Current and Emerging Approaches for Natural Product Biosynthesis in Microbial Cells

Kevin T. Watts, Benjamin N. Mijts, Claudia Schmidt-Dannert*

Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, 1479 Gortner Avenue, St. Paul, MN 55108, USA
Fax: (+1)-612-625-5780, e-mail: schmi232@tc.umn.edu

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Abstract: Microorganisms and plants synthesize a tremendous diversity of chemical compounds. For centuries, these compounds have been used as medicines, foods and other useful materials. The still largely unexplored structural and chemical diversity of natural products is unmatched by synthetic methodology and continues to be the most successful source for the discovery of novel scaffolds with important biological activities. Thus, exploiting the selectivity and specificity of the biosynthetic machineries that make these complex compounds can provide ways of synthesizing diverse natural products or their core scaffolds for further synthetic modification. Microbial cells can be fitted with new biosynthetic abilities using metabolic and genetic engineering strategies to overproduce desired compounds. Efforts in genome sequencing give access to an incredible number of genes from microorganisms and, more recently, from plants that can be *in silico* screened for new biosynthetic functions allowing tapping into the synthetic potential of microorganisms, and especially plants. In addition, by exploiting natural biodiversity by using traditional screening methods or metagenomics approaches, novel biosynthetic pathways and genes can be discovered for the synthesis of additional structures in engineered microbial cells. In this review, we will describe some of the recent developments in natural product biosynthesis, and also describe some of the emerging approaches to harness the chemical diversity that lies hidden in nature.

Keywords: carotenoids; directed evolution; natural products; polyketides; terpenoids

1 Introduction

Natural product chemistry deals with the isolation, identification, structure elucidation and study of the chemical characteristics of substances produced by living organisms. This broad definition encompasses a vast array of naturally synthesized compounds with an equally diverse array of applications. Traditionally, the natural products of greatest importance were those utilized as drugs, particularly antibiotics, and food additives. These natural products were isolated from native sources and production was improved by conventional approaches to strain development and optimization of growth conditions. Novel natural products were supplied by the isolation and identification of new source organisms. However, the isolation of new natural products from natural

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Kevin Watts (born 1977) got a B.S. in Microbiology from the University of Wisconsin-Madison in 1999, and since the fall of 2001 has been doing graduate work in Dr. Claudia Schmidt-Dannert's laboratory at the University of Minnesota. His project is primarily focused on engineering and manipulation of the flavonoid biosynthetic pathway in *Escherichia coli*.

Ben Mijts (born 1975) completed his PhD at Griffith University, Australia in Dr. Bharat Patel's laboratory in 2002. His doctoral research investigated industrial enzymes from a thermophilic, halophilic bacterium. Since that time he has worked as a postdoctoral associate in Dr. Claudia Schmidt-Dannert's laboratory at the University of Minnesota. His postdoctoral research is focused on the combinatorial biosynthesis and *in vitro* evolution of carotenoid biosynthetic pathways.

Claudia Schmidt-Dannert (born 1966) is an Associate Professor in the Department of Biochemistry, Molecular Biology and Biophysics at the University of Minnesota. After obtaining her PhD in the Department of Enzyme Technology at the National Research Center for Biotechnology (GBF) in Braunschweig under Rolf D. Schmid, she went to the University of Stuttgart as leader of the Molecular Biotechnology Group in the Institute of Technical Biochemistry headed by Rolf D. Schmid. In 1998 she obtained a habilitation-fellowship from the German Science Foundation (DFG) for research on *in vitro* pathway evolution and joined Frances Arnold's Group at the California Institute of Technology. From California she went to Minnesota to take a faculty position. Her research interests are biosynthetic pathway engineering, natural products biosynthesis and protein engineering using rational and evolutionary design strategies.

sources has slowed considerably, and in many cases natural products of potential cannot be produced in economical quantities from native producers. Synthetic chemistry can in many cases supply structural analogues of natural products or modified structures that are useful. Still, living organisms remain an important source and production means for useful compounds where chemical synthesis is difficult or less desirable. The emergence of molecular biology has transformed the basis of natural product discovery and production. Genetic manipulation of host strains and heterologous expression can permit economical production of natural products produced in trace quantities in source organisms. Protein engineering and directed evolution can also be utilized to improve natural product yields. In addition, these techniques can allow the complete manipulation of biosynthetic pathways, and genes responsible for biosynthetic steps from a number of source organisms can be combined and modified to produce desired natural products and also produce natural products with modified chemical structures that are rare or not observed in nature. These novel products with unique chemical properties may have improved efficacy or allow completely new applications. In addition, emerging technologies such as metagenomics provide a new approach to access genes responsible for desired biosynthetic steps, and have great potential for improved production of known natural products or the isolation of useful, novel compounds. In this review we will summarize recent advances in engineering microbial cells for natural product biosynthesis. This will be illustrated by examples from a number of compound classes that utilize genetic manipulation, heterologous expression, protein engineering or directed evolution. A second section will describe emerging approaches in natural product biosynthesis such as genomics, metagenomics and scaffold modification.

2 Polyketides

Polyketides are an extensively studied and modified class of natural products synthesized on large enzyme complexes called polyketide synthases (PKS). These large modular multi-enzyme complexes synthesize complex aromatic (typically produced by type II PKSs) or macrolide polyketides (type I PKSs) *via* the step-wise condensation of acyl-CoAs. Starter and extender units are bound within the PKS *via* an enzyme-bound acyl carrier protein (ACP). In the case of modular type I PKSs, further modification of the growing acyl chain can take place within ketoreductase (KR), enoyl reductase (ER) or dehydratase (DH) domains before finally being released from the enzyme complex *via* a thioesterase (TE) domain. Type II PKS systems produce a linear tetraketide by the action of an iteratively used ketosynthase (KS) domain together with chain length factor

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(CLF) to make the unmodified aromatic polyketide. The vast amount of work that has been done in the past two decades on polyketide isolation, characterization, genetics and metabolic engineering is well beyond the scope of this review, but has been summarized elsewhere.[1–3] Instead, this section will focus on recent developments in polyketide engineering.

2.1 Heterologous Expression in *Escherichia coli*

Heterologous expression of PKSs have been accomplished for a number of hosts, and is thoroughly reviewed elsewhere,[4] A recent example of note is the development of *E. coli*, which has become the workhorse of molecular biology and biotechnology, as a host for the heterologous expression of 6-deoxyerythronolide B synthase (DEBS), the modular type I polyketide synthase (PKS) responsible for production of 6-deoxyerythronolide B (6dEB) (Figure 1), an erythromycin precursor.[5] For *E. coli*, a non-polyketide producing organism, extensive metabolic engineering had to be employed to overcome several obstacles, including lack of a phosphopantetheinyl transferase and (2*S*)-methylmalonate for polyketide extension. The ability of these obstacles to be easily overcome demonstrates the power and flexibility of *E. coli* as a system for heterologous natural product synthesis.

The first challenge to 6dEB production in *E. coli* was producing fully active, soluble protein. In order to do this, growth conditions had to be modified and a phosphopantetheinyl transferase needed to be introduced in order to post-translationally modify the ACP domains

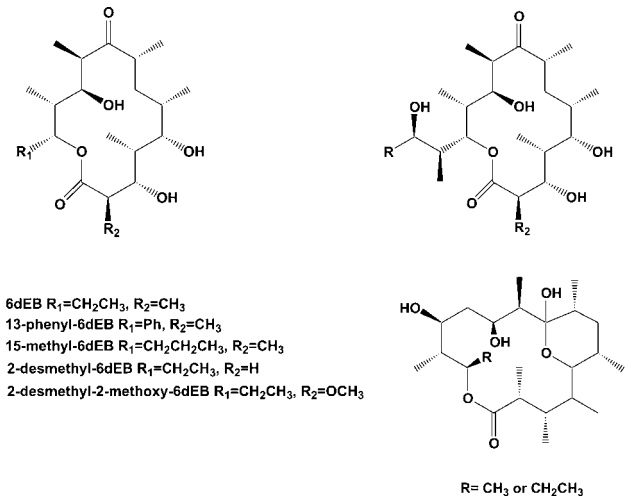

Figure 1. Examples of novel 6-deoxyerythronolide B structures produced by metabolic engineering of precursor pathways in heterologous hosts, feeding of substrate analogs, and manipulation of the 6-deoxyerythronolide B synthase enzyme complex.[5,13,14,17,21]

of the PKS, since *E. coli* cannot perform this activity. The *Bacillus subtilis sfp* gene was chosen as it has been shown to have a broad range of proteins that it pantetheinylates. This gene was then integrated into the *E. coli* chromosome within the propionate catabolic genes to knock out propionate catabolism in this strain. To overcome this loss, and still utilize propionate as the DEBS starter unit, the native propionyl-CoA ligase (PrpE) was overexpressed, thus supplying propionyl-CoA from exogenous sources of propionate. The second obstacle to 6dEB production in *E. coli* was that the host does not produce the DEBS extender unit, (2*S*)-methylmalonyl-CoA. A pathway for methylmalonyl-CoA biosynthesis was created by expressing propionyl-CoA carboxylase (PCC) from a *Streptomycete*. This enzyme synthesizes racemic methylmalonyl-CoA, but apparently enough of the *S*-isomer is synthesized to see the correct product, 6dEB, produced.[5] Further efforts to engineer a (2*S*)-methylmalonyl-CoA pathway in this strain involved using a methylmalonyl mutase from *Propionibacterium shermani* and an epimerase from *S. coelicolor* to produce methylmalonyl-CoA from succinate,[6] thus eliminating the production of both starter and extender units from exogenous propionate. Enhancements in 6dEB titers were also seen when a soluble thioesterase (TEII) from *S. erythraea* was included, raising the yield of 6dEB produced in *E. coli* to about 185 mg/L in a fed batch fermentation.[7] Further optimization has now raised that to about 1 g/L, near the level of heterologous production in *S. coelicolor*.[8]

The *E. coli* system has already proven robust enough to begin producing novel 6dEB analogues. A non-ribosomal peptide synthetase (NRPS, see below) loading module that can accept a benzoate starter unit, was fused to the first module of DEBS in lieu of the normal DEBS loading module. Using this approach, a phenyl-substituted 6dEB analogue was produced[5] (Figure 1), the same compound that was previously produced in a heterologous host *via* feeding of phenyl-substituted diketide *N*-acetylcysteamine (NAC) thioester.[9] That same approach of NAC thioester feeding that was so successful in the generation of new 6dEB analogues in *S. coelicolor*[9–11] has now been applied to *E. coli* as well,[12] with similar results. Further metabolic engineering of the original 6dEB strain has now made it possible to produce 15-methyl-6dEB[13] (Figure 1). Overexpression of a positive regulator (AtoC) of the short-chain fatty acid utilization operon resulted in production of butyryl-CoA, the preferred starter unit for 15-methyl-6dEB, once exogenous butyrate was added to growing cultures. However, it was not until a host decarboxylase (YgfG) was deleted that background production of 6dEB was eliminated and 15-methyl-6dEB accumulated as the main product.[13] Another 6dEB analogue, 2-desmethyl-6dEB (Figure 1), was produced by inactivation of the AT domain in module 6 of DEBS, and co-expressed with a malonyl-CoA:ACP transacylase (MAT)

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from *S. coelicolor*.[14] The MAT is able to functionally complement the mutated AT6 domain, but loads a malonate instead of methylmalonate, thus eliminating the methyl group at position 2. It was also shown that *E. coli* has some MAT activity as well since elimination of the *S. coelicolor* MAT did not eliminate 2-desmethyl-6dEB production.[14] This illustrates some of the pitfalls of working in a heterologous host such as *E. coli*, where products can be metabolized in unexpected ways. While *E. coli* does have some advantages over *S. coelicolor* for heterologous production of 6dEB, specifically faster doubling times and higher volumetric productivity and, most importantly, ease of genetic manipulation for the generation of structural diversity,[8] the technology is still in its infancy.

2.2 Module/Domain Swaps, Insertions and Deletions

Module and domain swaps, insertions and deletions continue to be a powerful tool for generation of novel polyketide structures, with perhaps the most striking example being the work of McDaniel et al.,[15] where over 50 new modified 6dEB structures were produced by domain swapping alone. Swapping the usual loading module of 6dEB for the avermectin loading module from *S. avermitilis*, resulted in production of 6dEB analogues with branched chains (isopropyl, *sec*-butyl) instead of the usual ethyl side chain at position 13.[16] Swapping of the DEBS AT6 domain with a methoxymalonate specific AT domain from the FK520 PKS resulted in production of 2-desmethyl-2-methoxy-6-dEB (Figure 1), when expressed with the cognate enzymes for methoxymalonate biosynthesis.[17] Novel pikromycin analogues were produced by swapping modules from both DEBS and tylosin PKSs in *S. venezuelae*.[18] Numerous structures were produced, including 12- and 14-membered macrolactones that were hydroxylated and glycosylated, with some showing antibiotic activity against *B. subtilis*.[18] Novel 6dEB structures have been modified in the same way even though there are subtle changes in the structure, indicating some degree of promiscuity in the modifying enzymes.[9,16,19] Incorporation of alternative starter units for a type II PKS system has been achieved with the R1128 loading domain priming the *tcm* and *act* PKSs, generating aromatic polyketides with alkyl priming units rather than the typical acetate.[20]

Insertion of entire modules, from the rapamycin PKS, into DEBS resulted in the production of novel 14-membered as well as 16-membered macrolactone structures[21] (Figure 1) depending on the position of the inserted modules. The module swapping technique has recently been employed in the generation of new desmethyl- and saturated geldanamycin analogues by *Streptomyces hygroscopicus*.[22] While the yields of the new structures seemed to be quite low, the beauty of using the natural geldanamycin-producing host was that modified compounds could be tested for normal post-synthetic modification and biological activity. Engineering of a novel nystatin structure was done in the producing strain, *S. noursei*, by deletion of a module to produce a fully conjugated hexaene structure instead of a heptaene.[23]

2.3 Polyketide Post-Synthetic Modifications

Polyketides often require a high degree of post-synthetic modification in order to become biologically active. Due to the high potential of novel product generation by manipulation of these pathways, this topic has been of special attention and is reviewed elsewhere.[24]

Some of the most interesting recent developments in polyketide modification have come from novel sugar pathways and incorporation of these new structures into the polyketide structure. A tylosin-producing strain of *S. fradiae* was engineered to produce a new dimethylamino sugar by introducing two genes from *S. narbonensis*.[25] The new sugar, D-desosamine, is produced by shunting an intermediate in the natural D-mycaminose pathway. The engineered strain was thus able to produce the natural sugar D-mycaminose, but was also able to produce D-desosamine and incorporate both onto protylonolide, the tylosin aglycone, to produce novel glycosylated tylosin analogues[25] (Figure 2). A deoxy sugar biosynthetic pathway (L-oleandrose) has been engineered into a plasmid specifically designed for synthesizing libraries for polyketide aglycone modification.[26] Using the approach of substituting different modifying enzymes (ketoreductases, epimerases, etc.) from different deoxysugar biosynthetic pathways, a library can be created if the right combination of promiscuous enzymes is utilized. Specifically, the plasmid was used to create L-rhamnose, D-olivose, L-olivose and L-rhodinose from the L-oleandrose pathway by substitution. Inclusion of a glycosyltransferase (GT) that is known to have a broad substrate specificity (ElmGT), generated tetracenomycin structures incorporating the new sugars.[26] A GT mutant library was constructed by recombination of a 10 amino acid region conferring substrate specificity to two urdamycin GTs responsible for consecutive glycosylations of the aglycone moiety.[27] Within the library were found enzymes with parental activity, as well as enzymes having dual activity, that is, performing both glycosylation reactions of the parents with only a single enzyme. A new enzymatic activity was also found, producing a novel urdamycin with a branched sugar side chain, urdamycin P[27] (Figure 3).

Beside glycosylation, hydroxylation is also an import post-synthetic modification to polyketide structures. A recent report has shown that a cytochrome P450, responsible for hydroxylation of pikromycin (PikC), can successfully be immobilized on a solid support and used as a biocatalyst.[28] This report shows that a complex

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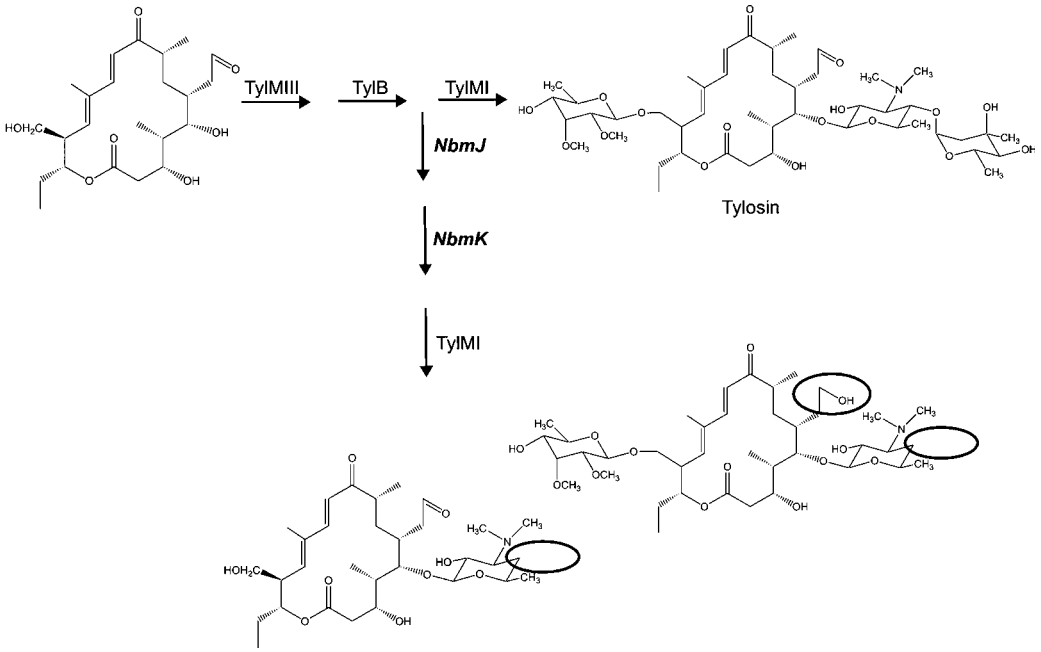

Figure 2. Tylosin analogues observed in *Streptomyces fradiae* expressing NbmJ and NbmK, two enzymes for desmosine production from *S. narbonensis,* in addition to the normal mycaminose pathway, indicated by TylMIII, TylB and TylMI.[25] Both novel compounds incorporate desmosine, a 3-deoxy derivative of the usual mycaminose sugar, and are indicated by circle regions. Additionally, one of the new compounds showed reduction of a ring aldehyde to an alcohol, also indicated by a circle.

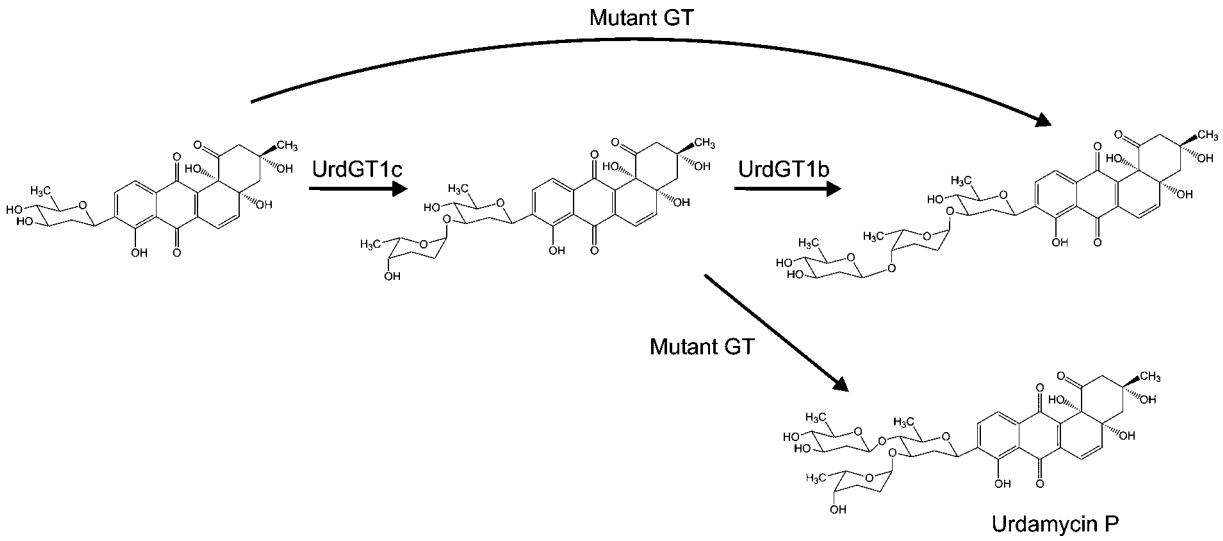

Figure 3. Production of a novel urdamycin by engineering host glycosyltransferases (GTs).[27] A library was created around a 10 amino acid region found to be critical for substrate specificity. Library members were screened and found to have activity similar to both parental types, UrdGT1c and UrdGT1b, as well as enzymes performing both reactions and enzymes that had new activity, resulting in production of a new compound, urdamycin P.

reaction involving several cofactors, and modifying a relatively complicated chemical structure, can be accomplished in a microfluidic system. This is a significant result, as it opens the door to modification of polyketides *in situ*, where the libraries of novel structures could be tested against an array of immobilized enzymes to generate fully decorated structures.

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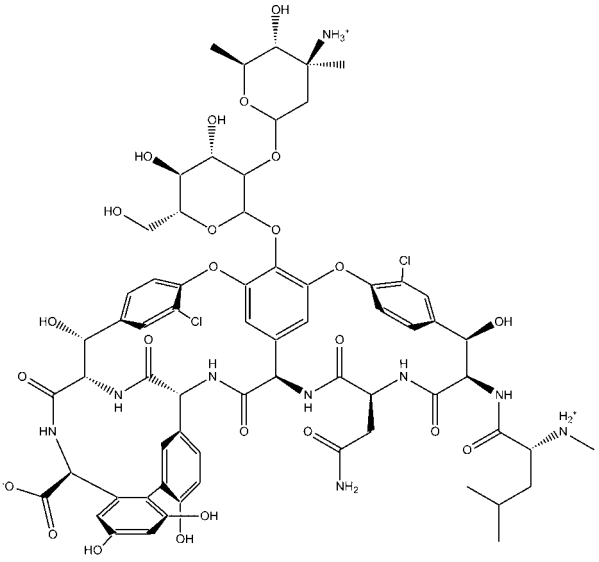
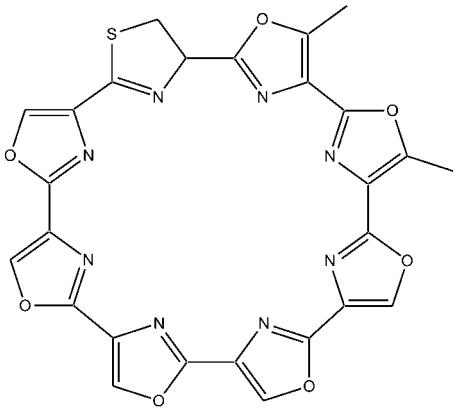


Vancomycin



Telomestatin

Figure 4. Examples of two non-ribosomal peptides, illustrating the structural diversity in this natural product class.[29]

3 Non-Ribosomal Peptides

Non-ribosomal peptides (NRPs) are natural products having important antibiotic, cytotoxic and anti-proliferate activities, whose biosynthesis is similar to that of modular type I PKSs.[3] Their structures range from the very complex glycopeptide antibiotic vancomycin, which incorporates non-proteinogenic amino acids and sugars in addition to typical L-amino acids, to the relatively simple telomestatin, which is synthesized from five L-serines, three L-threonines and one L-cysteine[29] (Figure 4).

3.1 Isolated Thioesterase Domains for Cyclic Peptide Formation

One of the most interesting new discoveries in the NRP field has been that the isolated TE domain of tyrocidine synthase can catalyze peptide cyclization.[30] The cyclization ability of isolated TE domains does seem to be a general property, although it does appear that some TE domains show more desirable cyclization abilities than others.[31] Normally the tyrocidine TE domain is responsible for release and cyclization of the fully formed tyrocidine decapeptide product within the NRP complex itself. The fact that this domain can be used independently to make fully cyclized peptides from peptide SNAC thioesters has been exploited to make tyrocidine analogues having sequences altered from the natural peptide substrate, some of which display enhanced antibiotic activity compared to tyrocidine.[32] This method is especially amenable to scale-up since the altered peptides can also be synthesized on a solid support, which mimics the natural substrate for the TE, which is an acylated peptidyl carrier protein (PCP).[32] The TE domain itself also shows a remarkably broad substrate specificity and can make a fully formed dipeptide, gramicidin S, from two pentapeptide SNAC thioesters.[30] Further work has shown that the TE domain is also able to cyclize semi-synthetic products of mixed NRP/PK origin.[33] This engineering strategy should enable the production of novel NRP and NRP/PK libraries in the near future.

3.2 Heterologous Production of Non-Ribosomal Peptides

The first two modules of the tyrocidine synthase, TycA and TycB1, were functionally expressed in *E. coli*.[34] These two modules are normally responsible for synthesis of a D-phenylalanine-L-proline dipeptide that is further assembled to make the decapeptide, but when expressed alone, the product is a D-phe-L-pro-diketopiperazine (D-phe-L-pro-DKP), created by autocatalytic cyclization. Extensive investigation into media composition, growth conditions and gene dosage resulted in an optimized strain for production of the desired product.[34] *E. coli* has also been engineered for production of yersiniabactin, which marks the first time that a complete NRPS has been functionally expressed in *E. coli*.[35] Yersiniabactin synthase was chosen for heterologous expression due to its relatively simple biosynthetic scheme. The peptide is synthesized from a salicylate starter unit, 3 cysteines and a malonyl-CoA. While cysteine and malonyl-CoA are compounds found in abundant quantities throughout the cell, salicylate is not produced by *E. coli* and had to be added upon culture induction.[35] The fact that *E. coli* is able to take up this compound and utilize it in the engineered pathway is exciting because it can

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mean that alternative compounds may be able to be fed in the same way to create slightly modified structures depending on the substrate tolerance of the yersiniabactin loading module.

3.3 Novel NRP and Mixed PK/NRP Structures

Novel NRPs can be generated by construction of a hybrid NRPS, assembled from sequential or non-sequential modules in tyrocidine synthase.[36] It was also demonstrated that the domains responsible for activating amino acids within the NRPS have relaxed substrate specificity, accepting a variety of structurally related amino acids *in vitro*.[36] While only tripeptides were produced in this initial report, with further development, the possibility exists for construction of more complex, fully cyclized natural or novel NRPs. Epothilone is a mixed PK/NRP compound of significant biological activity that has been the subject of intense scrutiny since the first report on the sequencing of the biosynthetic cluster from *Sorangium cellulosum*.[37] One of the first efforts at epothilone modification was to eliminate the hydroxy group at position C13 to produce the more biologically active epothilone D. This was accomplished by elimination of a post-synthetic cytochrome P450, EpoK.[37] Since then, a new epothilone derivative has been generated *in vivo* by elimination of an enoyl reductase domain to generate 10,11-didehydroepothilone D,[38] which shows similar biological activity to epothilone D. The loading module of epothilone has been exchanged with loading modules from rapamycin, yersiniabactin and enterobactin in order to investigate the ability of the epothilone NRPS domain to tolerate different modules.[39] These experiments showed clearly the importance of linker regions, as the foreign domains with epothilone linkers performed well and those with native linkers did not. The report also showed that by using the enterobactin loading module, a 4-methyl-2-pyridinecarboxylic acid could be loaded, creating a methylpyridine epothilone derivative.[39]

4 Flavonoids

Flavonoids are a class of plant specific natural products involved in nodulation, UV protection and host defense in plants, and various health-promoting effects in humans.[40] Flavonoids are synthesized from phenylpropionyl-CoAs, abundant plant metabolites derived from phenylalanine and involved in lignin and other secondary metabolic pathways, and malonyl-CoA, *via* the action of a plant type III PKS, chalcone synthase (CHS), which has been nicely reviewed elsewhere.[41]

4.1 Rational Mutagenesis of Chalcone Synthase for Production of Novel and Altered Flavonoid Products

Following the publication of the crystal structure of chalcone synthase from *Medicago sativa*,[42] it became increasingly possible to rationally engineer CHS, and other plant type III PKSs, for the formation of altered products. Experiments to determine the effect of active site cavity volume on product formation showed that decreases in cavity size correspond to a shortening of the polyketide intermediate.[43] Likewise, alteration in the substrate binding pocket also lead to changes in the profile of the substrates that would be accepted as starter units.[44] In this particularly striking example, changing a conserved phenylalanine (F215) in the active site of CHS resulted in the enzyme preferring a substrate that was not previously accepted, *N*-methylanthranile-CoA, and caused a loss of acceptance for the natural substrate, 4-coumaroyl-CoA, effectively turning the enzyme into a related type III PKS, acridone synthase (ACS).[44] This example is not the first instance of CHS being effectively converted into another enzyme, as a CHS triple mutant (T197L, G256L and S338I) transformed it into a 2-pyrone synthase (2-PS), an enzyme that uses an acetyl-CoA starter unit and only uses two malonyl-CoA extensions instead of three.[45] ACS itself has also been converted into CHS.[46] Using the model of CHS as a guide, the structure of ACS was predicted and certain amino acids were targeted for substitution, demonstrating the broad utility of the CHS crystal structure in rational engineering of related type III PKSs for altered substrate or polyketide chain length.[46] With the addition of another crystal structure of a plant type III PKS, stilbene synthase (STS),[47] the ability to accurately predict structure and engineer these enzymes will greatly improve.

4.2 *In Vitro* Exploration of Chalcone Synthase Substrate Specificity for Production of Novel Polyketides

Besides rational engineering of CHS to explore its potential substrate and product profiles, extensive work has been done to explore these same properties *in vitro* with enzymes known to be promiscuous with substrates. Using CHS from *Scutellaria baicalensis* and STS from *Arachis hypogaea*, novel products were synthesized using aromatic, aliphatic and fluorinated starter units to create an array of tri- and diketide by-products as well as the expected fully formed flavanone and stilbene type structures.[48–50] This work has continued with the use of alternative extender units (such as methylmalonyl-CoA and succinyl-CoA)[51] and with both non-physiological starter and extender units.[52] In both cases at least one novel product was formed. While the above re-

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sults have been somewhat limited, all the experiments have been performed with recombinant wild-type enzyme that shows broad substrate specificity. Undoubtedly, with incorporation of knowledge gleaned from the rational engineering approaches described above, the engineering of new compounds with these same substrates will improve.

4.3 Biotransformation and Heterologous Production of Flavonoids and Related Compounds

New approaches in the production of novel flavonoid compounds have been through biocatalysis and heterologous production methods. While it is known that some intestinal commensal microorganisms can extensively metabolize flavonoids,[53,54] recently, bacteria expressing heterologous biphenyl dioxygenases have been used to transform flavonoids and isoflavonoids for production of novel compounds.[55,56] In the same way, cells expressing plant methyl transferases and glycosyl transferases can also be used to modify a range of flavonoid structures that have been added to *E. coli* fermentation media.[57] These methods will prove to be very powerful tools for the modification of pre-existing flavonoid structures as new enzymes are investigated or evolved to function in this role.

New approaches for generation of the flavonoid structure itself have focused on heterologous production in *E. coli* and yeast. The stilbene compound resveratrol has been produced in *Saccharomyces cerevisiae* using a combined poplar and grapevine pathway, making it the first report of a heterologously produced stilbene.[58] (Figure 5). Using a pathway consisting of enzymes from a yeast, *Streptomycete* and plant, the first engineered flavonoid pathway in *E. coli* was created.[59] While production levels were relatively low, the use of bifunctional enzymes in the pathway allowed for production of two compounds, pinocembrin and naringenin, from phenylalanine and tyrosine, respectively (Figure 5). Another report described the creation of the same flavonoid pathway, but with a *Rhodobacter sphaeroides* enzyme and two plant enzymes.[60] Using this approach, much higher levels of naringenin were seen and a new compound, phloretin, was produced in *E. coli* for the first time. This work showed that *E. coli* is able to take up phenylpropionic acids directly from the media to produce flavonoids when expressing a shortened (2 en-

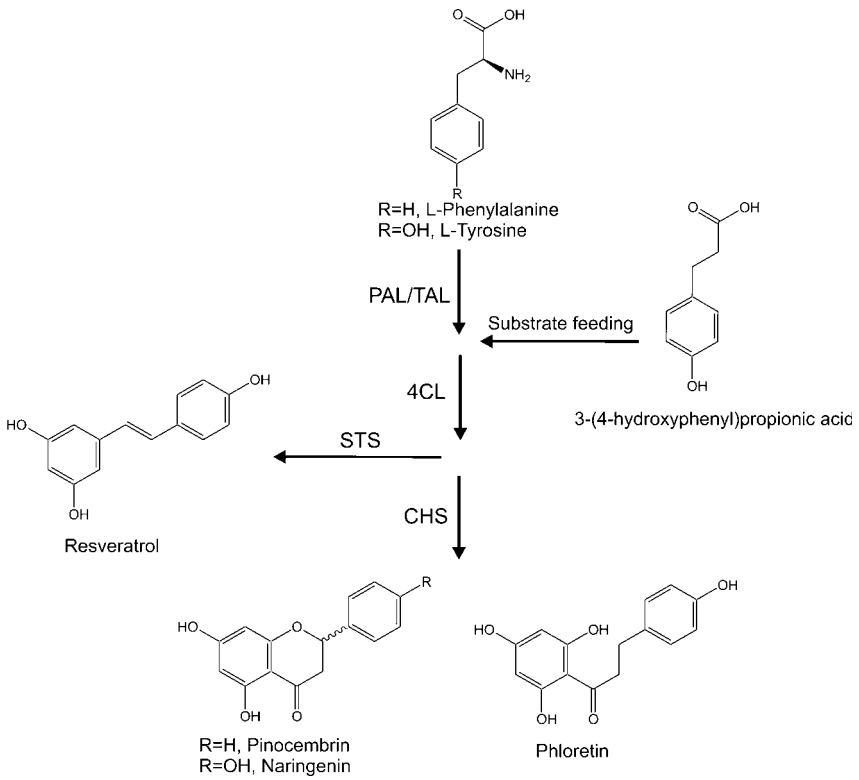

Figure 5. Heterologous production of flavonoid compounds by yeast and *E. coli*. Yeast expressing 4-coumaroyl-CoA ligase (4CL) and stilbene synthase (STS), and fed exogenous 4-coumaric acid, were shown to make resveratrol.[58] *E. coli* has been shown to make flavonoid compounds by substrate feeding as well, either with 4-coumaric acid or 3-(4-hydroxyphenyl)-propionic acid, to make naringenin and phloretin, respectively, *via* 4CL and chalcone synthase (CHS).[60] Additionally, naringenin and pinocmebrin have been made with a three-enzyme pathway starting with phenylalanine, for pinocembrin, or tyrosine, for naringenin.[59]

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zyme) pathway. By feeding 4-coumaric acid to growing *E. coli* cultures, naringenin was produced, and feeding 3-(4-hydroxyphenyl)propionic acid resulted in production of phloretin[60] (Figure 5). Considering the wealth of knowledge that exists about flavonoid pathway enzymes, metabolic engineering of the many diverse flavonoid pathways into *E. coli* and yeast should be possible in the near future.

5 Isoprenoids

Isoprenoids are a broad class of compounds, derived from the five-carbon isoprene (2-methylbuta-1,3-diene) unit. These compounds are important molecules in maintaining membrane fluidity (sterols), light absorption (carotenoids) and as intra- and extracellular signaling molecules (steroid hormones and terpenoids). Carotenoids and terpenoids are also of significant biotechnological value as natural food colorants and antioxidants, as well as antiparasitic and anticancer compounds.[61–63]

5.1 Novel Carotenoids

Typically, carotenoids have a 40-carbon backbone (C_{40}), although 30-carbon (C_{30}) and 50-carbon (C_{50}) chains are known. Their chemistry and biosynthesis is reviewed elsewhere,[64] but briefly, C_{40} carotenoids are synthesized by a head-to-tail condensation of four isoprene units to form C_{20} geranyl geranyl diphosphate (GGDP), followed by the head-to-head condensation of two GGDP molecules by phytoene synthase (CrtB). Desaturation of the C_{40} carbon chain by phytoene desaturase (CrtI) results in the first colored carotenoid, lycopene, with 11 *trans* double bonds. Lycopene can be further modified by lycopene cyclase (CrtY) to produce β,β-carotene, a bicyclic, fully desaturated carotenoid. Linear and cyclic carotenoids can then be further modified by a diverse array modifying enzymes such as oxygenases and glycosyl transferases. C_{30} carotenoid biosynthesis occurs by a similar condensation and desaturation of two C_{15} farnesyl diphosphate molecules while C_{50} carotenoids are synthesized by extension of linear C_{40} carotenoids with a prenyl-transferase-like enzyme.

Carotenoid expression in recombinant hosts such as *E. coli* is well established and the pigmented phenotype of recombinant cells makes carotenoid biosynthesis an ideal biosynthetic pathway for genetic manipulation. Directed evolution by DNA shuffling of phytoene desaturase CrtI has resulted in an enzyme that carries out additional rounds of desaturation of the carbon backbone to produce 3,4-didehydrolycopene and the fully conjugated 3′,4′,3,4-tetradehydrolycopene.[65] When coupled with a mutant CrtY, a novel pathway to the monocyclic carotenoid torulene was produced. By combination of these *in vitro* evolved pathways with a diverse array of microbial carotenoid modifying enzymes, a large number of novel carotenoid structures have been produced in recombinant *E. coli*.[66]

Recently, carotenoids with unusual C_{35} backbone chains have been generated by a combination of directed evolution and combinatorial biosynthesis.[67] When biosynthetic genes from C_{30} and C_{40} carotenoid pathways were combined in recombinant *E. coli*, such that both C_{15} and C_{20} precursor molecules were supplied to carotenoid synthase enzymes, unusual C_{35} backbone structures were observed. C_{30} and C_{40} carotenoid desaturases readily accepted these C_{35} substrates and combinatorial expression with carotene cyclases resulted in monocyclic C_{35} derivatives. Directed evolution of carotenoid desaturase enzymes was also utilized to modulate desaturase step number. Novel carotenoids with longer carbon backbones have also been reported recently. The C_{30} diapophytoene synthase CrtM from *Staphylococcus aureus* was first modified by directed evolution in recombinant *E. coli* for activity in a C_{40} carotenoid biosynthetic pathway.[68] By examining the amino acid changes in these mutants, functionally important residues were subjected to saturation mutagenesis. When supplied with C_{20} GGDP and C_{25} farnesyl geranyl diphosphate (FGDP) precursor molecules by an engineered variant of farnesyl diphosphate synthase, some CrtM variants were able to synthesize C_{45} and C_{50} carotenoid backbones.[69] If carotenoid desaturase and modification enzymes can be modified to accept these longer chain carotenoid substrates, potentially useful products with extended chromophores could be produced.

5.2 Terpenoids

Terpenoids are synthesized by the condensation of a series of isoprene units, followed by enzymatic cyclization by a terpene cyclase, and subsequent modification. Terpenes are grouped based upon their carbon chain length: monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), sesterterpenes (C_{25}) and triterpenes (C_{30}). Although microorganisms produce terpenoids, many important terpene products are found in plants including important flavor and aroma compounds and therapeutic and antimicrobial compounds. Extraction of these compounds from plant sources often results in expensive, low-yield processes. This has led to considerable efforts towards engineering plant terpenoid biosynthesis in recombinant microorganisms. Economical production of plant terpenes in recombinant *E. coli* has been limited by two major obstacles – low precursor supply and low plant enzyme expression levels or activity. Artemisin is a sesquiterpene found in sweet wormwood (*Artemisia annua*) with great potential as an antimalarial drug. By engineering the mevalonate isoprenoid pathway from *S. cerevisae* into recombinant *E. coli*, and bypassing the microbial DXP pathway for isoprenoid biosynthesis,

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high yields of artemisin precursor amorphadiene were achieved.[70] Poor expression of the plant amorphadiene synthase enzyme in *E. coli* was overcome be synthesizing the gene from oligonucleotides incorporating *E. coli* codon bias. This extensive engineering effort provides an excellent platform for further development of recombinant terpenoid production.

Taxol is a potent anticancer diterpene from pacific yew (*Taxus bervifolia*) that is an attractive target for recombinant production as it is difficult to extract or chemically synthesize. Production of the taxol precurosor taxadiene has been achieved in *E. coli* by engineering the *E. coli* DXP isoprenoid pathway and utilizing a truncated form of the taxadiene synthase enzyme with improved solubility.[71] By PCR differential display, homology based cloning and random cDNA sequencing of *Taxus* cells induced for taxol production, a number of the modifying enzymes necessary for the *in vivo* production of taxol from taxadiene have been identified.[72–79] Eventually these efforts may lead to a recombinant microbial production process for this important drug.

6 Future Trends and Strategies

The discovery of useful natural products has until recently been limited to screening of readily cultivated source organisms. The biosynthesis of the next generation of useful natural products may require the genetic potential of both sequenced source organisms and unculturable environmental organisms to be fully utilized. These approaches, outlined in the next section, may eventually allow the biosynthesis of desired natural products and modified structures by rational combinations of genes from completely separate biosynthetic routes from many source organisms.

6.1 Metagenomics and Cultivation of "Unculturable" Microorganisms

One of the major limitations to continued isolation of novel natural products is cultivation of producing organisms, whether soil-dwelling microbes, marine microbes, endosymbionts or organisms populating extreme environments. It is from these unique environments that the next generation of antibiotic, antiparasitic and anticancer compounds will most likely originate. Difficulty in obtaining sufficient quantities of the compound of interest has placed a great deal of need on advanced techniques for isolation of these difficult to isolate organisms. In the instances where normal laboratory cultivation techniques do not produce pure cultures, or growth at all, a number of growth conditions has been described that have resulted in growth of organisms that were not seen previously.[80–82] Encapsulation of cells in agarose beads as a means for pure culture formation has also been reported.[83] Using flow cytometry, the authors were able to sort single cells into agarose beads and maintain them in several different media, creating a fully diffusible, but enclosed, microenviroment.[83] This example also highlights another trend that is emerging in cell culture, high-throughput cultivation.[82,83] Robotics for liquid handling and automated cell sorting systems can be used to perform the normally tedious task of preparing the large amounts of media and inoculation. Using high-throughput cell sorting may also be useful in evaluation of cultures for natural product formation. Cells can be sorted into distinct pools based upon novel product formation, using an intrinsic feature of the molecule, such as fluorescence. This technique has already been demonstrated with *E. coli* producing novel metal porphyrins using fluorescence,[84] and may be applicable in the future for other natural products. Metagenomics (reviewed recently elsewhere[85,86]) has attempted to solve the cultivation problem by removing the culturing step and simply cloning the gene or genes responsible for the natural product biosynthesis by isolation of genomic DNA *in situ*. This process has so far been highly successful in isolating novel enzymes such as xylanases, lipases/esterases and proteases,[87] but reports on novel natural products,[88] and natural product gene clusters,[89] has been limited. One of the inherent problems is in screening, where assays based on antibiotic activity or color result in a relatively high rediscovery rate, for instance, violacein.[90] The lack of novel natural products, and their cognate biosynthetic clusters found in metagenomic libraries, could also be due to the large size of typical polyketide gene clusters, a random distribution of secondary metabolic genes throughout the genome or the availability of substrates in the chosen host for library expression. Vectors for expression of environmental DNA libraries in multiple hosts have been designed and used for screening,[91,92] but often the size of the library and the average insert size can limit the diversity of products that can be isolated. Likewise, products may be toxic and kill the host organism, eliminating those clones from the pool of viable colonies to screen. There continues to be a wealth of novel natural product structures isolated from the environment, particularly marine environments,[93] and this will no doubt be the fuel that drives the future natural product discovery drive, either by cultivation or isolating metagenomic DNA, of the symbiotic producing organisms.

6.2 Modification of Natural Product Core Structures

Rather than creation of novel structures by altering pathways or construction of new pathways, future prospects for modification of natural products may rely on modification of the core structure either *in vitro*, or *in vivo via* biotransformation. As has already been described, modification of a pikromycin precursor can be done

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in vitro by the action of an immobilized cytochrome P450.[28] These P450 enzymes are an important component of the modification of most of the natural product classes discussed so far, and make up significant portions of the genome of both bacteria[94] and plants.[95] The specificity of cytochrome P450s is not always well defined, and there are several examples of P450s being used as biocatalysts.[96] Using immobilized P450s to modify an array of natural product scaffolds would be a quick and cost effective means of novel structure generation. Aside from P450s, any number of modifying enzymes could in theory be immobilized in the same way. The fact that a P450 was successfully immobilized suggests that the possibility of immobilizing many enzymes is not that far fetched, as cytochrome P450s are typically membrane-bound enzymes that require several cofactors and are difficult to work with. With several immobilized enzymes, one could envision a matrix like approach to scaffold modification, with multiple rounds of modification taking place rapidly and with multiple compounds examined.

Likewise, dioxygenases, and other modifying enzymes, can also be used *in vivo* for modification of core structures *via* biotransformation. Dioxygenases, methyltransferases and glycosyltransferases have all been used to modify a pool of flavonoid structures with success,[55–57] owing to the ability of *E. coli* to express the active, heterologous enzymes, and the ability of the substrate to diffuse into the cell. Biotransformation, either by expression of heterologous enzymes (as above), or simply by allowing a certain compound to be modified in fermentation conditions by native enzymes,[97] will prove a powerful technique in natural product generation.

Perhaps the most intriguing possibility for modifying core structures is that non-pathway enzymes could be used for the modification. For example, an enzyme for catabolism of biphenyl was used to modify the flavonoid core structure,[55,56] which has only a superficial resemblance to biphenyl. Exploiting the broad substrate specificity of some enzymes could allow for production of completely novel compounds or new, more amenable biosynthetic routes to natural ones.

6.3 Genomic Bioprospecting

Bioprospecting refers to the search and discovery of biologically active metabolites in a range of diverse environments, such as a tropical rainforest, typically from plant and microbial sources. With genomic bioprospecting, the emphasis would be placed on going to diverse genomes and engaging in a search for only those important genes involved in secondary metabolite production. The rapid advances in genomic sequencing speed and efficiency have produced an abundance of sequence data. No longer are individual organisms being exclusively sequenced, but rather entire communities of organisms from diverse environments.[98,99] With few exceptions, large portions of finished, annotated genomes sit in the category of either uncharacterized or conserved hypothetical genes. Considering that upwards of 6% of the total genome of the avermectin producer *S. avermitilis* is involved in secondary metabolite production,[100] it would seem that a large degree of natural product biosynthetic machinery is being unnoticed. A recently sequenced, biocontrol strain of *B. amyloliquifaciens* FBZ42 showed up to 7% of the genome involved in polyketide and non-ribosomal peptide biosynthesis.[101] Comparative analysis to the previously sequenced genome of *B. subtilis* 168 showed the inclusion of an extra two unknown type I PKS clusters and one NRPS within the genome. Whether this amount of secondary metabolic diversity exists within the genomes of other environmental bacteria remains to be seen, however, it is entirely possible considering that so few have been completely sequenced.

New natural product clusters have been found using a new technique called genome scanning,[102] which is essentially screening a genome by sequencing large inserts that have homology to regions within known polyketide clusters. This way, only the PKS associated regions are sequenced without having to do an entire genome, saving time and money. With genome scanning, 16% of strains tested that were previously thought to not produce enediyne antibiotics actually had a enediyne PKS cluster, and were induced to produce the antibiotic.[102] This result is remarkable because it both demonstrates the hidden potential within the genome, and at the same time shows the power of different cultivation techniques. The technique, however, relies on databases of known biosynthetic enzymes and cannot really make predictions about genes that show no homology to known biosynthetic genes.

7 Conclusion

This review has highlighted recent advances in the engineering and manipulation of natural product biosynthetic pathways. The many examples of the heterologous expression and the manipulation of these pathways by combinatorial biosynthesis and *in vitro* evolution demonstrates the advantage of these methodologies for synthesizing both natural products and new compounds derived from natural products that are rare or not observed in nature. Heterologous expression in a genetically well-characterized host such as *E. coli* allows very precise engineering of biosynthetic pathways for improving yields and generating novel products. Although extensive engineering of *E. coli* is often necessary to successfully reconstitute some biosynthetic pathways, the potential to then manipulate this biosynthesis is a significant advantage when compared to engineering

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a poorly characterized source strain. Because of well-developed classical microbial fermentation technologies, e.g., for antibiotics produced by *Actinomycetes*, and the simpler cloning and expression of microbial genes, most biosynthetic pathways engineered so far into microbial cells are of microbial origin. However, plants are an extremely rich source of secondary metabolites (more than 100,000 plant natural products are known) and many important pharmaceuticals currently used are either directly or indirectly, *via* semi-synthesis, derived from plants. Considering that more than 400,000 higher plant species are known, harnessing their biosynthetic machineries that make these compounds for their large-scale biotechnological production represents therefore an attractive alternative to the cost-intensive extraction from plant cells.[103] When combined with new approaches to tap the potential of genome sequences and uncultured microorganisms, a wealth of novel natural products and derivatives could be synthesized in heterologous hosts, providing an enormous spectrum of chemical diversity to be exploited.

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