants constitutively emitted this sesquiterpene, resulting in substantially less root damage in field trials (30). A terpene synthase that makes a sesquiterpene that confers herbivore resistance is found in wild species of tomatoes but has been lost during the cultivation process. This terpene synthase was expressed in the trichomes of cultivated tomato, and the herbivore resistance of the wild species was restored (12). Recently, wheat was engineered to release the sesquiterpene (E)- $\beta$ -farnesene, the alarm pheromone for pest aphids (17). However, these plants showed no reduction of aphids in the field. Natural defense from aphids involves a pulsed emission of (E)- $\beta$ -farnesene, suggesting that aphids may become habituated to the continuous levels of the compound produced by the transgenic lines (17, 79). These experiments demonstrate how both the identity and expression profiles of secondary metabolism can be used to enhance the fitness of crop plants.

**Enzyme downregulation.** Improvements in the profile of peppermint oil were made by silencing a synthase that generates an undesirable monoterpene side product. Combined with overexpression of an MEP pathway gene, a 60% increase in oil with low levels of the undesirable side-product was achieved in field trials (80). To modify flower color in carnation, antisense suppression was used to block the expression of a key gene in the anthocyanin pathway. The transgenic plants exhibited a range of flower colors caused by slight decreases in anthocyanin production to flowers that lacked any color at all. Unexpectedly, transgenic plants with severe color modification were more fragrant than control plants because of higher levels of a biosynthetically unrelated compound, methyl benzoate (164). These unexpected effects often occur when engineering native metabolism. Genome editing methods, which are becoming routine for nonmodel plants (78), will allow faster and more selective knockdown of genes to impact secondary metabolism.

**Transcription factors.** Typically, transcription factors control production of a group of metabolites, as these regulators usually control expression of more than one gene (157). Expression of heterologous transcription factors has resulted in dramatic impacts on product profiles. In early experiments, when anthocyanin pathway-specific transcriptional activators from maize were transformed into *A. thaliana*, anthocyanins were produced in root, petal, and stamen tissues of the



transgenic plants (86). More recently, after a transcription factor from *A. thaliana* involved in anthocyanin biosynthesis was introduced into rose, both phenylpropanoids and terpenes that contribute to scent and color were upregulated in the resulting transgenic rose plants (165). When a regulatory gene that represses several signaling pathways controlled by light was silenced in tomato using RNA interference, flavonoid and carotenoid (terpene) content increased significantly (29). Anthocyanin production has been enhanced in tomato fruits by overexpressing two transcription factors involved in upregulating anthocyanin biosynthesis in snapdragon (19). Tomatoes that were colored purple throughout the fruit tissue resulted, and they contained the highest levels of anthocyanins ever reported in tomato. The search for transcription factors that control levels of high-value natural products is a burgeoning area in plant metabolism (142).

### ENGINEERING LOCATION OF SECONDARY METABOLISM

Many eukaryotic biosynthetic pathways, particularly in plants, are highly compartmentalized at the inter- and intracellular levels (50, 53). In addition to the linear design of metabolic pathways, successful engineering must consider this third dimension (45). This is a relatively new area of engineering within secondary metabolism, but early successes indicate that this approach should be further explored.

An emerging approach is to synthesize secondary metabolites in compartments where the metabolic process does not normally occur (150). For example, localizing terpene biosynthetic pathways to the plastids of plants has been shown to result in high levels of product (53). In plants, terpene biosynthesis generally occurs in either the cytosol via the mevalonic acid (MVA) pathway to make sesquiterpenes and triterpenes, or in the plastid via the methylerythritol phosphate (MEP) pathway to make monoterpenes and diterpenes. In a pioneering study, a sesquiterpene synthase, along with an upstream enzyme that makes the terpene precursor, was expressed in tobacco plastids, resulting in up to 1,000-fold higher accumulation of sesquiterpene levels when compared to levels achieved when the enzymes were expressed in the cytosol (151). Similar experiments were performed for triterpenes, which are also naturally produced in the cytosol. When two triterpene biosynthetic enzymes were expressed in tobacco plastids, up to 90-fold higher triterpene levels were observed (150). For reasons that are not clear, terpene production in the cytosol appears to be much more tightly regulated than it is in the plastid (1). In a subsequent study, the entire six-gene MVA pathway (normally expressed in the cytosol) was transferred to the plastid of tobacco, and this appeared to stimulate higher levels of terpene production in the plant compared to negative controls (77). Again, whether the MEP or MVA pathways would generally yield higher titers remains unclear. Notably, multiple genes for expression in the plastid can be stacked in an operon and coregulated by transcription from the same promoter as a polycistronic pre-mRNA. Use of such plasmids has resulted in up to tenfold improvement in titer of the plastid-produced product compared to controls (87).

Targeting biosynthetic enzymes to the mitochondria has also resulted in improved yields of terpenes and other compounds—up to a 20-fold improvement in levels of a sesquiterpene was found when the pathway was expressed in yeast mitochondria (38). New profiles of terpene products were also observed after changing localization of certain pathway enzymes to the mitochondria in the model plant *Arabidopsis thaliana* (69). It is speculated that these increases in titer after compartmentalizing a specific pathway may be due to higher local enzyme concentrations, increased availability of intermediates, the prevention of intermediates being diverted into other pathways, or interference of these intermediates with other cellular pathways (6).

The spatial separation of pathway enzymes implies that dedicated transporters exist to shuttle the intermediates to the correct location (112). A number of transporters involved in secondary



metabolism have been identified (112), but more study is required to evaluate whether approaches involving transporters will be a generally useful approach in metabolic engineering. One highly successful example of transport engineering involves the glucosinolates, which are synthesized in roots and then shuttled to seeds. Two transporters involved in moving glucosinolates to the seeds were identified in *A. thaliana*, and when knocked down, glucosinolates could no longer be detected in seeds (111). Because glucosinolate-free seeds from certain crops are highly desirable for feedstocks, this is an important demonstration of how transport engineering can introduce a desirable trait into plants.

## ENGINEERING APPROACHES TO PRODUCE UNNATURAL PRODUCTS

Modifying the chemical structures of secondary metabolites can often improve or change the biological activity of the parent compound (26). Genetic manipulation of biosynthetic pathways is a powerful way to make unnatural analogs, or completely novel molecules, with new or improved biological properties (101).

### Mutasynthesis

Unnatural products are produced when the biosynthesis of the natural starting material is genetically blocked, and the producing organism is forced to utilize exogenously supplied precursors exclusively for product biosynthesis. This strategy, termed mutasynthesis, was first applied several decades ago to yield novel antibiotics in *Streptomyces* (134). This strategy has been applied to countless microbial systems with great success, with too many examples to list individually (71). Precursor feeding is not practical for plants, but an example of mutasynthesis with plant cultures has been reported (127).

### Swapping Pathway Enzymes

Enzymes can be swapped among pathways to yield new metabolic processes that produce novel products, provided that downstream enzymes can recognize unnatural intermediates. One of the earliest examples showed that gene transfer between strains producing actinorhodin and several other related aromatic polyketides could be used to make actinorhodin analogs (55). These experiments have been expanded over the decades, largely facilitated by powerful heterologous expression hosts. In one recent example, fungal synthases that produce aromatic polyketides were expressed as random pairs in a yeast heterologous host to create a diverse library of molecules, including one member with an unprecedented skeleton and unique biological activity (153).

The saccharides that decorate the core scaffold of secondary metabolites can also be readily modified by enzyme swapping. For example, the saccharides that decorate erythromycin can be changed by substituting in saccharide pathways from other related polyketides, albeit at reduced titer levels (67). Again, these experiments are greatly facilitated by the heterologous expression systems that allow different biosynthetic genes to be switched in and out rapidly. Furthermore, glycosyltransferases from secondary metabolite pathways can act in reverse, allowing the sugar and aglycone moieties to be readily exchanged, allowing a wide diversity of scaffold-saccharide combinations to be synthesized (158). Many examples of this glyco-randomization approach have been successfully reported (42).

Plant pathways that use stand-alone enzymes are particularly amenable to pathway swapping. For example, libraries of flavonoid compounds using heterologous production systems



in *S. cerevisiae* (see Plant-Derived Phenylpropanoids) have been developed. Combinations of flavonoid biosynthetic genes along with unnatural substrates lead to new analogs, with some having new biological activities (22). The assembly process has been further expanded by using seven flavonoid pathways randomly combined on yeast artificial chromosomes, with a combination of natural and unnatural precursors (107). Plant-derived triterpenes, which can also be reconstituted in *S. cerevisiae* (27, 72), can be combined in different combinations with tailoring enzymes (P450s, glycosyltransferases) to produce both natural and unnatural triterpenes (41, 103).

Specialized enzymes decorate secondary metabolites with unusual functional groups that impart unique biological activity, with halogenation being one important example. To introduce this important functional group more widely into secondary metabolism, a foreign chlorinase gene was transformed into *Streptomyces coeruleorubidus* to yield a chlorinated analog of the native antibiotic product (124). A similar strategy was reported in which a halogenase from a bacterial pathway was transformed into medicinal plant cultures to yield unnatural chlorinated and brominated alkaloids (128). Although fluorinated compounds are known in nature, the only known natural fluorinated product is fluoroacetate. To add the fluoro functionality to polyketide secondary metabolites, a pathway to synthesize the required substrate, fluoromalonyl-CoA, was developed and successfully interfaced with a polyketide pathway to generate a fluorinated analog of a 6-deoxyerythronolide B biosynthetic intermediate (144). These examples highlight how metabolic engineering can take a relatively rare chemical modification (e.g., halogenation) and allow it to be incorporated into a broader array of metabolic products.

Instead of secondary metabolite analogs, completely novel compounds can be generated by randomly combining biosynthetic enzymes from a wide range of sources. This approach was implemented in a yeast host to create a library of more than 70 compounds, most of which have not been described previously (74).

## Enzyme Engineering

Enzyme engineering is a critical element to metabolic engineering. It is beyond the scope of this review to cover the applications of enzyme engineering to secondary metabolism in detail (9), but a few key examples are discussed below.

A major focus has been on polyketide biosynthetic pathways that generate macrolide-type polyketides such as erythromycin (**Figure 2**) (149). The enzymes that generate these polyketides are large, multidomain enzymes, with one module dedicated to each substrate that is added to the product. Polyketide analogs can be generated by cutting out an acyltransferase (AT) domain and replacing it with another having different substrate specificity (94, 120, 159). Although the downstream modules appear to be highly tolerant of the resulting unnatural biosynthetic intermediates, crucial protein-protein interactions are disrupted by this cut-and-paste approach. Better protein designs have been enabled by understanding the structural interactions that occur within and between modules (35, 46). These interactions are also responsible for polyketide size: Additional changes in polyketide structure can be achieved by mutation at the protein-protein interface so that two successive rounds of chain elongation are catalyzed, resulting in a bigger polyketide product (70).

Nonribosomal peptide synthetases, which construct peptides, function much like polyketide synthases. However, instead of an AT domain selecting an acyl substrate, an adenylation (A) domain selects an amino acid substrate. Nonribosomal peptide synthetases have been used to demonstrate how directed evolution can be used to restore the activity of the enzyme after foreign A domains are cut and pasted into the protein (39). The problems created by the cut-and-paste

approach can be avoided altogether by using point mutations to modify the substrate specificity of the native A domain. By mutating active site residues, coupled with a rapid mass spectrometry-based screening technique, a large number of A domain variants with alternate substrate specificity profiles could be generated (36). Ribosomal-generated peptides such as lantibiotics have proven to be highly amenable to structural engineering. These pathways use a ribosomal encoded peptide that is subjected to a variety of posttranslational modifications to yield the final secondary product. A leader peptide sequence, which is cleaved after biosynthesis, provides the recognition elements required for peptide modification following translation. The structure of the peptide product can therefore readily be altered by standard site-directed mutagenesis, provided it is attached to the leader peptide (133). Strategies to eliminate the requirement for a leader peptide have recently been reported (115).

## CONCLUSIONS

Engineering of secondary metabolism, which aims to enhance yield, increase chemical diversity, and/or generate species with enhanced phenotypes, poses both substantial challenges and opportunities. If the goal is to isolate the secondary metabolite, then the choice of host is framed in terms of the best titers in the highest purity. Alternatively, metabolic engineering may be employed to alter the properties of the host: for example, engineering the secondary metabolism of a plant might confer increased disease resistance. In these cases, the host for which the application is being designed must be used. The host may not be sequenced, or have appropriate genetic tools available, and embedded regulatory networks can tightly and redundantly control the levels of product production, usually to low levels. Nevertheless, a number of spectacularly successful outcomes for engineering native host metabolism are highlighted. Innovative solutions such as disrupting regulatory elements or moving the location of the metabolic pathway can be sufficient to override tight regulatory controls.

The use of heterologous hosts for production of secondary metabolites has often been framed in the terminology of synthetic biology, in which a neutral chassis is available for the installation of metabolic parts that can be rapidly combined to make the desired molecule. However, expressing pathways in heterologous hosts to commercially viable titers has a high failure rate and typically requires substantial optimization (and innovation) before success is achieved. Nevertheless, the advancements that have been made over the past two decades suggest that this approach has enormous potential to harness metabolism that would otherwise be out of reach. The choice of a heterologous host is still largely empirical, with the general assumption that the closer the host is to the native producer, the higher the titers, although there are exceptions. As more examples are reported, more reliable guidelines for which host is best suited for which metabolite may become clearer. Culturing combinations of hosts, with each one encoding the portion of the metabolic pathway for which it is best suited, may also be an option (162).

Artemisinin is an exemplar of a successful heterologous production platform; semisynthetic artemisinin derived from an *S. cerevisiae* host has been produced at titers high enough to enable commercialization. However, it is important to note that the native artemisinin producer, the plant *Artemisia annua*, remains an important source of this compound, highlighting that natural production sources can also be efficient production platforms. The artemisinin example has also brought to light broader social issues that can arise in the wake of a successful metabolic engineering project; it is not clear how the economic situation of the farmers who grow *A. annua* in the developing world will be impacted by an alternative production source. Despite all of these challenges, engineering of secondary metabolism is enabling much wider applications of these high-value, complex molecules.



## DISCLOSURE STATEMENT

The author is not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

## LITERATURE CITED

1. Aharoni A, Giri AP, Deuerlein S, Griepink F, de Kogel WJ, et al. 2003. Terpenoid metabolism in wild-type and transgenic *Arabidopsis* plants. *Plant Cell* 15:2866–84
2. Ajikumar PK, Xiao W-H, Tyo KEH, Wang Y, Simeon F, et al. 2010. Isoprenoid pathway optimization for taxol precursor overproduction in *Escherichia coli*. *Science* 330:70–74
3. Allen RS, Millgate AG, Chitty JA, Thisleton J, Miller JA, et al. 2004. RNAi-mediated replacement of morphine with the nonnarcotic alkaloid reticuline in opium poppy. *Nat. Biotechnol.* 22:1559–66
4. Anthony JR, Anthony LC, Nowroozi F, Kwon G, Newman JD, Keasling JD. 2009. Optimization of the mevalonate-based isoprenoid biosynthetic pathway in *Escherichia coli* for production of the anti-malarial drug precursor amorpha-4,11-diene. *Metab. Eng.* 11:13–19
5. Ashihara H, Sano H, Crozier A. 2008. Caffeine and related purine alkaloids: biosynthesis, catabolism, function and genetic engineering. *Phytochemistry* 69:841–56
6. Avalos JL, Fink GR, Stephanopoulos G. 2013. Compartmentalization of metabolic pathways in yeast mitochondria improves the production of branched-chain alcohols. *Nat. Biotechnol.* 31:335–41
7. Bach SS, King BC, Zhan X, Simonsen HT, Hamberger B. 2014. Heterologous stable expression of terpenoid biosynthetic genes using the moss *Physcomitrella patens*. *Methods Mol. Biol.* 1153:257–71
8. Baltz RH. 2011. Strain improvement in actinomycetes in the postgenomic era. *J. Ind. Microbiol. Biotechnol.* 38:657–66
9. Bar-Even A, Salah Tawfik D. 2013. Engineering specialized metabolic pathways: Is there a room for enzyme improvements? *Curr. Opin. Biotechnol.* 24:310–19
10. Becker J, Armstrong G, Vandermerwe M, Lambrechts M, Vivier M, Pretorius I. 2003. Metabolic engineering of *Saccharomyces cerevisiae* for the synthesis of the wine-related antioxidant resveratrol. *FEMS Yeast Res.* 4:79–85
11. Bergmann S, Schumann J, Scherlach K, Lange C, Brakhage A, Hertweck C. 2007. Genomics-driven discovery of PKS-NRPS hybrid metabolites from *Aspergillus nidulans*. *Nat. Chem. Biol.* 3:213–17
12. Bleeker P, Mirabella R, Diergaarde P, VanDoorn A, Tissier A, et al. 2012. Improved herbivore resistance in cultivated tomato with the sesquiterpene biosynthetic pathway from a wild relative. *PNAS* 109:20124–29
13. Blin K, Medema MH, Kazempour D, Fischbach MA, Breitling R, et al. 2013. antiSMASH 2.0: a versatile platform for genome mining of secondary metabolite producers. *Nucl. Acids Res.* 41:W204–12
14. Bok JW, Chiang YM, Szewczyk E, Reyes-Dominguez Y, Davidson AD, et al. 2009. Chromatin-level regulation of biosynthetic gene clusters. *Nat. Chem. Biol.* 5:462–64
15. Bok JW, Hoffmeister D, Maggio-Hall LA, Murillo R, Glasner JD, Keller NP. 2006. Genomic mining for *Aspergillus* natural products. *Chem. Biol.* 13:31–37
16. Brown S, Clastre M, Courdavault V, O'Connor SE. 2015. De novo production of the plant-derived alkaloid strictosidine in yeast. *PNAS* 112:3205–10
17. Bruce TJ, Aradottir GI, Smart LE, Martin JL, Caulfield JC, Doherty A, Sparks CA, Woodcock CM, Birkett MA, Napier JA, Jones HD, Pickett JA. 2015. The first crop plant genetically engineered to release an insect pheromone for defence. *Sci. Rep.* 5:11183
18. Bunet R, Song L, Mendes MV, Corre C, Hotel L, et al. 2011. Characterization and manipulation of the pathway-specific late regulator AlpW reveals *Streptomyces ambofaciens* as a new producer of kinamycins. *J. Bacteriol.* 193:1142–53
19. Butelli E, Titta L, Giorgio M, Mock HP, Matros A, et al. 2008. Enrichment of tomato fruit with health-promoting anthocyanins by expression of select transcription factors. *Nat. Biotechnol.* 26:1301–8
20. Cacho RA, Tang Y, Chooi YH. 2014. Next-generation sequencing approach for connecting secondary metabolites to biosynthetic gene clusters in fungi. *Front. Microbiol.* 5:774

21. Chang MCYE, Eachus RA, Trieu W, Ro D-K, Keasling JD. 2007. Engineering *Escherichia coli* for production of functionalized terpenoids using plant P450s. *Nat. Chem. Biol.* 3:274–77
22. Chemler JA, Lim CG, Daiss JL, Koffas MA. 2010. A versatile microbial system for biosynthesis of novel polyphenols with altered estrogen receptor binding activity. *Chem. Biol.* 17:392–401
23. Chen X, Zhou L, Tian K, Kumar A, Singh S, et al. 2013. Metabolic engineering of *Escherichia coli*: a sustainable industrial platform for bio-based chemical production. *Biotechnol. Adv.* 31:1200–23
24. Chiang Y-M, Lee K-H, Sancheza J, Keller N, Wang C. 2009. Unlocking fungal cryptic natural products. *Nat. Prod. Commun.* 4:1505–10
25. Coyle CM, Panaccione DG. 2005. An ergot alkaloid biosynthesis gene and clustered hypothetical genes from *Aspergillus fumigatus*. *Appl. Environ. Microbiol.* 71:3112–18
26. Cummings M, Breitling R, Takano E. 2014. Steps towards the synthetic biology of polyketide biosynthesis. *FEMS Microbiol. Lett.* 351:116–25
27. Dai Z, Liu Y, Zhang X, Shi M, Wang B, et al. 2013. Metabolic engineering of *Saccharomyces cerevisiae* for production of ginsenosides. *Metab. Eng.* 20:146–56
28. Davidovich-Rikanati R, Sitrit Y, Tadmor Y, Iijima Y, Bilenko N, et al. 2007. Enrichment of tomato flavor by diversion of the early plastidial terpenoid pathway. *Nat. Biotechnol.* 25:899–901
29. Davuluri GR, van Tuinen A, Fraser PD, Manfredonia A, Newman R, et al. 2005. Fruit-specific RNAi-mediated suppression of DET1 enhances carotenoid and flavonoid content in tomatoes. *Nat. Biotechnol.* 23:890–95
30. Degenhardt J, Hiltpold I, Kollner TG, Frey M, Gierl A, et al. 2009. Restoring a maize root signal that attracts insect-killing nematodes to control a major pest. *PNAS* 106:13213–18
31. DeLoache WC, Russ ZN, Narcross L, Gonzales AM, Martin VJ, Dueber JE. 2015. An enzyme-coupled biosensor enables (S)-reticuline production in yeast from glucose. *Nat. Chem. Biol.* 11:465–71
32. Dietrich J, Yoshikuni Y, Fisher KJ, Woolard FX, Ockey D, et al. 2009. A novel semi-biosynthetic route for artemisinin production using engineered substrate-promiscuous P450BM3. *ACS Chem. Biol.* 4:261–67
33. Dubock A. 2014. The politics of Golden Rice. *GM Crops Food* 5:210–22
34. Duge de Bernonville T, Clastre M, Besseau S, Oudin A, Burlat V, et al. 2014. Phytochemical genomics of the Madagascar periwinkle: unravelling the last twists of the alkaloid engine. *Phytochemistry* 113:9–23
35. Dutta S, Whicher JR, Hansen DA, Hale WA, Chemler JA, et al. 2014. Structure of a modular polyketide synthase. *Nature* 510:512–17
36. Evans BS, Chen Y, Metcalf WW, Zhao H, Kelleher NL. 2011. Directed evolution of the nonribosomal peptide synthetase AdmK generates new andrimid derivatives in vivo. *Chem. Biol.* 18:601–7
37. Farhi M, Marhevka E, Ben-Ari J, Algamas-Dimantov A, Liang Z, et al. 2011. Generation of the potent anti-malarial drug artemisinin in tobacco. *Nat. Biotechnol.* 29:1072–74
38. Farhi M, Marhevka E, Masci T, Marcos E, Eyal Y, et al. 2011. Harnessing yeast subcellular compartments for the production of plant terpenoids. *Metab. Eng.* 13:474–81
39. Fischbach MA, Lai JR, Roche ED, Walsh CT, Liu DR. 2007. Directed evolution can rapidly improve the activity of chimeric assembly-line enzymes. *PNAS* 104:11951–56
40. Fossati E, Ekins A, Narcross L, Zhu Y, Falgoutyret JP, et al. 2014. Reconstitution of a 10-gene pathway for synthesis of the plant alkaloid dihydrosanguinarine in *Saccharomyces cerevisiae*. *Nat. Commun.* 5:3283
41. Fukushima EO, Seki H, Sawai S, Suzuki M, Ohyama K, et al. 2013. Combinatorial biosynthesis of legume natural and rare triterpenoids in engineered yeast. *Plant Cell Physiol.* 54:740–49
42. Gantt RW, Peltier-Pain P, Thorson JS. 2011. Enzymatic methods for glyco(diversification)/randomization) of drugs and small molecules. *Nat. Prod. Rep.* 28:1811–53
43. Geu-Flores F, Nielsen MT, Nafisi M, Moldrup ME, Olsen CE, et al. 2009. Glucosinolate engineering identifies a gamma-glutamyl peptidase. *Nat. Chem. Biol.* 5:575–77
44. Gimpel JA, Hyun JS, Schoepp NG, Mayfield SP. 2015. Production of recombinant proteins in microalgae at pilot greenhouse scale. *Biotechnol. Bioeng.* 112:339–45
45. Glenn WS, Runguphan W, O'Connor SE. 2013. Recent progress in the metabolic engineering of alkaloids in plant systems. *Curr. Opin. Biotechnol.* 24:354–65
46. Gokhale RS, Tsuji SY, Cane DE, Khosla C. 1999. Dissecting and exploiting intermodular communication in polyketide synthases. *Science* 284:482–85





47. Gomez-Escribano J, Bibb M. 2011. Engineering *Streptomyces coelicolor* for heterologous expression of secondary metabolite gene clusters. *Microb. Biotechnol.* 4:207–15
48. Gomez-Escribano JP, Bibb MJ. 2014. Heterologous expression of natural product biosynthetic gene clusters in *Streptomyces coelicolor*: from genome mining to manipulation of biosynthetic pathways. *J. Ind. Microbiol. Biotechnol.* 41:425–31
49. Gottelt M, Kol S, Gomez-Escribano JP, Bibb M, Takano E. 2010. Deletion of a regulatory gene within the *cpk* gene cluster reveals novel antibacterial activity in *Streptomyces coelicolor* A3(2). *Microbiology* 156:2343–53
50. Guirimand G, Guihur A, Poutrain P, Hericourt F, Mahroug S, et al. 2011. Spatial organization of the vindoline biosynthetic pathway in *Catharanthus roseus*. *J. Plant Physiol.* 168:549–57
51. Guo F, Xiang S, Li L, Wang B, Rajasarkka J, et al. 2015. Targeted activation of silent natural product biosynthesis pathways by reporter-guided mutant selection. *Metab. Eng.* 28C:134–42
52. Hawkins KM, Smolke CD. 2008. Production of benzylisoquinoline alkaloids in *Saccharomyces cerevisiae*. *Nat. Chem. Biol.* 4:564–73
53. Heinig U, Gutensohn M, Dudareva N, Aharoni A. 2013. The challenges of cellular compartmentalization in plant metabolic engineering. *Curr. Opin. Biotechnol.* 24:239–46
54. Hofer R, Dong L, Andre F, Ginglinger JF, Lugan R, et al. 2013. Geraniol hydroxylase and hydroxygeraniol oxidase activities of the CYP76 family of cytochrome P450 enzymes and potential for engineering the early steps of the (seco)iridoid pathway. *Metab. Eng.* 20:221–32
55. Hopwood DA, Malpartida F, Kieser HM, Ikeda H, Duncan J, et al. 1985. Production of “hybrid” antibiotics by genetic engineering. *Nature* 314:642–44
56. Hwang EI, Kaneko M, Ohnishi Y, Horinouchi S. 2003. Production of plant-specific flavanones by *Escherichia coli* containing an artificial gene cluster. *Appl. Environ. Microbiol.* 69:2699–706
57. Hwang K, Kim H, Charusanti P, Palsson B, Lee S. 2014. Systems biology and biotechnology of *Streptomyces* species for the production of secondary metabolites. *Biotechnol. Adv.* 32:255–68
58. Ignea C, Pontini M, Maffei ME, Makris AM, Kampranis SC. 2014. Engineering monoterpene production in yeast using a synthetic dominant negative geranyl diphosphate synthase. *ACS Synth. Biol.* 3:298–306
59. Ishizaki K, Chiyoda S, Yamato KT, Kohchi T. 2008. Agrobacterium-mediated transformation of the haploid liverwort *Marchantia polymorpha* L., an emerging model for plant biology. *Plant Cell Physiol.* 49:1084–91
60. Jakubczyk D, Caputi L, Hatsch A, Nielsen CAF, Diefenbacher M, et al. 2015. Discovery and reconstitution of the cycloclavine biosynthetic pathway: enzymatic formation of a cyclopropyl group. *Angew. Chem. Int. Ed.* 54:5117–21
61. Jeandet P, Vasserot Y, Chastang T, Courrot E. 2013. Engineering microbial cells for the biosynthesis of natural compounds of pharmaceutical significance. *BioMed Res. Int.* 2013:780145
62. Jenke-Kodama H, Muller R, Dittmann E. 2008. Evolutionary mechanisms underlying secondary metabolite diversity. *Prog. Drug Res.* 65:119, 121–40
63. Jiang H, Wood KV, Morgan JA. 2005. Metabolic engineering of the phenylpropanoid pathway in *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* 71:2962–69
64. Jiang M, Fang L, Pfeifer BA. 2013. Improved heterologous erythromycin A production through expression plasmid re-design. *Biotechnol. Prog.* 29:862–69
65. Jiang M, Pfeifer BA. 2013. Metabolic and pathway engineering to influence native and altered erythromycin production through *E. coli*. *Metab. Eng.* 19:42–49
66. Jiang M, Stephanopoulos G, Pfeifer BA. 2012. Toward biosynthetic design and implementation of *Escherichia coli*-derived paclitaxel and other heterologous polyisoprene compounds. *Appl. Environ. Microbiol.* 78:2497–504
67. Jiang M, Zhang H, Park SH, Li Y, Pfeifer BA. 2013. Deoxysugar pathway interchange for erythromycin analogues heterologously produced through *Escherichia coli*. *Metab. Eng.* 20:92–100
68. Kao CM, Katz L, Khosla C. 1994. Engineered biosynthesis of a complete macrolactone in a heterologous host. *Science* 265:509–12
69. Kappers IF, Aharoni A, van Herpen TWJM, Luckerhoff LLP, Dicke M, Bouwmeester HJ. 2005. Genetic engineering of terpenoid metabolism attracts bodyguards to *Arabidopsis*. *Science* 309:2070–72

5.20

O'Connor





70. Kapur S, Lowry B, Yuzawa S, Kenthirapalan S, Chen AY, et al. 2012. Reprogramming a module of the 6-deoxyerythronolide B synthase for iterative chain elongation. *PNAS* 109:4110–15
71. Kennedy J. 2008. Mutasynthesis, chemobiosynthesis, and back to semi-synthesis: combining synthetic chemistry and biosynthetic engineering for diversifying natural products. *Nat. Prod. Rep.* 25:25–34
72. Kirby J, Romanini DW, Paradise EM, Keasling JD. 2008. Engineering triterpene production in *Saccharomyces cerevisiae*-beta-amyrin synthase from *Artemisia annua*. *FEBS J.* 275:1852–59
73. Kitaoka N, Lu X, Yang B, Peters RJ. 2015. The application of synthetic biology to elucidation of plant mono-, sesqui-, and diterpenoid metabolism. *Mol. Plant* 8:6–16
74. Klein J, Heal JR, Hamilton WD, Boussemghoune T, Tange TO, et al. 2014. Yeast synthetic biology platform generates novel chemical structures as scaffolds for drug discovery. *ACS Synth. Biol.* 3:314–23
75. Koopman F, Beekwilder J, Crimi B, van Houwelingen A, Hall R, et al. 2012. De novo production of the flavonoid naringenin in engineered *Saccharomyces cerevisiae*. *Microb. Cell Fact.* 11:155
76. Krivoruchko A, Nielsen J. 2014. Production of natural products through metabolic engineering of *Saccharomyces cerevisiae*. *Curr. Opin. Biotechnol.* 35C:7–15
77. Kumar S, Hahn FM, Baidoo E, Kahlon TS, Wood DF, et al. 2012. Remodeling the isoprenoid pathway in tobacco by expressing the cytoplasmic mevalonate pathway in chloroplasts. *Metab. Eng.* 14:19–28
78. Kumar V, Jain M. 2015. The CRISPR-Cas system for plant genome editing: advances and opportunities. *J. Exp. Bot.* 66:47–57
79. Kunert G, Reinhold C, Gershenzon J. 2010. Constitutive emission of the aphid alarm pheromone, (E)- $\beta$ -farnesene, from plants does not serve as a direct defense against aphids. *BMC Ecol.* 10:23
80. Lange BM, Mahmoud SS, Wildung MR, Turner GW, Davis EM, et al. 2011. Improving peppermint essential oil yield and composition by metabolic engineering. *PNAS* 108:16944–49
81. Laureti L, Song L, Huang S, Corre C, Leblond P, et al. 2011. Identification of a bioactive 51-membered macrolide complex by activation of a silent polyketide synthase in *Streptomyces ambofaciens*. *PNAS* 108:6258–63
82. Leonard E, Ajikumar PK, Thayer K, Xiao WH, Mo JD, et al. 2010. Combining metabolic and protein engineering of a terpenoid biosynthetic pathway for overproduction and selectivity control. *PNAS* 107:13654–59
83. Li W-H, Vederas J. 2009. Drug discovery and natural products: end of an era or an endless frontier? *Science* 325:161–65
84. Lim CG, Fowler ZL, Hueller T, Schaffer S, Koffas MA. 2011. High-yield resveratrol production in engineered *Escherichia coli*. *Appl. Environ. Microbiol.* 77:3451–60
85. Lim FY, Sanchez JF, Wang CC, Keller NP. 2012. Toward awakening cryptic secondary metabolite gene clusters in filamentous fungi. *Methods Enzymol.* 517:303–24
86. Lloyd AM, Walbot V, Davis RW. 1992. *Arabidopsis* and *Nicotiana glauca* anthocyanin production activated by maize regulators R and C1. *Science* 258:1773–75
87. Lu Y, Rijzaani H, Karcher D, Ruf S, Bock R. 2013. Efficient metabolic pathway engineering in transgenic tobacco and tomato plastids with synthetic multigene operons. *PNAS* 110:E623–32
88. Lubertozzi D, Keasling JD. 2009. Developing *Aspergillus* as a host for heterologous expression. *Biotechnol. Adv.* 27:53–75
89. Luo Y, Huang H, Liang J, Wang M, Lu L, et al. 2013. Activation and characterization of a cryptic polycyclic tetramate macrolactam biosynthetic gene cluster. *Nat. Commun.* 4:2894
90. Ma SM, Li JWH, Choi JW, Zhou H, Lee KKM, et al. 2009. Complete reconstitution of a highly reducing iterative polyketide synthase. *Science* 326:589–92
91. Malpartida F, Hopwood DA. 1984. Molecular cloning of the whole biosynthetic pathway of a *Streptomyces* antibiotic and its expression in a heterologous host. *Nature* 309:462–64
92. Martin VJJ, Pitera DJ, Withers ST, Newman JD, Keasling JD. 2003. Engineering the mevalonate pathway in *Escherichia coli* for production of terpenoids. *Nat. Biotechnol.* 21:796–802
93. McCranie EK, Bachmann BO. 2014. Bioactive oligosaccharide natural products. *Nat. Prod. Rep.* 31:1026–42
94. McDaniel R, Thamchaipenet A, Gustafsson C, Fu H, Betlach M, et al. 1999. Multiple genetic modifications of the erythromycin polyketide synthase to produce a library of novel “unnatural” natural products. *PNAS* 96:1846–51



95. McDaniel R, Welch M, Hutchinson CR. 2005. Genetic approaches to polyketide antibiotics. *Chem. Rev.* 105:543–58
96. Metten KL, Aschehoug ET, Callaway RM. 2009. Plant behavioural ecology: dynamic plasticity in secondary metabolites. *Plant Cell Environ.* 32:641–53
97. Meyer P, Heidmann I, Forkmann G, Saedler H. 1987. A new petunia flower colour generated by transformation of a mutant with a maize gene. *Nature* 330:677–78
98. Miettinen K, Dong L, Navrot N, Schneider T, Burlat V, et al. 2014. The seco-iridoid pathway from *Catbaranthus roseus*. *Nat. Commun.* 5:3606
99. Mikkelsen MD, Olsen CE, Halkier BA. 2010. Production of the cancer-preventive glucoraphanin in tobacco. *Mol. Plant* 3:751–59
100. Minami H, Kim JS, Ikezawa N, Takemura T, Katayama T, et al. 2008. Microbial production of plant benzylisoquinoline alkaloids. *PNAS* 105:7393–98
101. Mora-Pale M, Sanchez-Rodriguez SP, Linhardt RJ, Dordick JS, Koffas MA. 2013. Metabolic engineering and in vitro biosynthesis of phytochemicals and non-natural analogues. *Plant Sci.* 210:10–24
102. Morrone D, Lowry L, Determan MK, Hershey DM, Xu M, Peters RJ. 2010. Increasing diterpene yield with a modular metabolic engineering system in *E. coli*: comparison of MEV and MEP isoprenoid precursor pathway engineering. *Appl. Microbiol. Biotechnol.* 85:1893–906
103. Moses T, Pollier J, Almagro L, Buyst D, Van Montagu M, et al. 2014. Combinatorial biosynthesis of saponins and saponins in *Saccharomyces cerevisiae* using a C-16 $\alpha$  hydroxylase from *Bupleurum falcatum*. *PNAS* 111:1634–39
104. Mugford ST, Louveau T, Melton R, Qi X, Bakht S, et al. 2013. Modularity of plant metabolic gene clusters: a trio of linked genes that are collectively required for acylation of triterpenes in oat. *Plant Cell* 25:1078–92
105. Muir SR, Collins GJ, Robinson S, Hughes S, Bovy A, et al. 2001. Overexpression of petunia chalcone isomerase in tomato results in fruit containing increased levels of flavonols. *Nat. Biotechnol.* 19:470–74
106. Murli S, Kennedy J, Dayem LC, Carney JR, Kealey JT. 2003. Metabolic engineering of *Escherichia coli* for improved 6-deoxyerythronolide B production. *J. Ind. Microbiol. Biotechnol.* 30:500–9
107. Naesby M, Nielsen SV, Nielsen CA, Green T, Tange TO, et al. 2009. Yeast artificial chromosomes employed for random assembly of biosynthetic pathways and production of diverse compounds in *Saccharomyces cerevisiae*. *Microb. Cell Fact.* 8:45
108. Nakagawa A, Minami H, Kim J-S, Koyanagi T, Katayama T, et al. 2011. A bacterial platform for fermentative production of plant alkaloids. *Nat. Commun.* 2:326
109. Nekrasov V, Staskawicz B, Weigel D, Jones JDG, Kamoun S. 2013. Targeted mutagenesis in the model plant *Nicotiana benthamiana* using Cas9 RNA-guided endonuclease. *Nat. Biotechnol.* 31:691–93
110. Newman D, Cragg G. 2012. Natural products as sources of new drugs over the 30 years from 1981 to 2010. *J. Nat. Prod.* 75:311–35
111. Nour-Eldin HH, Andersen TG, Burow M, Madsen SR, Jorgensen ME, et al. 2012. NRT/PTR transporters are essential for translocation of glucosinolate defence compounds to seeds. *Nature* 488:531–34
112. Nour-Eldin HH, Halkier BA. 2013. The emerging field of transport engineering of plant specialized metabolites. *Curr. Opin. Biotechnol.* 24:263–70
113. Nuttmann HW, Osbourn A. 2014. Gene clustering in plant specialized metabolism. *Curr. Opin. Biotechnol.* 26:91–99
114. Olano C, Garcia I, Gonzalez A, Rodriguez M, Rozas D, et al. 2014. Activation and identification of five clusters for secondary metabolites in *Streptomyces albus* J1074. *Microb. Biotechnol.* 7:242–56
115. Oman TJ, Knerr PJ, Bindman NA, Velasquez JE, van der Donk WA. 2012. An engineered lantibiotic synthetase that does not require a leader peptide on its substrate. *J. Am. Chem. Soc.* 134:6952–55
116. Paddon CJ, Keasling JD. 2014. Semi-synthetic artemisinin: a model for the use of synthetic biology in pharmaceutical development. *Nat. Rev. Microbiol.* 12:355–67
117. Paddon CJ, Westfall PJ, Pitera DJ, Benjamin K, Fisher K, et al. 2013. High-level semi-synthetic production of the potent antimalarial artemisinin. *Nature* 496:528–32
118. Patton GC, van der Donk WA. 2005. New developments in lantibiotic biosynthesis and mode of action. *Curr. Opin. Microbiol.* 8:543–51



119. Peiru S, Menzella HG, Rodriguez E, Carney J, Gramajo H. 2005. Production of the potent antibacterial polyketide erythromycin C in *Escherichia coli*. *Appl. Environ. Microbiol.* 71:2539–47
120. Pfeifer B, Admiraal S, Gramajo H, Cane D, Khosla C. 2001. Biosynthesis of complex polyketides in a metabolically engineered strain of *E. coli*. *Science* 291:1790–92
121. Pickens LB, Tang Y, Chooi YH. 2011. Metabolic engineering for the production of natural products. *Annu. Rev. Chem. Biomol. Eng.* 2:211–36
122. Putignani L, Massa O, Alisi A. 2013. Engineered *Escherichia coli* as new source of flavonoids and terpenoids. *Food Res. Int.* 54:1084–95
123. Ro DK, Paradise EM, Ouellet M, Fisher KJ, Newman KL, et al. 2006. Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature* 440:940–43
124. Roy A, Gruschow S, Cairns N, Goss R. 2010. Gene expression enabling synthetic diversification of natural products: chemogenetic generation of pacidamycin analogs. *J. Am. Chem. Soc.* 132:12243–45
125. Rudd BAM, Hopwood DA. 1980. A pigmented mycelial antibiotic in *Streptomyces coelicolor*: control by a chromosomal gene cluster. *J. Gen. Microbiol.* 119:333–40
126. Rugbjerg P, Naesby M, Mortensen UH, Frandsen RJ. 2013. Reconstruction of the biosynthetic pathway for the core fungal polyketide scaffold rubrofusarin in *Saccharomyces cerevisiae*. *Microb. Cell Fact.* 12:31
127. Rungtaphan W, Maresh JJ, O'Connor SE. 2009. Silencing of tryptamine biosynthesis for production of nonnatural alkaloids in plant culture. *PNAS* 106:13673–78
128. Rungtaphan W, Qu X, O'Connor SE. 2010. Integrating carbon-halogen bond formation into medicinal plant metabolism. *Nature* 468:461–64
129. Ryan KL, Moore CT, Panaccione DG. 2013. Partial reconstruction of the ergot alkaloid pathway by heterologous gene expression in *Aspergillus nidulans*. *Toxins* 5:445–55
130. Sainsbury F, Saxena P, Geisler K, Osbourn A, Lomonossoff GP. 2012. Using a virus-derived system to manipulate plant natural product biosynthetic pathways. *Methods Enzymol.* 517:185–202
131. Santos CN, Koffas M, Stephanopoulos G. 2011. Optimization of a heterologous pathway for the production of flavonoids from glucose. *Metab. Eng.* 13:392–400
132. Schilmiller AL, Pichersky E, Last RL. 2012. Taming the hydra of specialized metabolism: how systems biology and comparative approaches are revolutionizing plant biochemistry. *Curr. Opin. Plant Biol.* 15:338–44
133. Shi Y, Yang X, Garg N, van der Donk WA. 2011. Production of lantipeptides in *Escherichia coli*. *J. Am. Chem. Soc.* 133:2338–41
134. Shier W, Rinehart K, Gottlieb D. 1969. Preparation of four new antibiotics from a mutant of *Streptomyces fradiae*. *PNAS* 63:198–204
135. Siddiqui MS, Thodey K, Trenchard I, Smolke CD. 2012. Advancing secondary metabolite biosynthesis in yeast with synthetic biology tools. *FEMS Yeast Res.* 12:144–70
136. Takahashi S, Yeo Y, Greenhagen B, McMullin T, Song L, et al. 2007. Metabolic engineering of sesquiterpene metabolism in yeast. *Biotechnol. Bioeng.* 97:170–81
137. Thodey K, Galanie S, Smolke CD. 2014. A microbial biomanufacturing platform for natural and semisynthetic opioids. *Nat. Chem. Biol.* 10:837–44
138. Ting HM, Wang B, Ryden AM, Woittiez L, van Herpen T, et al. 2013. The metabolite chemotype of *Nicotiana benthamiana* transiently expressing artemisinin biosynthetic pathway genes is a function of CYP71AV1 type and relative gene dosage. *New Phytol.* 199:352–66
139. Trantas E, Panopoulos N, Ververidis F. 2009. Metabolic engineering of the complete pathway leading to heterologous biosynthesis of various flavonoids and stilbenoids in *Saccharomyces cerevisiae*. *Metab. Eng.* 11:355–66
140. Trantas EA, Koffas MA, Xu P, Ververidis F. 2015. When plants produce not enough or at all: metabolic engineering of flavonoids in microbial hosts. *Front. Plant Sci.* 6:7
141. van Dijk JM, Hecker M. 2013. *Bacillus subtilis*: from soil bacterium to super-secreting cell factory. *Microb. Cell Fact.* 12:3
142. Van Moerkercke A, Steensma P, Schweizer F, Pollier J, Gariboldi I, et al. 2015. The bHLH transcription factor BIS1 controls the iridoid branch of the monoterpenoid indole alkaloid pathway in *Catharanthus roseus*. *PNAS* 112:8130–35



143. Vogt T. 2010. Phenylpropanoid biosynthesis. *Mol. Plant* 3:2–20
144. Walker MC, Thuronyi BW, Charkoudian LK, Lowry B, Khosla C, Chang MC. 2013. Expanding the fluorine chemistry of living systems using engineered polyketide synthase pathways. *Science* 341:1089–94
145. Wang Y, Pfeifer BA. 2008. 6-Deoxyerythronolide B production through chromosomal localization of the deoxyerythronolide B synthase genes in *E. coli*. *Metab. Eng.* 10:33–38
146. Wenzel SC, Muller R. 2005. Formation of novel secondary metabolites by bacterial multimodular assembly lines: deviations from textbook biosynthetic logic. *Curr. Opin. Chem. Biol.* 9:447–58
147. Westfall PJ, Pitera DJ, Lenihan JR, Eng D, Woollard FX, et al. 2012. Production of amorphadiene in yeast, and its conversion to dihydroartemisinic acid, precursor to the antimalarial agent artemisinin. *PNAS* 109:E111–18
148. Winter JM, Tang Y. 2012. Synthetic biological approaches to natural product biosynthesis. *Curr. Opin. Biotechnol.* 23:736–43
149. Wong FT, Khosla C. 2012. Combinatorial biosynthesis of polyketides: a perspective. *Curr. Opin. Chem. Biol.* 16:117–23
150. Wu S, Jiang Z, Kempinski C, Eric Nybo S, Husodo S, et al. 2012. Engineering triterpene metabolism in tobacco. *Planta* 236:867–77
151. Wu S, Schalk M, Clark A, Miles RB, Coates R, Chappell J. 2006. Redirection of cytosolic or plastidic isoprenoid precursors elevates terpene production in plants. *Nat. Biotechnol.* 24:1441–47
152. Xu W, Gavia DJ, Tang Y. 2014. Biosynthesis of fungal indole alkaloids. *Nat. Prod. Rep.* 31:1474–87
153. Xu Y, Zhou T, Zhang S, Espinosa-Artiles P, Wang L, et al. 2014. Diversity-oriented combinatorial biosynthesis of benzenediol lactone scaffolds by subunit shuffling of fungal polyketide synthases. *PNAS* 111:12354–59
154. Yadav VG, De Mey M, Giaw Lim C, Ajikumar PK, Stephanopoulos G. 2012. The future of metabolic engineering and synthetic biology: towards a systematic practice. *Metab. Eng.* 14:233–41
155. Yin WB, Chooi YH, Smith AR, Cacho RA, Hu Y, et al. 2013. Discovery of cryptic polyketide metabolites from dermatophytes using heterologous expression in *Aspergillus nidulans*. *ACS Synth. Biol.* 2:629–34
156. Yonekura-Sakakibara K, Saito K. 2013. Transcriptome coexpression analysis using ATTED-II for integrated transcriptomic/metabolomic analysis. *Methods Mol. Biol.* 1011:317–26
157. Yuan L, Grotewold E. 2015. Metabolic engineering to enhance the value of plants as green factories. *Metab. Eng.* 27:83–91
158. Zhang C, Griffith BR, Fu Q, Albermann C, Fu X, et al. 2006. Exploiting the reversibility of natural product glycosyltransferase-catalyzed reactions. *Science* 313:1291–94
159. Zhang H, Wang Y, Wu J, Skalina K, Pfeifer BA. 2010. Complete biosynthesis of erythromycin A and designed analogs using *E. coli* as a heterologous host. *Chem. Biol.* 17:1232–40
160. Zhang L, Ding R, Chai Y, Bonfill M, Moyano E, et al. 2004. Engineering tropane biosynthetic pathway in *Hyoscyamus niger* hairy root cultures. *PNAS* 101:6786–91
161. Zhou H, Qiao K, Gao Z, Vederas JC, Tang Y. 2010. Insights into radicicol biosynthesis via heterologous synthesis of intermediates and analogs. *J. Biol. Chem.* 285:41412–21
162. Zhou K, Qiao K, Edgar S, Stephanopoulos G. 2015. Distributing a metabolic pathway among a microbial consortium enhances production of natural products. *Nat. Biotechnol.* 33:377–83
163. Ziegler J, Facchini PJ. 2008. Alkaloid biosynthesis: metabolism and trafficking. *Annu. Rev. Plant Biol.* 59:735–69
164. Zuker A, Tzfira T, Ben-Meir H, Ovadis M, Shklarman E, et al. 2002. Modification of flower color and fragrance by antisense suppression of the flavanone 3-hydroxylase gene. *Mol. Breed.* 9:33–41
165. Zvi MM, Shklarman E, Masci T, Kaleb H, Debener T, et al. 2012. PAP1 transcription factor enhances production of phenylpropanoid and terpenoid scent compounds in rose flowers. *New Phytol.* 195:335–45