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Advances in pathway engineering for natural product biosynthesis

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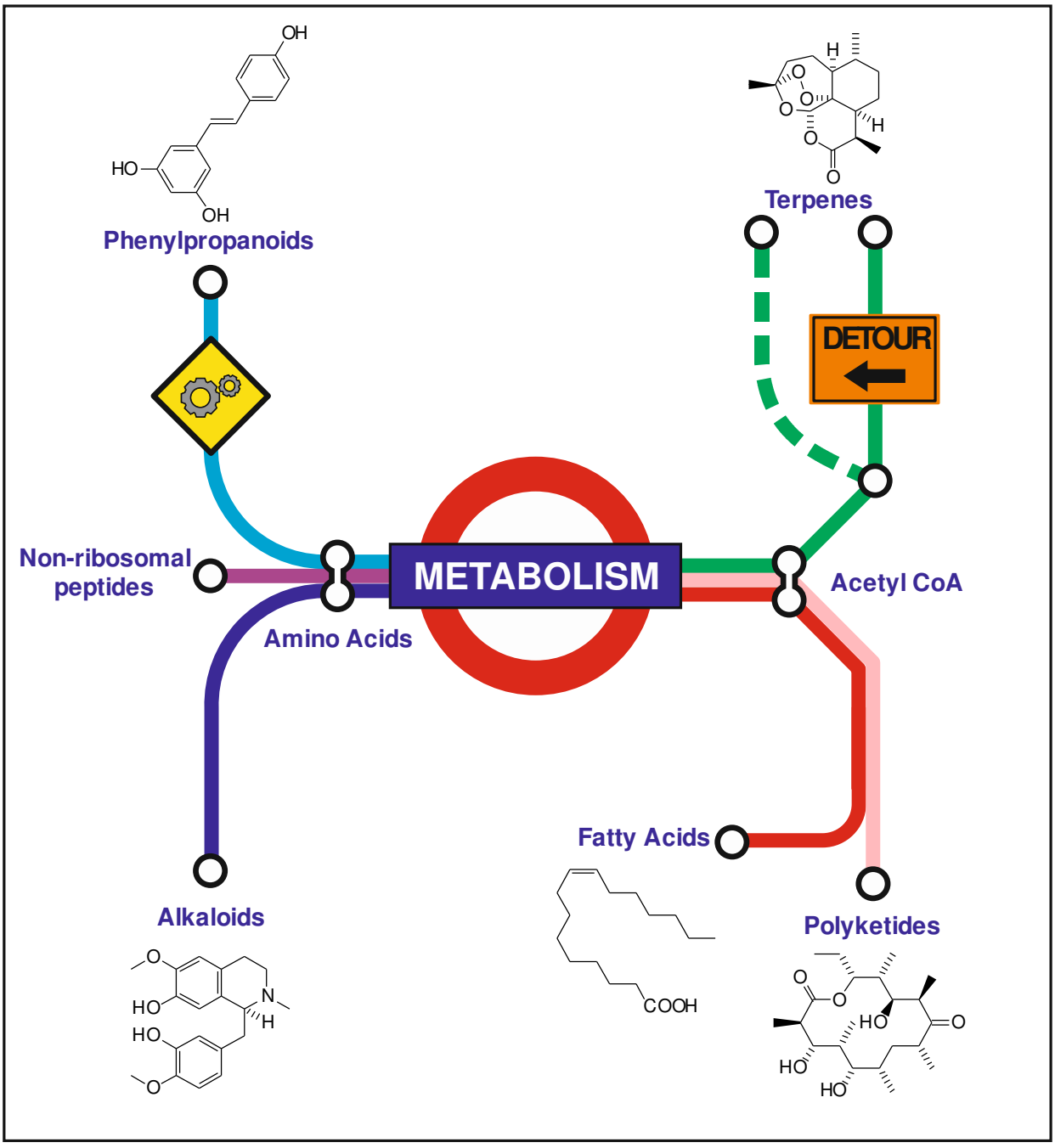
Dedication: This article is dedicated to Prof Wolf-Dieter (Woody) Fessner on the occasion of his 60th birthday



Abstract: Biocatalysts provide an efficient, inexpensive, and environmentally friendly alternative to traditional organic synthesis, especially for compounds with complex stereochemistries. The past decade has seen a significant rise in the use of biocatalysts for the synthesis of compounds in an industrial setting; however the incorporation of single enzymatically catalyzed steps into organic synthesis schemes can be problematic. The emerging field of synthetic biology has sparked interest in the development of whole-cell factories that can convert simple, common metabolites into complex, high-value molecules with a range of applications such as pharmaceuticals and biofuels. This review summarises conventional methods and recent advances in metabolic engineering of pathways in microorganisms for the synthesis of natural products.

1. Beginnings of metabolic engineering

Synthetic biology integrates two closely related disciplines - biochemistry and genetic engineering - to artificially design and manipulate biological systems in organisms with the aim of remodelling their metabolic pathways to change their function and behaviours.[1] This scientific approach began to emerge in the 1980s and in 1991, Bailey formulated the expression “metabolic engineering” to describe the techniques and systems devised to enhance metabolite synthesis in organisms.[2] The rationale behind metabolic engineering of natural product biosynthetic pathways predominantly focusses on enhancing the synthesis of a required target product that is either insufficiently, or not naturally, produced by the native host. Establishing new synthetic routesmay be achieved by manipulating the endogenous pathway or alternatively by introducing components of a heterologous pathway[3] designing a range of new enzyme activities to create products non-native to the host.[4] This “combinatorial biosynthesis” method involves expressing genes from various sources in one organism, and has been practised using the enzymes of plant metabolic pathways for over 30 years.[5] Excellent microbial hosts for metabolic engineering are *Escherichia coli, Saccharomyces cerevisiae* and the *Pseudomonas* species, because their amenability allows numerous optimisation approaches using the broad set of genetic tools available for engineering. Moreover, their fast growth and prolific protein production means they are able to produce rich quantities of natural products, especially when fermented in large cultures [6].

Enhancement of the production of natural products may involve the import of heterologous enzymes and over-expression of required native enzymes, increasing the cells‘ uptake of essential substrates, optimising pathways by minimising alternative product formation, increasing cofactor availability and augmenting substrate usage, optimisation of enzyme activity and specificity by protein engineering or directed evolution, and optimising access to the final products by secretion or cell lysis methods.[7] This pipeline is summarised in Figure 1 and examples of successful methodologies which are typically used to tackle these aspects including over-expression of enzymes mediating rate-limiting reactions, introduction of regulatory transcription factors, heterologous pathway construction, optimising codons for efficient protein expression and exclusion of opposing pathways to minimise by-product formation will be given throughout the review.[2b, 8] Our metabolic engineering capability has advanced considerably, especially with the development of novel multivariate modular metabolic

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Alan Berry graduated from the University of Southampton (BSc 1979, PhD 1983). After his PhD on the mechanism of enzymes involved in porphyrin biosynthesis, he worked for two years at ETH-Zurich on the stereochemistry of methyl group insertion in corrin biosynthesis. His interest in protein engineering developed during a Royal Society University Research Fellowship at the University of Cambridge. In 1994 he joined the University of Leeds as lecturer and was promoted to Senior Lecturer in 1997, Reader of Enzymology in 2006 and Professor of Molecular Enzymology in 2009. He works on the design and redesign of enzymes, particularly aldolases, as biocatalysts by both rational design and directed evolution.

engineering (MMME) techniques.[9]. The intention of MMME is to exclude pathway regulatory mechanisms and increase flux through rate-limiting reactions by assembling a modified pathway composed of separate modules. Moreover, the ever-decreasing costs associated with synthesising genes and the refinement of analytical tools has supported the manipulation of whole cells, as opposed to simply removing and inserting individual genes.[7] In the context of natural product biosynthesis, metabolic engineering has been successfully applied towards terpenoid, flavonoid, phenylpropanoid, alkaloid and polyketide pathways, with well documented evidence available in the literature.[8b, 10]

This review summarises conventional methods and recent advances in metabolic pathway engineering for the synthesis of natural products, focusing on how particular strategies have been applied to enhance isoprenoid, alkaloid, polyketide, and phenylpropanoid biosynthesis. Although exact approaches are specific to the natural product in question, general challenges and metabolic engineering principles are highlighted. We conclude by contemplating future perspectives and developments in this progressing research field.

1.1. Importance of metabolic engineering as a tool for enhanced natural product biosynthesis

Natural products belong to an extensive family of diverse organic molecules with in excess of 200,000 discovered and extracted from higher plant species. Plant natural products comprise of two classes: the primary class, which are ubiquitous and fundamental for plant viability, such as hormones, photosynthetic chlorophylls, carotenoids, cytokinins, ubiquinones and sterols constituting the bilayer.[8a, 11] The secondary class are significantly more diverse and are named secondary because they have no established role in growth and development.[12] The importance of natural products is emphasised by their significance to the pharmaceutical industry for over 60 years, delivering novel antibiotics, hormones and anti-tumour agents to the therapeutic drug repertoire.[6a, 13] Over the last 25 years, one quarter of pharmaceuticals are isolated natural products, or designed based on their structure, which is reflected by their complexity and chirality, a predominant feature of most approved drugs.[6c, 14] The advantages of plant natural products to modern society are however diminished by their relatively poor abundance in nature, restricting their use in research and development as extraction produces low yields. To further complicate this problem, the structural complexity of approved natural product drugs presents challenges for chemists because synthesis by conventional methods is impractical and the presence of multiple chiral centres generates stereoisomers which have to be purified accordingly.[6c] It is therefore essential that novel approaches are designed to enhance the accumulation of these valuable molecules that are naturally synthesised in small amounts.[3]

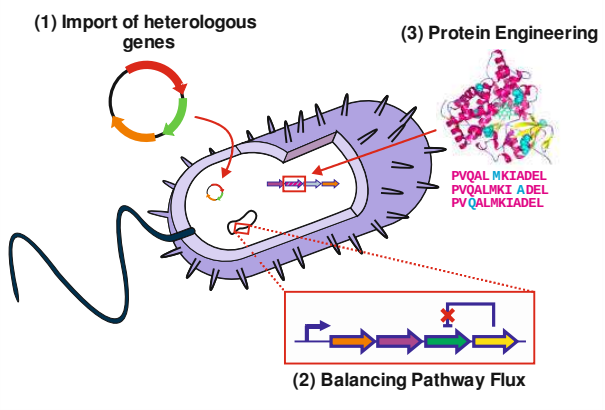

Figure 1. Strategies for the enhancement of natural product production in microorganisms. (1) Import of heterologous genes to assemble a non-native biosynthetic pathway in the host organism. (2) Balancing pathway flux, for example by increasing substrate availability, down regulating competing pathways, or increasing expression of key pathway enzymes. (3) Protein engineering to enhance or modify individual pathway enzymes.

1.2. Natural Product Biosynthetic Pathways

Despite the vast richness of natural products, they are derived from limited primary metabolites and are synthesised by relatively few central biosynthetic pathways (Figure 2). Briefly, polyketides and non-ribosomal peptide natural products are generated from simple substrates such as acetyl-CoA, propionyl-CoA, malonyl-CoA, methylmalonyl-CoA or amino acids through the action of huge PKS or NRPS megasythases. The nitrogenous alkaloids are a diverse class of natural products usually derived from amino acids such as histidine, phenylalanine, tyrosine, and tryptophan, amongst other substrates such as purines and caffeine.[15] Phenylpropanoids, comprising of the flavonoids, coumarins, lignans, and stilbenes, are phenolic natural products comprised of tyrosine and phenylalanine amino acids condensed with malonyl-CoA units.[16] and isoprenoid biosynthesis features the terpene synthases which cyclise poly-isoprene units to generate over 40000 distinct isoprenoids.[17]

The diversity of natural products in each of these classes is also attributed to tailoring enzymes which facilitate glycosylation, hydroxylation, methylation, halogenation, and reduction reactions to generate the final unique compounds.[18] Although natural products display distinctive structural and chemical characteristics, similar metabolic engineering strategies may be employed to enhance their yield and to provide routes to engineer new natural products. We begin by considering traditional strain improvement methods before discussing techniques for heterologous biosynthesis and pathway optimisation and future perspectives in this research field.

1.3. Traditional Strain Improvement

In nature, sufficient quantities of secondary metabolites may only be synthesised when absolutely necessary to confer a selective advantage.[19] Their relatively low abundance therefore presents a problem not only with extraction yields, but also with industrial scale production of the natural organism. Initial strategies to improve yields involved traditional strain improvement where native producing strains were screened in numerous rounds of natural selection for their elevated ability to synthesise the desired natural product. This is a reasonable approach when no prior knowledge of the biosynthetic pathway is known.[19]

Early examples of conventional strain improvement are demonstrated in the isolation of *Penicillium chryogenum*; a penicillin producing strain with titres 100-fold higher than the original strain isolated by Fleming.[20] Industrial strains are now predicted to be producing penicillin at exceptional titres with a 100,000-fold enhancement.[20] Although natural penicillin production represents a successful attempt at strain improvement, this method can be laborious and time consuming, with only 1 in 10,000 organisms isolated with each round selection.[19] With advances in our understanding of metabolic pathwaysand expansion of our genetic toolkit, enhancement of natural product yields in native strains is promising. This technique however is limited, as not all organisms are fermentable or genetically tractable. Heterologous biosynthesis and optimisation therefore represents a more successful approach to improve natural product yields, assuming the necessary biosynthetic pathway is known.

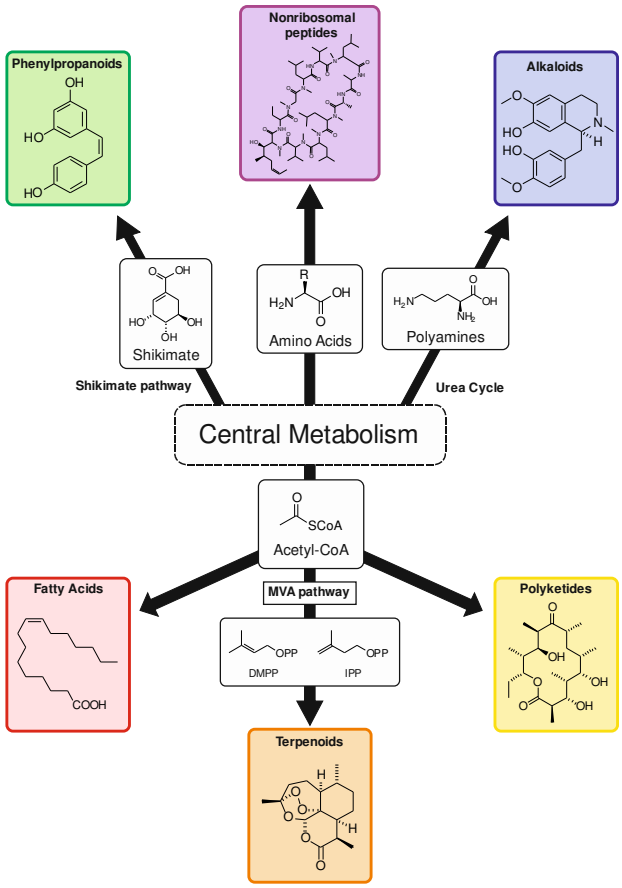

Figure 2. The vast number of natural products are generated from a surprisingly small number of building blocks from primary metabolism. The figure illustrates the synthesis of a number of the major families of natural product.

2. Import of heterologous enzymes and overexpression of required native enzymes

The significance of plant natural products to human healthcare have resulted in considerable interest towards their manufacture in microbial hosts.[10g, 21] Enzyme biocatalysts in microbial systems have considerable advantages over extraction and chemical manufacturing, primarily because of their remarkable selectivity, so their products are enantiomerically distinct, but also because of their proficiency under mild conditions.[22] However, our potential to reconstruct metabolic pathways is limited, not only by our understanding of the biosynthetic route, the enzymes catalysing these reactions and their regulatory mechanisms, but also by physical obstacles such as native expression and functionality of eukaryotic enzymes, and preventing toxicity in the host due to the build-up of intermediates.[4, 21a] Heterologous production of plant natural products in microbes represents two complicated challenges; firstly, identification of the distributed genes responsible for the biosynthesis of the desired compound, and secondly achieving functional expression of large, complex enzymes in lower organisms.[19] An example of expression difficulties is demonstrated by the P450 modification enzymes, featuring in many of the plant natural product biosynthetic pathways.[23] Although heterologous expression of P450's in eukaryotes, for example *Saccharomyces*, is fully functional, expression in prokaryotic microorganisms, such as *E. coli*, is challenging due to lack of post-translational modifications, inability to translate this membrane bound enzyme on membranes, and incorrect protein folding.[24] Moreover, following elucidation of the central biosynthetic pathways in plants and bacteria, it is necessary to understand complex regulatory mechanisms.[25] Nevertheless, natural product biosynthetic pathways have been successfully translated into heterologous systems, including those encoding precursors for the significant isoprenoids, paclitaxel and artemisinin, and the alkaloid precursor reticuline. Assembly of artificial pathways has also been successful in flavonoid, phenylalanine and phenylalkaloid biosynthesis, amongst other natural products.[19]

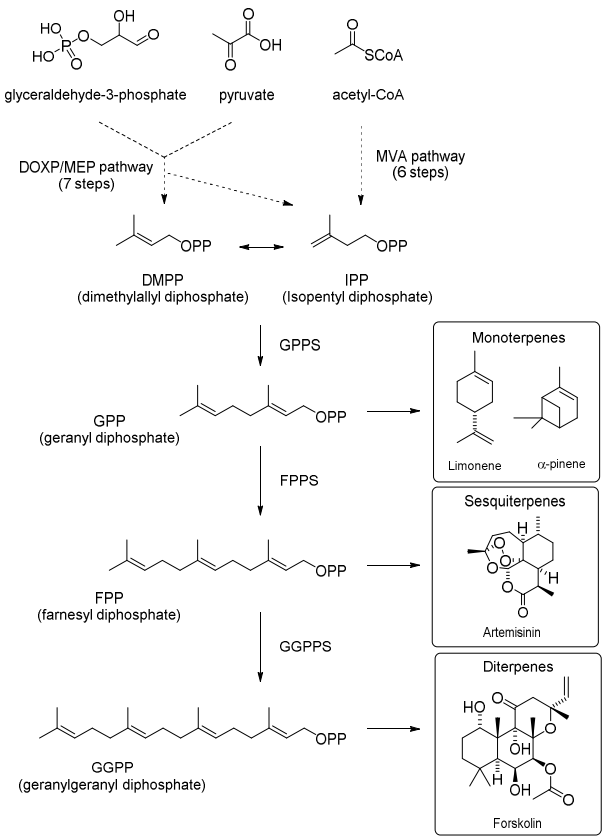

Figure 3. Biosynthesis of the isoprenoid family of natural products. The universal isoprenoid precursors, IPP (isopentenyl diphosphate) and DMPP (dimethylallyl diphosphate), can be synthesised by (I) the MVA (mevalonate) pathway by conversion form acetyl-CoA involving 6 enzymatic steps or (II) the DOXP (1-deoxy-D-xylulose-5-phosphate) pathway (also referred to as the MEP pathway) by a 7 enzymatic step conversion from glyceraldehyde-3-phosphate and pyruvate. Condensation of IPP and DMPP generate the first intermediate, geranyl diphosphate (C10), and sequential appendages of IPP create further intermediates: farnesyl diphosphate (C15) and geranylgeranyl diphosphate (C20). Downstream enzymes catalyse a range of chemical modifications to cyclise, oxidise, reduce, isomerise and hydroxylate the prenyldiphosphate intermediates to generate the respective terpenoid product.

The diverse nature and multi-functional properties of natural products have inspired significant interest in their microbial factory production and optimisation of their biosynthetic pathways.[6c, 21a-c] Over the past 20 years a substantial number of metabolic engineering approaches, with emphasis on heterologous expression, have been applied to various natural product biosynthetic pathways in microbes and plants, to maximise their synthesis far beyond what is achievable naturally, and produce a yield considerably higher than simple extraction.

Deciding to use a heterologous biosynthesis approach begins with selecting an appropriate chassis in which to express the pathway enzymes. Technical convenience is a highly influencing factor when considering a heterologous host. Well characterised organisms requiring simple culturing techniques, with the possibility of industrial fermentation are advantageous; *E. coli* boasts all of these characteristics and is an excellent host for metabolic engineering. Secondly, the natural product class and complexity must be evaluated. Natural product pathways in eukaryotic organisms are more likely to be reconstituted successfully in eukaryotic hosts, especially since functional gene expression is favoured. Thirdly, one needs to consider transformation and metabolic engineering techniques available to manipulate the chosen host. For example, particular hosts present technical barriers to introduction of genetic material. Finally, it is necessary to understand metabolism native to the chosen heterologous host, not only to assess the hosts capability to synthesise a compound, but also to anticipate interplay between metabolic pathways that could complicate production.[18] These criteria will be further elaborated using specific examples to demonstrate achievements in heterologous biosynthesis of natural products.

Isoprenoids are derived from the five-carbon metabolite precursors, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMPP), and can be synthesised by two different metabolic pathways depending on the organism: the mevalonate (MVA) pathway or the methyl-*D*-erythritol 4-phosphate/1-deoxy-*D*-xylulose 5-phosphate (MEP/DOXP) pathway (Figure 3).[6b, 26] Optimisation of precursor concentrations either by native pathway optimisation or transportation of a heterologous pathway, in combination with heterologous expression of a specific terpene synthase enzyme is necessary to successfully produce isoprenoids at relevant concentrations. Efforts to optimise the endogenous DOXP pathway in *E. coli* to increase the supply of isoprenoid precursors required for high-level production of lycopene have been described.[21d, 27] Simply overexpressing native DOXP pathway enzymes, or balancing concentrations of the DOXP pathway substrates, pyruvate and glyceraldhyde-3-phosphate, has resulted in enhanced lycopene production.[21d, 27] Similarly, enzymes of the native DOXP pathway and prenyldiphosphate synthases, catalysing the synthesis of GPP and GGPP, were overexpressed to increase the production of monoterpenes and diterpenes.[28] For example, transforming *E. coli* with multiple gene operons encoding these pathway enzymes, provides a system for enhanced production of terpenoids when coupled with expression of the corresponding terpene synthase.[28] Although these approaches have slightly increased isoprenoid production, one fundamental aspect limiting higher yields is the endogenous regulation mechanism present in the *E. coli* host. In order to maximise terpene yields, heterologous import of the yeast MVA pathway needs to be considered.[29] This approach has been successfully applied to E. *coli* strains, now capable of functioning as platform hosts for the production of any terpenoid compound. Circumventing the native, highly regulated DOXP pathway has generated considerably higher concentrations of the IPP and DMPP precursors, so abundant in fact, that the accumulation of toxic prenyldiphosphate intermediates halts the growth of *E. coli*. Overcoming this toxicity is however possible by overexpressing downstream pathway enzymes, for example amorphadiene synthase, to relieve toxic intermediate build-up by shuttling

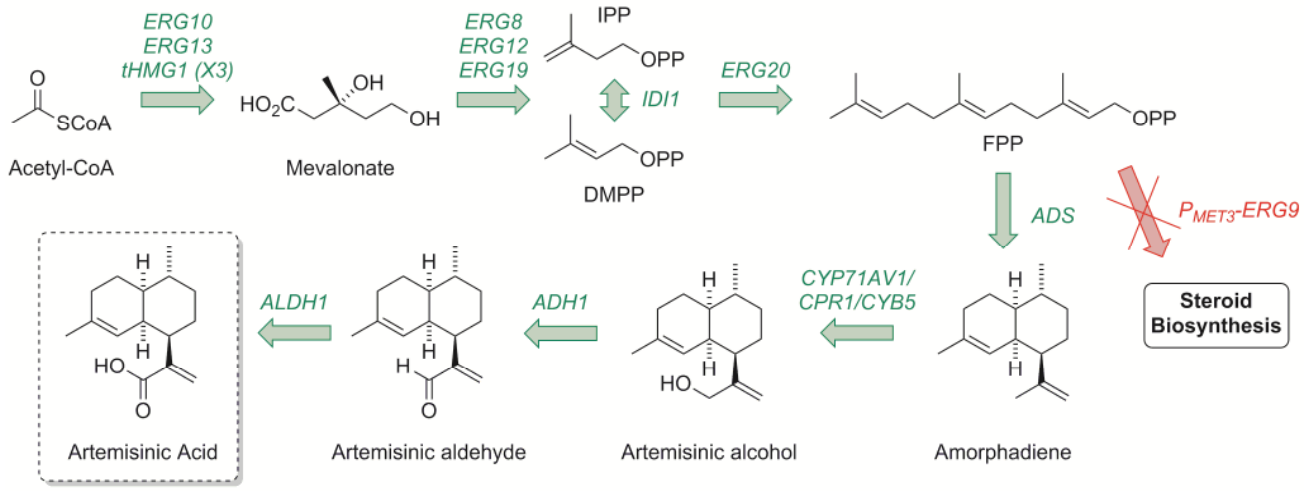

Figure 4. Schematic representation of the engineered artemisinic acid biosynthetic pathway in a genetically modified strain of *S. cerivisiae*. Overexpressed genes are shown in green. Heterologous expression of the amorphadiene synthase (ADS), amorphadiene oxidase (CYP71AV1), NADPH:cytochrome P450 reductase (CPR1), cytochrome b_5 (CYB5) artemisinic aldehyde dehydrogenase (ALDH1) and alcohol dehydrogenase (ADH1) from *Artemisia annua* resulted in production of artemisinic acid. Squalene synthase (ERG9) expression was restricted using a methionine repressible promoter (P_{MET3}) to reduce competition for farnesyl diphosphate. IPP, isopentenyl diphosphate; DMPP, dimethylallyl diphosphate; FPP, farnesyl diphosphate; tHMG1, truncated HMG-CoA reductase;. ERG10 - acetyl-CoA C-acetyltransferase; ERG13 - hydroxymethylglutaryl-CoA synthase; ERG12 mevalonate kinase; ERG8 - phosphomevalonate kinase; ERG19 mevalonate diphosphate decarboxylase; ERG20 (encoding yeast farnesyl diphosphate synthase)

through sequential enzymes.[10a] This aspect has also been used for α-pinene production, where co-expression of the α-pinene synthase and import of the yeast MVA pathway enzymes has boosted titres of α-pinene in *E. coli* to 0.97 g L^{-1}.[30] Similarly, farnesol production in *E. coli* is significantly boosted when the overexpression of farnesyl pyrophosphate synthase (*isp*A) is supplemented by supplying higher levels of DMPP and IPP from the heterologously expressed yeast MVA pathway.[31]

A further example of successful natural product production from heterologous gene expression is the microbial production of the antimalarial drug artimisinin (Figure 4).[32] Isolation of this sesquiterpene lactone from the plant sweet wormwood is inadequate, prompting a microbial engineered manufacturing approach.[33] To synthesise the early artemisinin precursor amorphadiene, a codon optimised amorphadiene synthase was co-expressed in *E. coli* with the high flux MVA pathway genes.[10a] Subsequently, expression systems have moved to the more functional *Saccharomyces* as a microbial host, prompting the expression of the amorphadiene synthase gene in yeast by chromosomal integration and plasmid transformation techniques.[34] Further optimisation of the native yeast MVA pathway, along with expression of the P450 enzyme catalysing the synthesis of artemisinic acid yields high levels of this compound that can be used to semi-synthetically derive artemisinin.[35] and a breakthrough in rational artemisinin synthesis has been demonstrated, where a comprehensive biosynthetic pathway has been expressed in *Saccharomyces* to produce artemisinic acid, and a feasible process for its chemical conversion to artemisinin outlined (Figure 4).[36] Although there has been enormous success in this field, innovative ideas for metabolic engineering in *Bacillus* are currently being studied, optimising the expression of individual pathway operons using separate promoter elements and balancing inducer concentrations.[37]

Heterologous expression also plays an important role in the engineered production of phenylpropanoids including the flavonoid and stilbene molecule classes (Figure 5). These products constitute a diverse group of plant secondary metabolites derived from the condensation of phenylalanine or tyrosine with numerous malonyl-CoA molecules. Significant interest has been directed towards microbial synthesis of phenylpropanoids due to their natural health benefits as excellent antioxidants with potential as pharmacological agents with antiviral, antibacterial, anticancer and estrogenic activities[16a], but prokaryotes do not possess the phenylpropanoid pathways of plants.[38]

The phenylpropanoid core is synthesised by non-oxidatively eliminating the amino group by tyrosine ammonia-lyase (TAL) or phenylalanine ammonia-lyase (PAL), depending on the incorporated amino acid. Successive modification reactions mediate reduction, hydroxylation and methylation reactions to generate the precursors for the subfamilies of the phenylpropanoids: flavonoids, coumarins, lignans and stilbenes.[16b] Flavonoids are derived from a universal chalcone precursor; synthesised by the enzyme chalcone synthase, condensing one molecule of 4-coumaroyl CoA with three molecules of malonyl-CoA. Divergent branching pathways originating from the chalcone precursor are responsible for the multitude of flavonoid subfamilies.[38] Recombinant

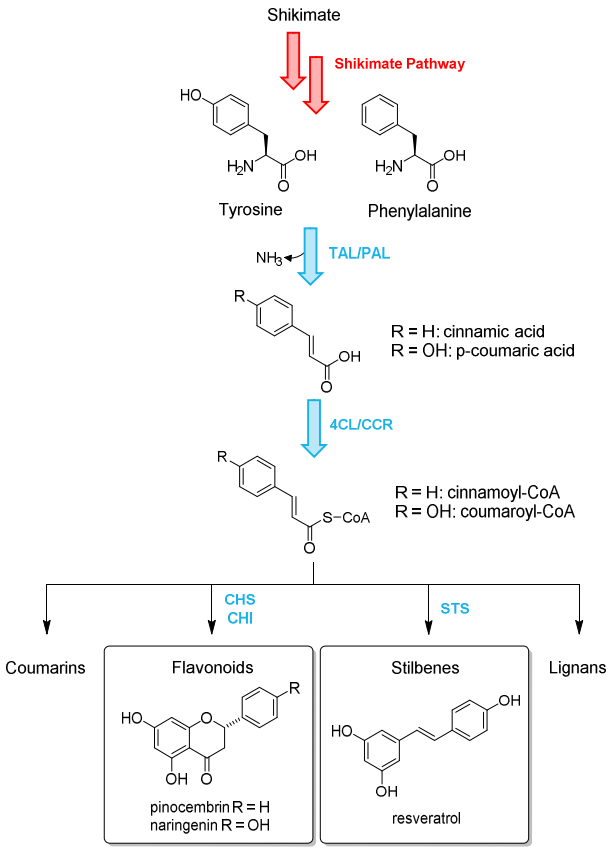

Figure 5. . Biosynthesis of the phenylpropanoids. The phenylpropanoid core is generated via elimination of the amino group from the aromatic amino acids tyrosine and phenylalanine by the enzymes tyrosine ammonia lyase (TAL) and phenylalanine ammonia lyase (PAL), respectively. 4-coumaroyl-CoA ligase (4CL), chalcone synthase (CHL) and chalcone isomerase (CHI).

phenylpropanoid pathways have been reconstituted in *E. coli* and *S. cerevisiae*, expressing PAL to derive phenylalanine based flavonoids,[39] and TAL to derive tyrosine based flavonoids.[40] This represents an entry point into phenylpropanoid metabolism, providing the appropriate precursors for the flavonoids and stilbenes. The first production of flavonoids in *E. coli* was reported recently, by also expressing 4-coumaroyl-CoA ligase (4CL) and chalcone synthase (CHS), along with PAL, as part of an artificial gene cluster from *Streptomyces ceolicolor*. When supplied with the appropriate precursors, tyrosine and phenylalanine, naringenin chalcone, a cyclised intermediate in the flavonoid pathway, and pinocembrin chalcone were synthesised, respectively (Figure 5).[41] Similarly, mixtures of flavonoid compounds have been reported in yeast, expressing similar heterologous enzymes from *Rhodosporidium, Arabidopsis* and *Hypercium* plant species.[42] Heterologous expression of the successive enzyme in the flavonoid biosynthetic pathway, chalcone reductase (CHR), generated the derivative molecules 5-deoxyflavones and 5-hydroxy flavonones.[43] Expression of further heterologous enzymes, flavonol 3β-hydroxylase (FLT), dihydroflavonol reductase (DFR) and leucocyanidin reductase (LAR) yielded the cathechins,[44] whilst expression of FLT with flavonol synthase (FLS) yielded the flavonols.[10e, 45] Moreover, the flavanones have been generated in *E. coli* [46] and *Saccharomyces* [47] by expression of the essential precursor flavanone pathway genes together with a flavanone synthase (FNA). Although substantial efforts have improved yields of various flavonoids, scalable fermentation methods pose one significant disadvantage; the supplementation of expensive phenylpropanoic substrates in the media. Therefore, recent advances in flavonoid microbial synthesis have focused on engineering an *E. coli* strain which has already been optimised for tyrosine production. This strain directly synthesises tyrosine from glucose, without supplementation of the amino acid in the medium. With concomitant expression of CHS and three codon optimised flavonoid pathway enzymes, TAL, 4CL and CHI, the resultant strain generated 29 mg/L naringenin,[48] a 64-fold improvement on the first naringenin producing strain by simply expressing three pathway enzymes.[41]

Stilbenes are derived via the same central pathway to the flavonoids, synthesising the universal chalcone precursor molecule, except shuttling this intermediate through stilbene synthase (STS) as opposed to chalcone synthase (CHS) for the flavonoids. An interesting stilbene of particular interest is resveratrol; a potent antioxidant with potential cancer chemopreventive properties.[49] Synthesis of resveratrol has been observed in yeast strains, heterologously expressing PAL, 4CL and STS, with the latter two enzymes expressed as enzymatic fusions.[50] Protein fusions will be discussed in the optimisation of pathway flux section, as a method to augment substrate channeling.

The alkaloids represent another family of natural products where heterologous expression of enzymes is absolutely necessary for their synthesis in microbes.[15] Alkaloids, with substantial pharmacological applications as analgesics, stimulants and chemotherapeutics, are a family of nitrogenous, low molecular weight compounds derived from amino acids such as histidine, phenylalanine, tyrosine and tryptophan, and other substrates such as purines and caffeine.[16b] Biosynthesis of the isoquinoline alkaloids involves the essential precursor molecule *S*-norcoclaurine, formed by the condensation of dopamine and 4-hydroxyphenyl acetaldehyde (4HPAA) in a reaction catalysed by norcoclaurine synthase (NCS). *S*-norcoclaurine is consequently converted to *S*-reticuline by four sequential enzymes; three transferases and a hydroxylase.[10g] Difficulties in the production of initial intermediates in the benzyl isoquinoline alkaloid pathway are presented in microbial systems, due to the tedious expression of eukaryotic P450 mono-oxygenases. Since this pathway is difficult to reconstruct for efficient product of benzyl isoquinoline alkaloids, subversion using a microbial monoamine oxidase simplified the production of reticuline. Reconstructing the benzyl isoquinoline alkaloid pathway in *E. coli*, consisting of the three transferases and hydroxylase enzymes from *Coptis japonica,* and monoamine oxidase enzyme

from *Micrococcus luteus*, yielded a strain producing 55 mg/L *S*-reticuline.[10g] Moreover, by integrating enzymatic activities from different plant species, increasing diversity can be generated in such alkaloid natural products. This is demonstrated by the notable work synthesising sanguinarine/berberine and morphinan subfamilies of the benzyl isoquinoline alkaloids by assimilating enzymes from three different plants.[10f]

3. Optimising pathway flux

Natural biosynthetic pathways in the native host are regulated by exact mechanisms, ensuring metabolites are synthesised in sufficient amounts for cell growth and survival without wasting important resources on dispensable processes. However, reprogramming metabolic pathways in heterologous organisms presents the challenge of reconstructing regulatory control to mimic endogenous metabolism. Without fundamental control points, heterologous pathways are excessive, leading to retardation of growth, accumulation of toxic intermediates and metabolic imbalance.[51] Deletion of competitive pathways, creation of protein fusions and scaffolds, optimising cofactor regeneration and manipulating pathway regulatory elements are useful methods to optimise pathway flux and maximise natural product titres.

3.1. Deletion of competing pathways

Understanding the complex metabolism native to the host organism is essential to assess interplay between metabolic pathways that could otherwise complicate production to obtain high yields.[18] Natural endogenous capabilities of the host can deplete essential precursors, intermediates and cellular resources if occupying roles in competing pathways. By eliminating or down-regulating enzymes in major opposing pathways, the synthesis of desired natural products is favoured as an alternative to energy depleting by-product formation.[19] For example, increased production of the sesquiterpenes farnesol and patchulol[52], and amorphadiene [53] in yeast was accomplished by replacing the endogenous promoters in the major opposing pathway enzyme squalene synthase, encoded by *erg9*, by the methionine repressible promoter MET3 (Figure 4). Squalene synthase uses the central sesquiterpene intermediate farnesyl pyrophosphate (FPP), and an optimal concentration of methionine could be established to inhibit the activity of squalene synthase sufficiently to increase synthesis of farnesol, patchulol and amorphadiene without interrupting essential endogenous processes.[52] More recently a similar technique has been applied towards α-santalol synthesis, using the glucose repressible promoter HXT1 to modulate the expression of ERG9.[54] A similar problem with supply of precursors arises for the synthesis of flavonoid and polyketide natural products where the levels of the precursor malonyl-CoA naturally present in *E. coli* is limited.[55] Malonyl-CoA is a central precursor for many endogenous pathways and inhibition of competitive malonyl-CoA consuming routes is therefore a suitable strategy to increase availability. Multiple engineering methods have been devised to accumulate the malonyl-CoA substrate for high-level flavanone and anthocyanin production in *E. coli* (Figure 6).

A significant (15-fold) enhancement in the cellular pool of malonyl-CoA has been demonstrated by over-expressing acetyl-CoA carboxylase (ACC), irreversibly converting acetyl-CoA to malonyl-CoA, and secondly improving ACC conversion activity by increasing acetyl-CoA availability. This was achieved by deleting opposing pathways forming ethanol and acetate by-products and overexpressing an acetate-forming enzyme. This modified strain of *E. coli* with improved cellular concentrations of malonyl-CoA provides a platform for enhanced production of natural products, such as flavonoids and polyketides, where central malonyl-CoA substrates are rate-limiting.[55]

Fatty acid biosynthesis also competes for the supply of malonyl-CoA and hinders flavonoid production by converting the malonyl-CoA substrate to acetyl-CoA via fatty acid synthase enzymes. Following assembly of the heterologous biosynthetic pathways directing flavanone and anthocyanin synthesis, the intracellular concentration of the essential metabolite malonyl-CoA was increased up to 250% by introducing an alternative malonate assimilation pathway. Inhibition of the competitive fatty acid synthesis pathway using sub-lethal concentrations of the antibiotic inhibitor cerulenin enhanced flavonoid production levels to a remarkable 900%. Using this approach, the yield of flavonones was reported up to 700 mg/L and anthocyanins up to 113 mg/L, when supplemented with their appropriate phenylpropanic acid and flavan-3-ol precursors, respectively. Moreover, the results reinforce the idea that carbon metabolites are predominantly channelled towards fatty acid biosynthesis, which is therefore a key competitive pathway in flavonoid biosynthesis.[56] Integrating multiple pathway optimisation techniques has also been useful in *S. cerevisiae* engineering for α-santalene production; a highly valuable constituent of sandalwood oil. The approach previously described, regulating the expression of ERG9 using the HXT1 glucose sensing promoter, was firstly applied together with the deletion of two lipid phosphate phosphatases (LPP) in order to increase available FPP for α-santalene synthesis. Secondly, two central enzymatic reactions catalysed by the tightly controlled FPPS and HMG-CoA reductase (HMGR) enzymes were optimised, by deleting the regulatory domain of HMGR and over-expressing FPPS; this increased the sesquiterpene FPP precursor. Thirdly, the concentration of the necessary NADPH reductive cofactor was increased by deletion of an NADPH utilising dehydrogenase whilst expressing an NADP consuming dehydrogenase, regenerating NADPH. The final engineering involved the endogenous MVA pathway, introducing a continuously activated transcription factor known to be involved in expression of the corresponding genes.[57]

Expanding the concept of gene deletion presents the idea of creating a minimal host organism for the synthesis of secondary metabolites. One can envisage an organism solely committed to synthesising the desired natural product, void of any

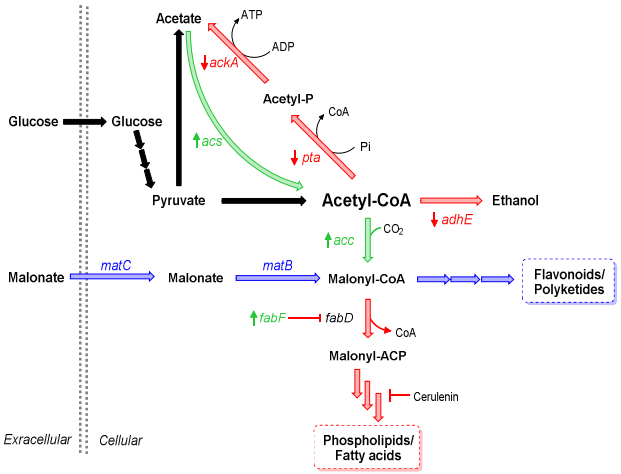

Figure 6. Engineered pathways for increased intracellular malonyl-CoA concentration as a precursor for flavonoid and polyketide biosynthesis. Two alternate strategies are shown: (1) In work by Zha *et al.* intracellular malonyl-CoA levels were increased by up- (green arrows) and down- (red arrows) regulating central metabolic enzymes in *E.coli* to funnel metabolism into malonyl-CoA production. (2) An alternative strategy used by Leonard *et al.* incorporated a malonate assimilation pathway (blue arrows) to allow direct synthesis of malonyl-CoA. The fatty acid synthase inhibitor cerulenin was used to downregulate the competing fatty acid pathway.

non-essential competing pathways and directing all metabolites only towards those pathways absolutely required for survival and natural product biosynthesis. Advanced genome engineering techniques have facilitated the creation of minimal-genome *E. coli*, displaying increased genome stability, improved transformation efficiency and importantly enhanced production of biological molecules corresponding to the pathway assembled. Remarkably, the streamlined *E. coli* strain with a genome reduction of 22% not only exhibits the enhanced characteristics above, but also has no growth deficiency, providing a promising novel approach to biochemical production.[58]

3.2. Protein Fusions and scaffolds

Although deleting competitive pathways and directing intermediates towards the desired route rather than an alternative one is advantageous, optimal efficiency of substrate usage can be facilitated by using protein fusions (Figure 7). A versatile technique which can be applied to numerous biosynthetic pathways; protein fusion combines the enzymes catalysing sequential reactions in a pathway. This provides several advantages. The substrate channeling allowed in such fusion proteins prevents loss to competing enzymes, decreases intermediate diffusion distance and loss to the environment, and protects labile intermediates from the solvent.[59] Creation of multi-enzymatic protein chimeras has been applied to elevate yields of several natural products. Yields of the sesquiterpene patchoulol were increased by fusing the sequential enzymes farnesyl pyrophosphate synthase (FPPS) and patchulol synthase (PTS) to maintain the required intermediates in close proximity and to reduce diffusion times.[60] Production of the diterpene miltiradiene, a Chinese medicine constituent, has also benefitted from fusion of consecutive pathway enzymes. When two fusion proteins, one of *Salvia mitiorhiza* kaurene synthase-like (SmKSL) and labdadienyl/copalyl diphosphate synthase (SmCPS) and one of FPP synthase and GGPP synthase, were expressed together an 8-fold increase in the yield of miltiradiene was observed (Figure 7A). This fusion of terpenoid synthases mimics the complex PKS mega-structure.[61] Application of protein fusion technology has also been demonstrated within the stilbene class of natural products, creating a 4-coumaroyl-CoA ligase (4CL) and stilbene synthase (STS) fusion to increase the synthesis of resveratrol. By channeling intermediates and ensuring the active sites are in close proximity, the efficiency of the 4CL:STS enzyme fusion increased by up to 15-fold in yeast.

Although protein fusions have demonstrated potential to optimise flux through metabolic pathways, they do present some limitations, dependent on the particular enzymes involved. Firstly, not all enzymes behave particularly well when fused to another enzyme in a chimera, for example, enzymes with catalytic functions residing in the termini are prone to misfolding when involved in a protein fusion. Secondly, the stoichiometry of the enzyme fusion is determined by the ability of the tethered proteins to functionally fold; inability to fold correctly when linked to multiple enzymes results in protein fusions limited to a 1:1 ratio. Consequently, this ratio may not be optimal for pathway flux, especially if the corresponding enzymes display different kinetics.[62] Therefore, novel approaches such as scaffolding are desirable to alleviate the enzyme requirements for substrate channeling (Figure 7B). The concept of engineering protein scaffolds involves creating synthetic complexes of pathway enzymes to balance metabolic flux and maximise efficiency in entirely heterologous systems.[63] Although assembly of a heterologous pathway circumvents endogenous regulatory mechanisms, flux imbalances are inevitable as the native regulatory mechanisms are not translated into the system.

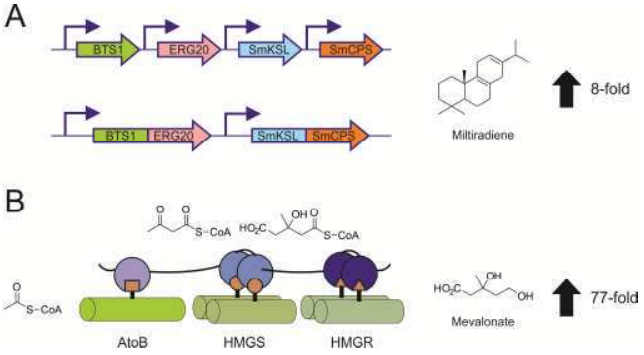

Figure 7. Substrate channelling using protein fusions and scaffolds. **A.** Creation of two fusion proteins, one of FPP synthase (ERG20) and GGPP synthase (BTS1), and one of kaurene synthase-like(SmKSL) and labdadienyl/copalyl diphosphate synthase (SmCPS) increased yields of the diterpene miltiradiene. **B.** The use of synthetic protein scaffolds can balance both substrate channelling and enzyme stoichiometry. Scaffolding of acetoacetyl-CoA thiolase (AtoB), hydroxy-methylglutaryl-CoA synthase (HMGS) and hydroxymethylglutaryl-CoA reductase (HMGR) in a 1:2:2 ratio led to a 77-fold increase in mevalonate production.

Therefore, engineering regulatory control of the introduced pathway needs to be considered to balance stoichiometry and minimise accumulation of metabolites that are toxic to the host.[64] Synthetic protein scaffolds spatially recruit pathway enzymes by engineering interactions between well-established protein-protein interaction domains of signalling proteins and their corresponding ligands. This balances flux through the metabolic pathway, increasing effective local concentrations of intermediates, and preventing intermediate loss and toxic accumulation. Furthermore, the number of interaction domains between the different enzymes can be modified to create novel scaffolds with varying numbers of enzymes. Inspired by natural modular machines, such as polyketide synthases and non-ribosomal peptide synthases, the scaffolding approach was applied to three enzymes of the mevalonate pathway, converting acetyl-CoA to mevalonate. Optimising the stoichiometry of the reaction using this method achieved a remarkable 77-fold enhancement in mevalonate, despite lower enzyme expression. Moreover, this strategy has proved applicable to other biosynthetic pathways to rapidly optimise and refine metabolic flux, recruiting specific numbers of enzymes into a functional complex.[63]

3.3. Cofactor regeneration and optimisation

An important feature of many natural product biosynthetic pathways is the use of redox cofactors such as NADH and NADPH by metabolic enzymes. Optimising cofactor availability, balancing redox potential and accelerating regeneration reduces the metabolic burden imposed on the system and increases flux through the metabolic pathway to generate higher yields of natural products.[51] These features can be established by engineering pathways to elevate concentrations of highly required cofactors,[65] or alternatively by modifying or exchanging key enzymes in the pathway to change their cofactor preference.[66]

Biosynthetic pathways dependent on NADPH cofactors require plentiful supply of nicotinamide and careful redox balance for maximal pathway efficiency.[67] The importance of NADPH availability is demonstrated in the biosynthesis of the flavonoid polyphenols, leucoanthocyanidins and (+)-cathechins, since their corresponding enzymes, dihydroflavonol 4-reductase (DFR) and leucoanthocyanidin reductase (LAR), are NADPH-dependent. After identifying the primary restriction factor for the high-level production of polyphenols as the availability of NADPH, a stoichiometric-based simulation method was designed where combinations of different genes were repressed to increase the intracellular concentration of NADPH. Appropriate combinations of glycolytic enzyme deletions were designed and constructed in *E. coli* to redirect the metabolic flux towards the NADPH producing pentose phosphate pathway, elevating the intracellular concentration of the essential redox cofactor. The optimal strain was a triple mutant deficient in three genes encoding phospholipase (*pld*A), phosphoenolpyruvate carboxylase (*ppc*), and phosphoglucose isomerase (*pgi*) to produce excellent yields of 817 mg/L leucocyanidin and 39 mg/L (+)-cathechin.[65a]

Exchanging key enzymes in central metabolism that consume essential cofactors for those that generate them, is another successful strategy to enhance intracellular NADPH and yields of natural products that require this specific redox factor during biosynthesis. Glycolytic metabolism has been engineered in *E. coli* to use an NADP-dependent enzyme instead of an NAD-dependent enzyme, generating NADPH during the oxidation of glyceraldehyde-3-phosphate to 1, 3-biphospho-glycerate as opposed to NAD. The efficacy of the engineered system was evaluated by also introducing lycopene and ε-caprolactone heterologous pathways, demonstrating higher productivity than parental strains.[65b] Furthermore, direct intra-conversion of NADH to NADPH through the expression of a transhydrolase has enhanced the productivity of an amorphadiene producing *E. coli* strain. The engineered strain, already transformed with the yeast MVA pathway enzymes, demonstrated a 120% increase in amorphadiene production with yields of 700 mg/L. This work highlights the importance of cofactor regeneration in maximising product yields, since the rate-limiting factor was NADPH availability due to presence of the high-flux MVA pathway.[66b] Alternative approaches aim to specifically modify central enzymes in the pathway by screening cofactor-dependent heterologous enzymes for optimal kinetic parameters,[66b] or by rational protein engineering to switch cofactor specificity, manipulate NAPDH metabolism and regenerate large amounts of the required cofactor.[66a]

3.4. Multivariate-modular pathway engineering

Metabolic engineering approaches have predominantly explored the optimisation of separate components of biosynthetic pathways. In the case of isoprenoids it seems rational to simplify the pathway into an upstream segment, providing the IPP and DMPP precursors, and a downstream segment, utilising these substrates to form specific isoprenoids. However, titres have reached a mere 10 mg/L using this limited approach, simply overexpressing upstream pathway enzymes and reconstructing downstream tailoring pathways to direct taxadiene synthesis in both *E. coli* and *S. cerevisiae*.[68] Although reasonable increases in metabolic flux are observed using this linear model, there are high-yield limitations such as toxic accumulation of intermediates and competitive pathways exhausting substrates. A novel combinatorial approach devised by Ajikumar et al., namely 'multivariate-modular pathway engineering' (Figure 8), organises the biosynthetic pathway into smaller modules, with the expression of each component fluctuated for optimal synthesis of taxadiene[6b], an intermediate in the biosynthetic pathway for the anti-cancer drug taxol.[69] In this example, the expression of two gene segments – an upstream MEP pathway and a downstream taxadiene synthesis pathway – were varied simultaneously to optimally balance the taxadiene synthesis pathway. In the upstream module, four rate-limiting enzymes were overexpressed to synthesise an abundant supply of the IPP and DMPP precursors. In the downstream module, the

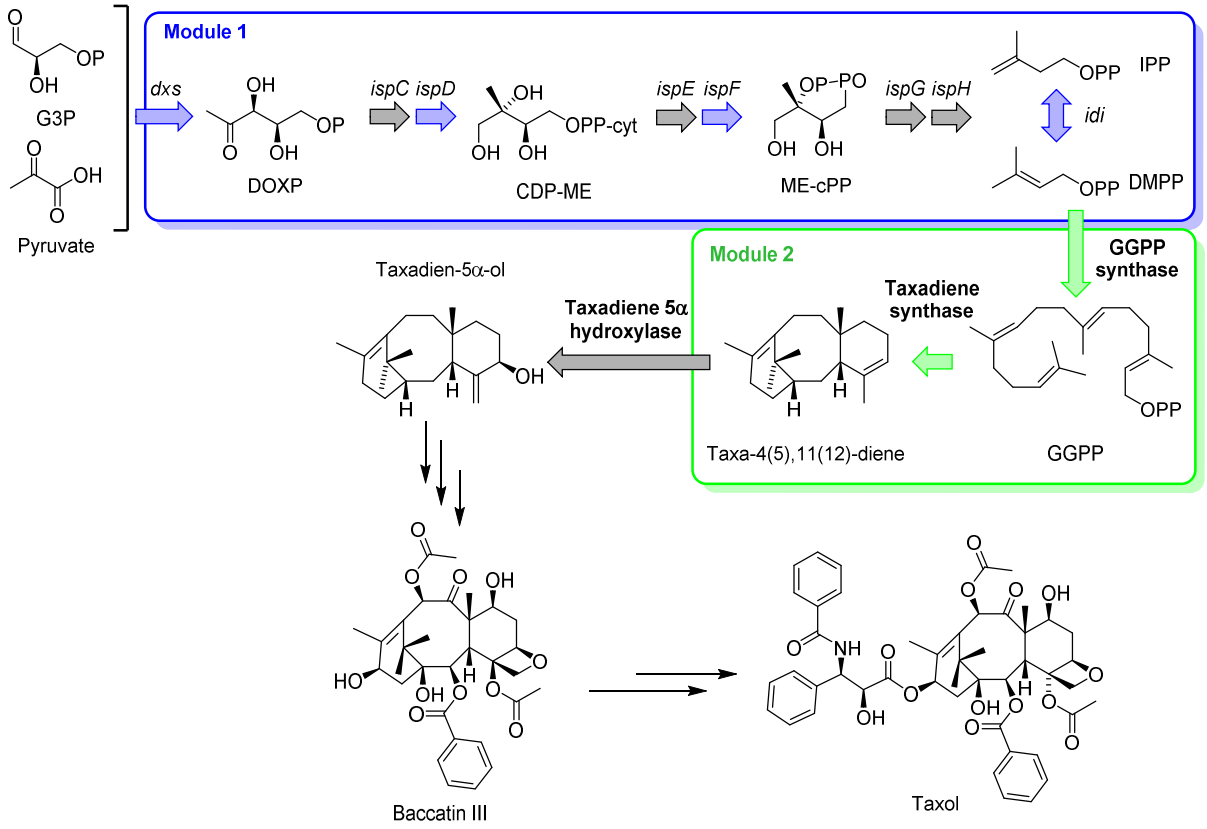

Figure 8. Multivariate-modular pathway engineering approach devised by Ajikumar *et al.* for the synthesis of taxa-4(5),11(12)-diene. The expression of two gene segments - an upstream MEP pathway and a downstream taxadiene synthesis pathway – were fluctuated for optimal taxadiene synthesis. In the upstream module, genes encoding the rate-limiting enzymes (dxs, ispD, ispF and idi) were overexpressed to synthesise an abundant supply of the IPP and DMPP precursors. In the downstream module, the heterologous enzymes GGPP synthase and taxadiene synthase were expressed to drive the pool of IPP and DMPP towards taxa-4(5), 11(12)-diene. The upstream and downstream modules were regulated by inducible promoters to allow fine tuning of enzyme expression. The complete synthesis of taxol from taxadiene involves acylation, benzoylation and oxidations in several enzymatic reactions.

heterologous enzymes GGPP synthase and taxadiene synthase were expressed to drive the pool of IPP and DMPP towards taxa-4(5),11(12)-diene. The upstream and downstream modules were regulated by inducible promoters to allow fine tuning of enzyme expression. Combinations of various promoters and gene-copy numbers were analysed to create 32 unique strains, representing a wide range of expression levels. Multivariate-modular pathway engineering has maximised taxadiene titres in *E. coli* to gram per litre volumes; an outstanding 15 000 fold improvement providing a platform for complete taxol production, by extracting the intermediate and subsequent further chemical modification.[6b]

3.5. Manipulating regulatory pathway elements

Reconstructing regulatory control to mimic natural metabolism is complemented by relieving endogenous control to optimise flux through the biosynthetic pathway. Engineers, however, must be careful to relieve control in such a way to enhance metabolism through the chosen pathway without disrupting central endogenous metabolism that could otherwise be detrimental to the host. An approaches which has been applied to sesquiterpene synthesis and also acts as a platform for the increased synthesis of other isoprenoids is relieving control mechanisms of the pyruvate dehydrogenase complex in *S. cerevisiae.* The pyruvate dehydrogenase complex creates a restriction on acetyl-CoA production, the precursor for isoprenoid IPP and DMPP substrates. Regulatory feedback control mechanisms were circumvented by introducing heterologous dehydrogenase and synthetase enzymes to by-pass the endogenous multi-enzyme complex. Conversion of pyruvate to acetyl-CoA was enhanced to obtain higher sesquiterpene yields and created a generalised system for production of other isoprenoids.[70] Attention has been drawn towards another essential regulatory step in the endogenous DOXP pathway mediated by HMG-CoA reductase (HMGR), controlling the levels of IPP and DMPP. A collection of variant HMGR enzymes were selected, expressed in *E. coli*, and their ability in amorphadiene synthesis assayed. Subsequently, highly performing enzymes were supplemented with their required redox cofactor, in this case NADH, to maximise amorphadiene production.[71]

Further examples of manipulating endogenous metabolism are demonstrated in phenylalanine synthesis. Phenylalanine and its hydroxylated derivative tyrosine are fundamental precursors for natural products such as flavonoids, alkaloids and non-ribosomal peptides. However, complex intrinsic feedback mechanisms prevent high-level accumulation of phenylalanine and tyrosine via the shikimate pathway.[72] Supplementation is therefore required in the media for their use in natural product biosynthetic pathways, making fermentation protocols expensive.[48] Consequently, research into developing an economical and efficient microbial system is desirable. Traditional methods have concentrated on eliminating the negative feedback mechanisms and facilitating carbon flux through the shikimate pathway.[73] Accumulation of phenylalanine is prevented by the feedback mechanisms of 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (AroG), chorismate mutase-prephenate dehydrogenase (TyrA), and transcriptional attenuation of the tyrosine repressor (TyrR).[72] Limited success has been observed by expressing feed-back inhibition resistant homologs of AroG and TyrA enzymes in combination with TyrR deletion, yielding tyrosine concentrations of 3.8 g/L. Additional engineering in this *E. coli* strain to enhance erythrose-4-phosphate (E4P) and phosphoenolpyruvate (PEP) substrates of the shikimate pathway, expressing *pps*A and *tkt*A genes, has only elevated titres to 9.2 g/L.[73a] However, recent advancements in tyrosine production have been described, alleviating shikimate pathway feedback inhibition to engineer an *E. coli* strain capable of accumulating significantly higher titres of 401 mg/L tyrosine. This approach effectively creates a phenylalanine sink, continuously draining phenylalanine from the system for synthesis of tyrosine, mediated by the enzyme phenylalanine 4-hydroxylase (PH4). Assuming that phenylalanine is constantly depleted, the negative feedback inhibition mechanisms of AroG, TyrA and TyrR are not repressed, relieving the shikimate pathway from inhibition and resulting in constitutive tyrosine accumulation.[74]

Outstanding yields of tyrosine have been observed in an *E. coli* strain using modular engineering techniques of multiple genes, maximising production to 80% of the potential yield. Proteomics and metabolomics aided the design of a modular system composed of two plasmids synthesising tyrosine from E4P and PEP. Three major bottlenecks in the shikimate pathway were identified and alleviated by optimising gene codons, plasmid copy numbers and promoter activity, and assembling synthetic operons, including homologs of bottleneck enzymes. Remarkable tyrosine titres of more than 2 g/L were reported, demonstrating once again the success of modular pathway engineering techniques to optimise natural product titres.[75]

4. Pathway optimisation by protein engineering

Despite progress in metabolic engineering strategies and synthetic biology in recent years, optimisation of engineered pathways to reach industrially relevant yields of product molecules is not trivial. As discussed above, modulating the expression levels of individual pathway enzymes through various approaches is a proven strategy for increasing synthetic pathway efficiency and product titre. However, these strategies fail to address inefficiencies associated with the individual pathway enzymes themselves. These inherent enzyme characteristics can result in bottlenecks, generate toxic/undesirable by-products, and ultimately reduce titres. It may, therefore, be desirable to engineer the properties of pathway enzymes to increase activity, stability, and modify substrate/product specificity. Furthermore, control of substrate/product inhibition and protein localisation can be engineered into individual enzymes to optimise overall pathway flux. Advances in protein engineering have resulted in notable improvements in the catalytic efficiency of existing enzymes for natural or novel substrates, and have even been applied to the generation of completely novel enzymes. Protein engineering, in combination with metabolic engineering approaches, is a powerful tool for the optimisation of synthetic pathways for secondary metabolite production.

4.1. Optimising catalytic efficiency

Several engineering methods have been developed that target transcription and translation to balance protein expression and, consequently, pathway flux. However, in systems with inherently inefficient enzymes, a concomitant increase in energy and resource expenditure can be a significant metabolic burden on the host cell, stifling optimal yields. One approach to increase the *in vivo* activities of enzymes in synthetic pathways is to increase specific activities under the required production conditions. In one example, the geranylgeranyl diphosphate (GGPP) synthase enzyme from the hyperthermophile *Archaeoglobus fulgidus* was engineered for optimal activity at ambient temperatures by directed evolution. Selected variants were identified that increased production of the astaxanthin precursor lycopene by 100%[76]. Additionally, production of levopimaradiene, the diterpenoid precursor to the pharmaceutically important ginkgolides, was improved markedly by site saturation mutagenesis of synthase enzymes. This resulted in increased levopimaradiene yield by 2600-fold using engineered GGPP synthase and levopimaradiene synthase in an optimised system.[77]

In some cases the *in vivo* activity of enzymes can also be improved by increasing the stability and thus proportion of functional protein expression, by avoiding the accumulation of inactive insoluble aggregates when over-expressed in heterologous host systems. This strategy was effectively applied to improve the *in vivo* properties of the *Abies grandis* γ-humulene synthase (HUM) when expressed in *E.coli.* An adaptive evolution model was used to analyse the sequences of over 30,000 homologs to over 200 *E.coli* enzymes involved in central metabolism and the design principles were then used to redesign the *in vivo* properties of humulene synthase to relieve pathway bottlenecks while leaving product distribution unaltered.[78]

4.2. Expanding substrate scope

Unlike their counterparts in central metabolic pathways, many enzymes utilised in secondary metabolism display broad substrate and product specificities. For example, many isoprenoid biosynthetic enzymes are known to accept a variety of both natural and unnatural substrates. Several examples exist that can synthesise a large range of compounds (>50 in some cases) from a single substrate. Control of specificity is generally through a small number of highly stringent enzymes upstream in the pathway that dictate backbone structures.

The tolerant nature of the terpene synthase active site presents engineering opportunities, modifying the enzyme structure to either synthesise different products or provide a preference for a desired product (Figure 9). For example, the various terpenoid products of the 5-epi-aristolochene synthase can be changed by mutating the less conserved residues present in the active site of the enzyme.[79] Moreover, preference for specific terpenoid products has been artificially engineered in the promiscuous γ-humulene synthase, biasing the reaction towards a selection of the 50 possible sesquiterpene products.[80] The proportion of terpenoid products can also be adjusted by mutating the metal ion cofactor binding residues in terpene synthases; as has been demonstrated for the trichodiene synthase from *Fusarium sporotrichioides*.[81] Rational engineering of terpene synthases represents an interesting approach to bias the formation of desired terpenes from single substrate compounds.

Several examples of engineered enzymes for the synthesis of carotenoids, natural pigments with interesting biological properties, have been reported. In the C_{40} carotenoid pathway, phytoene desaturase (CrtI) from *Erwinia uredovora* and lycopene cyclase (CrtY) from *Erwinia herbicola* were evolved by DNA shuffling to produce C_{40} carotenoids of higher and lower levels of saturation, respectively. The discovery of specific CrtI and CrtY mutants led to a novel biosynthetic route for production of the cyclic carotenoid torulene in *E.coli*.[82] In other work, the C_{30} carotene synthase, CrtM, from *Staphylococcus aureus* was engineered using a combination of directed evolution and site-directed mutagenesis to synthesise novel longer chain carotenoid backbones than previously found in nature.[83]

Cytochrome P450 monooxygenases (CYPs or P450s) catalyse a range of transformations including hydroxylation, epoxidation, dealkylation at unactivated carbons in a regioselective and

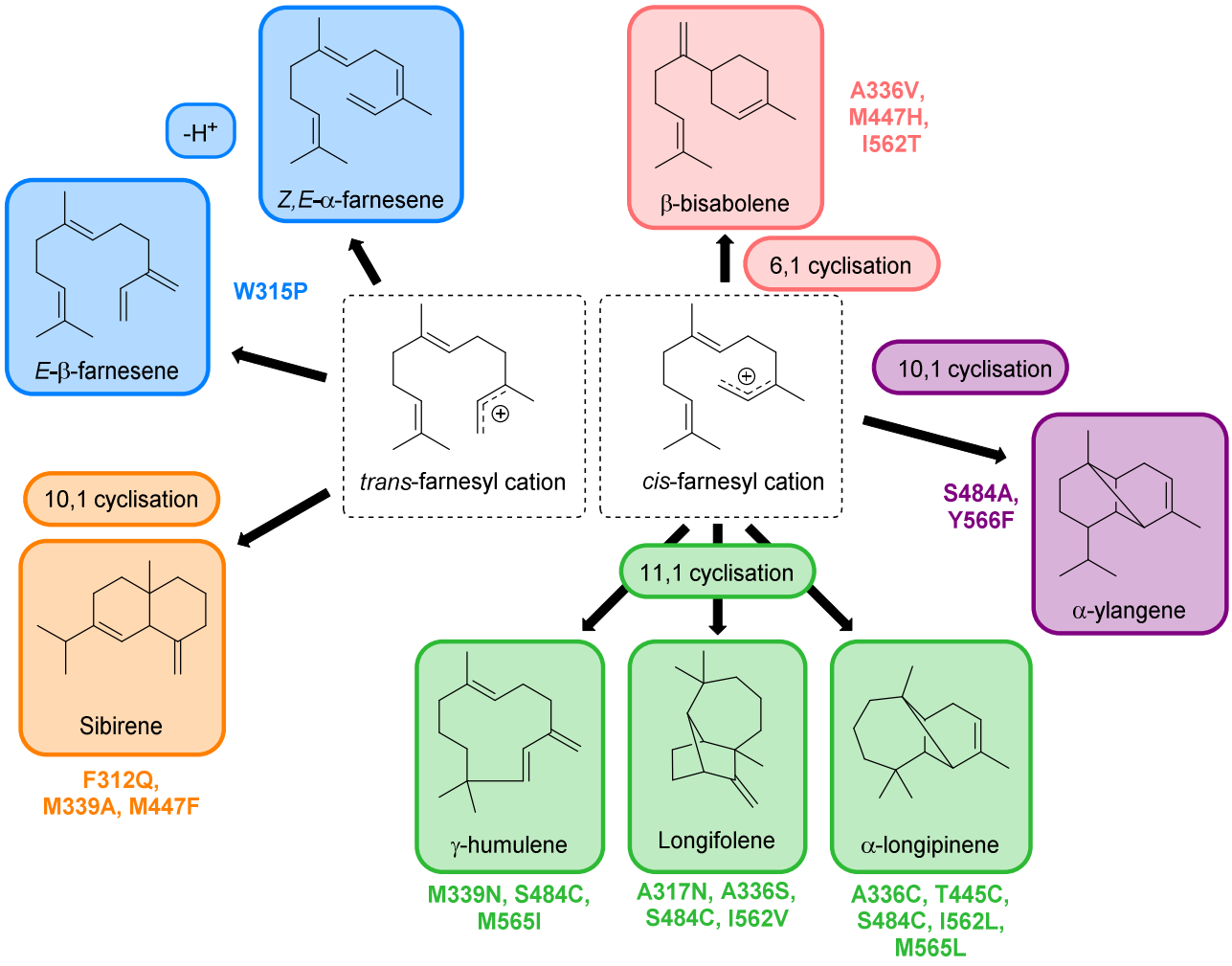

Figure 9. Engineering of the promiscuous γ-humulene synthase to show a preference for different cyclisation products. The substrate, farnesyl diphosphate, binds to the enzyme active site via a metal ion cofactor followed by cleavage of the diphosphate to yield either the *trans*- or *cis*- farnesyl cation. From here, a range of possible cyclisation or deprotonation reactions can occur to generate the final product(s). Yoshikuni *et al.* identified a range of mutations (shown) that alter product distributions to give enzyme variants that show a clear preference for each of the compounds shown.

stereoselective manner and are involved in a range of biosynthetic routes to useful natural products, generating a great deal of interest in their use and engineering for use in novel synthetic pathways. The most prominent examples of the use of P450s in metabolic engineering are the use of CYP71AV1 from *A. annua,* to catalyse three successive oxidation steps in the conversion of amorpha-4,11-diene to artemisinic acid;[35] and the use of a P450 enzyme from the *Taxus* species to catalyse the regioselective hydroxylation of taxadiene to taxadien-5a-ol in the synthesis of paclitaxel.[6b] Protein engineering has been extensively applied to the cytochrome P450 class of enzymes, making an extensive discussion on the subject beyond the scope of this review (see [84] and references therein). The apparent evolvability of P450 enzymes, demonstrated by the engineering of P450 BM3 from *Bacillusmegaterium* to catalyse a range of activities on an impressive range of substrates. Despite numerous successes in P450 engineering, examples of uses of engineered enzymes in metabolic pathways remains relatively rare, likely due to the additional challenges associated with optimal performance *in vivo*.

4.3. Bypassing feedback inhibition

As an alternative to engineering the kinetic parameters of pathway enzymes, flux through a pathway can be increased via the elimination of feedback regulation by substrates or products. For example, Liao and co-workers have engineered a novel pathway for the production of a range of long-chain alcohols – potential biofuel candidates – from keto-acid intermediates generated by native amino-acid biosynthesis.[85] Due to the highly regulated nature of amino-acid metabolism, a key hurdle to overcome to boost product titres was the relief of feedback inhibition in these pathways. Introduction of a previously identified feedback resistant mutant of the aspartate kinase/homoserine dehydrogenase enzyme (ThrA), which catalyses the first two steps in the conversion of aspartate to threonine, resulted in a nearly 4-fold higher titre of 1-propanol and 1-butanol.[86] In further work on the production of 3-methyl-1-butanol, incorporation of a feedback resistant mutant of the 2-isopropylmalate synthase (IMPS) resulted in an 8-fold increase in accumulation of intermediate 2-ketoisocaproate and enhanced production of the target product.[87]

Feedback inhibition can be also be eliminated by removing entire regulatory domains of bottleneck enzymes, as demonstrated for the optimisation of L-phenylalanine production in *E.coli*. In this pathway the key pathway enzyme chorismate mutase-prephenate dehydratase (CM-PDT) is allosterically inhibited by binding of the product L-phenylalanine. The *E.coli* CM-PDT enzyme contains three distinct domains; CM (residues 1–109), PDT (residues 101–285), and the regulatory-domain (residues 286–386). Therefore, to overcome the problem of feedback inhibition a truncated mutant CM-PDT lacking the entire regulatory domain was created. After further optimisation of the CM-PDT truncation mutant by directed evolution, a L-phenylalanine yield of 0.21 g/g glucose could be achieved – 38% of the theoretical maximum yield.[88]

4.4 De novo enzyme generation

In recent years computational design has become a powerful tool for the generation of tailor-made enzymes that catalyse unnatural reactions, exemplified by designed enzymes with retro-aldol, Kemp elimination, and Diels-Alderase activities.[89] These *in silico* designed enzymes are capable of impressive rate enhancements of up to 2×10^5 over the uncatalysed reaction.[89b] Moreover, directed evolution studies have indicated that the evolvability of enzymes generated using computational design methods appears to be high, with further rate enhancements of over 2000-fold appearing typical.[90] Not only does this strategy hold a great deal of promise for the rational design of *de novo* enzymes to perform novel chemical transformations, but also opens the door for the engineering of entire pathways not found in nature. In an exciting demonstration of the potential of this combinatorial approach, an enzyme, formolase, was designed to catalyse the carboligation of three one-carbon formaldehyde molecules into one three-carbon dihydroxyacetone molecule. Combining the designed enzyme with those from natural organisms allowed the construction of a novel carbon fixation pathway, designated the formolase pathway, converting formate into the central metabolite dihydroxyacetone phosphate *in vitro*.[91]

5. Summary and Outlook

The engineering of microorganisms into whole-cell factories for the synthesis of useful secondary metabolites is of great interest to chemists and biologists alike. Recent advances in the field have demonstrated the power of metabolic engineering and synthetic biology as tools towards acheiving these goals. Moreover, the complementary fields of metabolic engineering and protein engineering present the imminent prospect of expanding the range of metabolites beyond the repertoire of Nature. As an emerging discipline, one may expect that many challenges remain before widespread use of engineered microorganisms for synthesis of complex molecules. Progess is likely to be facilitated by developments in host organism optimisation, exmplified by the efforts towards standardised hosts with minimal genomes and well characterised properties. Furthermore, advances in efforts to standardise pathway components and assembly methods via synthetic biology should speed up novel pathway development significantly. Finally, development of protein engineering strategies in the context of metabolic pathways *in vivo* should augment the progess towards development of high-yielding pathways and novel natural products.

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Keywords:• Biosynthesis•Synthetic Biology•Natural Products• Pathway engineering

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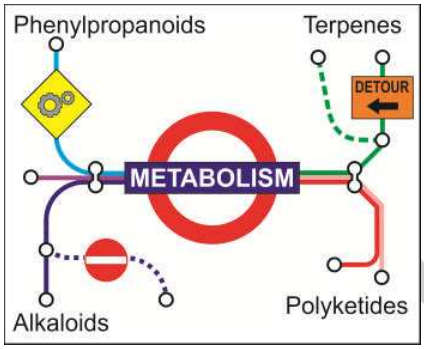
Entry for the Table of Contents

REVIEW

On the right track: Biocatalysis is an attractive alternative to traditional organic chemistry for the synthesis of natural products. In this review, we highlight recent developments in metabolic pathway engineering and synthetic biology towards the use of whole-cell microbial factories for the synthesis of these useful compounds.



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