TIMELINE

Metabolic engineering for drug discovery and development

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Metabolic engineering has been defined as the redirection of metabolic pathways using genetic manipulation. Since the emergence of metabolic engineering science in the early 1980s, the field has made notable strides not only at a conceptual level, but also with regard to translating these concepts into practical products and processes. Today, metabolic engineering plays an important role in the generation of fuels from renewable resources, the conversion of agricultural raw materials (for example, corn syrup) into bulk and specialty chemicals, and the discovery, development and scale-up of therapeutically useful products. This article focuses on recent advances in the last category. Specifically, we review the impact that converging developments in genetic engineering and biosynthetic chemistry are having on natural-product drug discovery.

It has been estimated that 5,000–10,000 compounds must be introduced into the drug discovery pipeline for every successful candidate molecule that makes it to market. On average, it takes 10–15 years to develop a successful drug. Given the low success rates, long timescales and consequent high costs associated with developing a drug, it is important for any drug company to introduce as many drug candidates into their pipeline as possible.

An important source for drug leads has been natural products. As many as 60% of successful drugs are of natural origin[1]. Some of the most potent anticancer, antibacterial and antifungal drugs are natural products (FIG. 1). However, most of these natural products have evolved for purposes other than the treatment of human disease. So even though these natural products function as human therapeutics, their pharmacokinetic and pharmacodynamic properties might not be optimal. Furthermore, many of these drugs are produced in small amounts in their native hosts, making the drugs expensive to harvest.

Organic chemistry methodologies are widely used to synthesize many pharmaceuticals (whether of natural origin or not) and to functionalize many of the pharmaceutically relevant natural products in use today. With considerable effort, chiral centres and functionalities can be introduced into molecules with precision. The advent of combinatorial chemical synthesis enables one to construct entire families of molecules substituted at several positions with several different substituents, allowing drug companies to rapidly sample variations of promising leads. More recently, enzymes have been used for *in vitro* combinatorial functionalization of complex molecules. The next logical step in the synthesis of chemotherapeutics is the use of enzymes for combinatorial synthesis inside the cell. This would allow one to produce drug candidates from inexpensive starting materials and avoid purification of the enzymes, which might be necessary for *in vitro* synthesis.

The production of a new or existing drug in a heterologous host generally involves the introduction of several genes in a biosynthetic cascade. In principle, metabolic engineering is much more than just introducing several genes into the cell — it often involves carefully balancing the genes in the new metabolic pathway so that no gene is drastically overexpressed, robbing the cells of precursors for growth and product biosynthesis, or so that no gene is drastically underexpressed, creating a bottleneck in the heterologous metabolic pathways and limiting product synthesis. Metabolic engineering also involves diverting resources from central metabolic pathways to the biosynthetic pathway for the desired molecule. As such, the control of gene expression — particularly the control of multiple genes simultaneously, and metabolic flux balances, both within the heterologous metabolic pathway and between the pathway and the host's native metabolism — is a core issue in the production of drugs using metabolic engineering.

During the past decade, metabolic engineering has started to have an effect on natural-product drug discovery in fundamentally new and practically useful ways (TIMELINE). For example, the recognition that secondary metabolite biosynthetic pathways in bacteria and fungi are intimately linked at the genetic level greatly simplifies the cloning and sequence analysis of biosynthetic, regulatory and self-resistance genes associated with a target natural product. The ability to produce these compounds in reagent quantities in biologically friendly heterologous hosts, such as *Streptomyces coelicolor*, *Escherichia coli* and *Saccharomyces cerevisiae*, simplifies protein engineering and metabolic engineering programmes. And the highly conserved mechanisms responsible for synthesizing individual members in any given class of natural products allows the emergence of generally applicable insights and technologies for biosynthetic manipulation. Last, the diversion of significant cellular resources into these biosynthetic pathways, and the efficient expression of the heterologous pathways within the cell, will eventually result in less expensive drugs, particularly for the treatment of diseases in developing countries where the cost of most drugs prohibits their widespread use. Below we

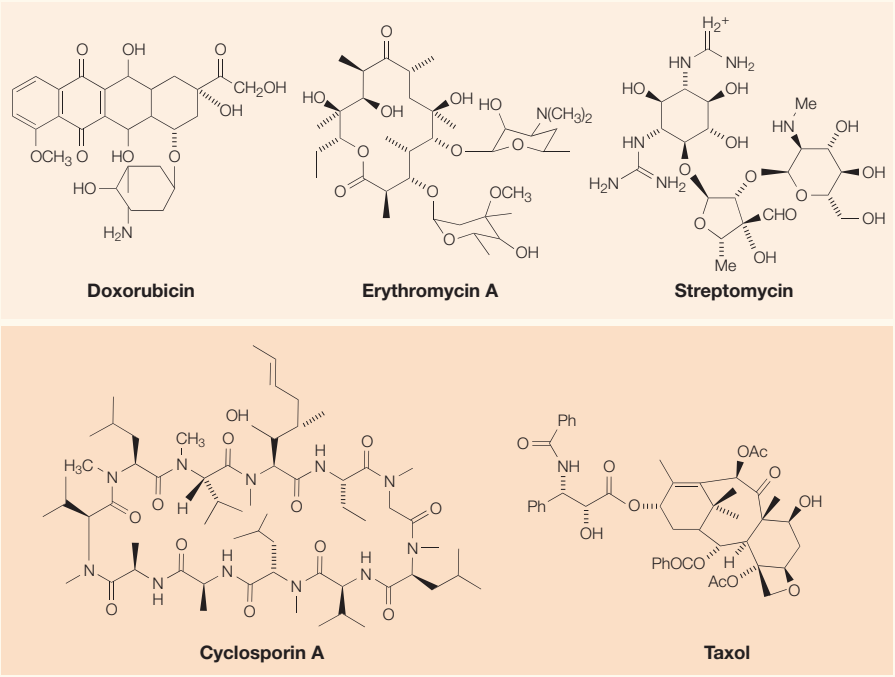


Figure 1 | **Examples of natural product drugs.** Doxorubicin (an anticancer agent) and erythromycin (an antibacterial) are glycosylated polyketides. Streptomycin (an antibacterial) is an aminocyclitol derived from unusual sugar moieties. Cyclosporin (an immunosuppressant) is a non-ribosomal peptide. Taxol (an anticancer agent) is a terpene.

examine selected classes of natural products for which such an effect is beginning to be felt, including isoprenoids, polyketides, non-ribosomal peptides and carbohydrates.

Isoprenoids

Isoprenoids are among the most diverse groups of compounds synthesized by biological systems; it has been estimated that there are approximately 50,000 known isoprenoids, which include the terpenoids and carotenoids[2]. Isoprenoids are important in maintaining membrane fluidity, electron transport, protein prenylation, cellular and organismal development, as fragrances and essential oils, and as antibacterial and antifungal agents[3]. Terpenes are a class of isoprenoids classified by the number of isoprene (C_5) units used to make them. Monoterpenes (C_{10}), such as menthol and camphor, and sesquiterpenes (C_{15}), such as zingiberene (ginger), are the principal constituents of herbs and spices. Other sesquiterpenes and diterpenes (C_{20}) are pheromones, defensive agents and signal-transduction agents[4,5]. Higher-molecular-mass isoprenoids stabilize membranes (cholesterol and other C_{30} compounds) and serve as photoreceptive agents (carotenoids and other C_{40} compounds).

There has been significant interest in terpenoids as antifungal, antibacterial and anticancer agents for the treatment of human disease. Indeed, worldwide sales of terpene-based pharmaceuticals in 2002 were approximately US $12 billion[6]. Several sesquiterpenes have found application as antiparasitic and anticancer agents[7–9]. For example, artemisinin, a sesquiterpene extracted from sweet wormwood (*Artimisia annua*), is one of the few antimalarial drugs to which there is no known resistance and, as such, has been hailed as a miracle drug. Unfortunately, its current cost is approximately 100 times that of chloroquine, sulfadoxine and pyrimethamine, the most widely used antimalarial drugs. Eleutherobin, a potential anticancer compound with taxol-like modes of action, was first isolated from a soft coral (*Eleutherobia sp. Alcyonacea Alcyoniidae*) discovered in the Indian Ocean near Bennett's Shoal in Western Australia in 1995 (REF. 10). Because the total chemical synthesis of eleutherobin[11,12] is costly, supply limitations still hamper efforts to bring it to the clinic.

Similarly, several diterpene and monoterpene derivatives are in use or are being investigated as anticancer agents. Taxol (FIG. 1), a diterpene extracted from the Pacific Yew (*Taxus brevifolia*), is effective in the treatment of certain cancers (ovarian, breast, lung and neck, bladder and cervix, melanoma and Kaposi's sarcoma)[13,14]. Unfortunately, the complexity of the molecule precludes commercial total chemical synthesis and its rarity makes it extremely expensive[14]. The monoterpene limonene and related derivatives are believed to inhibit farnesylation of the growth-promoting protein RAS, and therefore inhibit malignant cell proliferation[15–17].

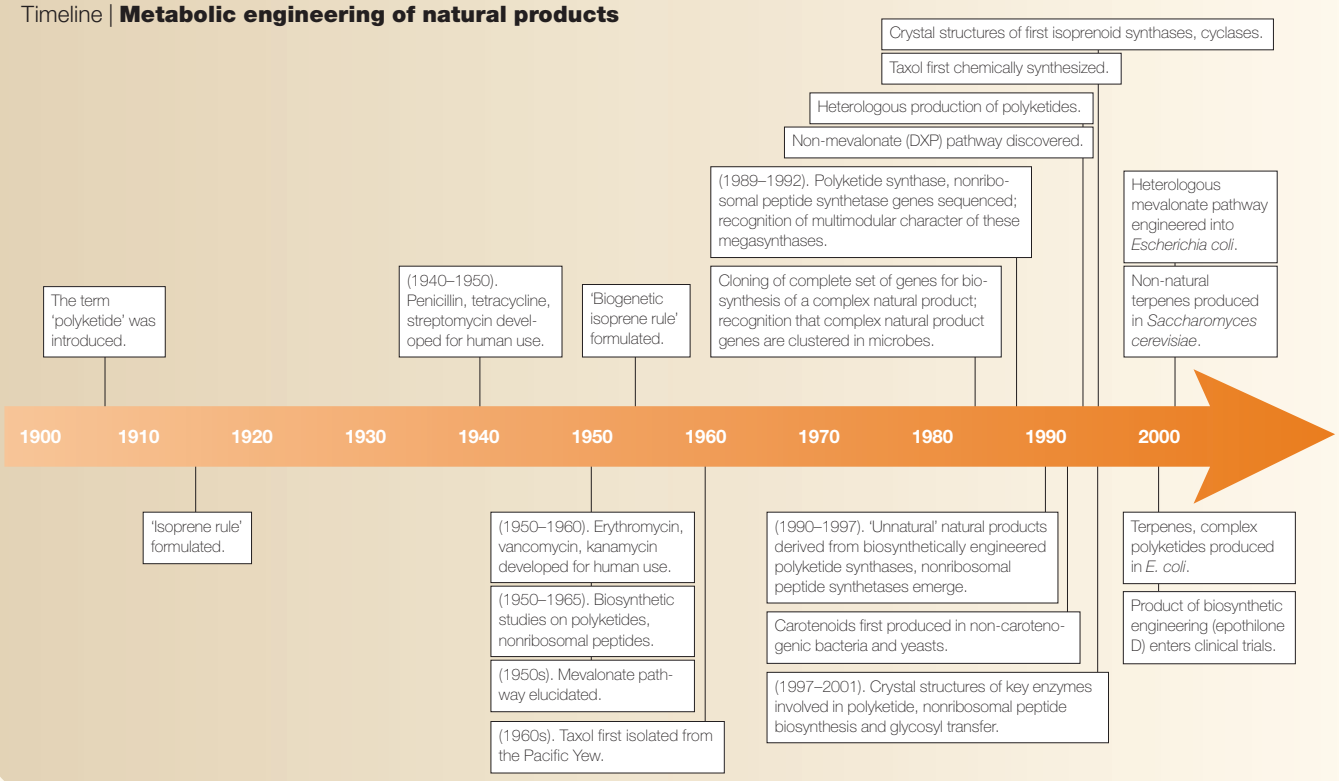
The ability to produce current drugs in bacteria could significantly reduce their production costs and increase their chances of reaching clinical trials and the market, not to mention making them affordable in the developing world. The ability to produce variants of the terpene backbone and to functionalize them with various substituents using enzymes would give rise to large libraries of compounds that could be screened for the treatment of human disease or that could be further functionalized using traditional organic chemistry.

Biosynthesis of terpene olefins. The primary building block (C_5 unit) for the synthesis of isoprenoids is isopentenyl pyrophosphate (IPP). IPP is synthesized via two different pathways: the mevalonate pathway and the non-mevalonate or 1-deoxyxylulose-5-phosphate (DXP) pathway (FIG. 2). The mevalonate pathway is found primarily in eukaryotes and archae (but has been found in a few prokaryotes), whereas the non-mevalonate pathway is found primarily in prokaryotes and in plastids of photosynthetic eukaryotes[18,19]. Dimethyl allyl pyrophosphate (DMAPP), an isomer of IPP, acts as a primer for the sequential additions of IPP by the isoprenyl pyrophosphate synthases to form C_{10} geranyl pyrophosphate (GPP), C_{15} farnesyl pyrophosphate (FPP), C_{20} geranylgeranyl pyrophosphate (GGPP), and larger isoprenyl pyrophosphates (FIG. 2).

Terpene olefins are synthesized from the linear terpene pyrophosphate esters GPP, FPP and GGPP. Cyclization of GPP by terpene cyclases (or synthases) forms the monoterpenes, cyclization of FPP forms the sesquiterpenes, and cyclization of GGPP forms the diterpenes. Although some of the terpene cyclases form one or a few products, there are some cyclases that produce a variety of products from a single substrate type; for example, the δ-selinene and γ-humulene synthases of the grand fir (*Abies grandis*) produce 34 and 52 total sesquiterpenes, respectively[20]. The incredible plasticity of these enzymes indicates their potential for generating novel terpene olefins that might be more effective than natural terpenes in treating human disease.

Terpene functionalization. Most terpenes of medical importance are functionalized in at least one position on the hydrocarbon backbone. Functionalization, which includes glycosylation, acetylation, hydroxylation and

Timeline | **Metabolic engineering of natural products**



benzoylation of the terpene backbone, further expands the diversity of possible terpenes. Relatively few of the enzymes responsible for terpene functionalization have been purified and/or characterized, and only a fraction of the genes encoding these enzymes have been cloned.

The monoterpenes menthol and carvone (from *Mentha* x *piperita* and *M. spicata*, respectively) are both derived from limonene but differ in the position of oxygenation. The cytochrome P450 hydroxylases (limonene-3-hydroxylase and limonene-6-hydroxylase) that form these two products from limonene have been cloned and expressed in *E. coli* and *S. cerevisiae*[21–24]. As is the case for most terpene hydroxylases, coexpression of a NADPH-cytochrome P450 reductase gene in *S. cerevisiae*, or addition of the purified NADPH-cytochrome P450 reductase to *E. coli* lysate, is required to reconstitute enzyme activity *in vitro*. Recently, it was shown that a single amino-acid substitution could convert limonene-6-hydroxylase to limonene-3-hydroxylase[23], indicating the potential of protein engineering to produce novel enzymes for hydroxylating novel terpenes or existing terpenes in novel locations.

Of the functionalized diterpenes, taxol, a highly substituted molecule containing multiple acetyl and benzoyl esters, has the best-known biosynthetic pathway. Two taxadiene hydroxylating cytochrome P450s (taxadiene-5α-hydroxylase and taxane-10β-hydroxylase) have been purified and characterized; the gene encoding taxane-10β-hydroxylase has been cloned and functionally expressed in *S. cerevisiae*[25–27]. A third P450, 13α-hydroxylase, has been expressed using a baculovirus-based system and purified[28]. In addition, two acyltransferases and a benzoyltransferase that act on taxanes have been isolated and their corresponding genes have been cloned[27,29–32]. All these genes were isolated from complementary DNA libraries derived from plant cells producing taxol. This identification was followed by laborious and time-consuming *in vitro* work spanning a decade. Elucidation of the final steps in the biosynthetic pathway might one day enable production of taxol or derivatives in microorganisms or easily cultivable plants.

Metabolic engineering of terpene production. There have been a number of reports of metabolic engineering of carotenoid production in *E. coli*, *S. cerevisiae* and other organisms[33–44]. However, there have been few reports describing the production of terpenes *in vivo*, in part due to the poor expression of terpene cyclase genes in *E. coli* and *S. cerevisiae*, the slow turnover of terpene cyclases and insufficient supplies of prenyl pyrophosphate precursors[45–47]. Recently, high-level *in vivo* production of terpenes in *E. coli* was demonstrated[47]. Problems with the expression of the terpene cyclase gene were overcome by synthesis of genes optimized for heterologous expression, and the addition of a completely heterologous pathway greatly improved production of prenyl pyrophosphate precursors supplied to the cyclase. Interestingly, it was found that IPP, DMAPP and/or FPP accumulated in the cell and were toxic when a terpene cyclase was not present, pointing to the importance of balancing precursor availability and the expression of genes in the heterologous biosynthetic pathway. Similar efforts to improve terpene production in *S. cerevisiae* have been hampered, in part, by an active phosphatase that dephosphorylates prenyl pyrophosphates before they can be captured and cyclized by the terpene cyclases.

The eventual incorporation of P450s and decorating enzymes (glycosyl transferases, acetylases and so on) will enable the large-scale production of inexpensive terpene drugs in microbial fermentations. Not surprisingly, there will be several challenges in decorating terpenes in a heterologous host: the choice of redox partner, the ability to functionally express redox partners in a heterologous host and the supply of haem, to name a few. Beyond hydroxylation of the terpene olefin, the functionalizing group will need to be supplied either in the growth medium, in which case it will need to be transported into

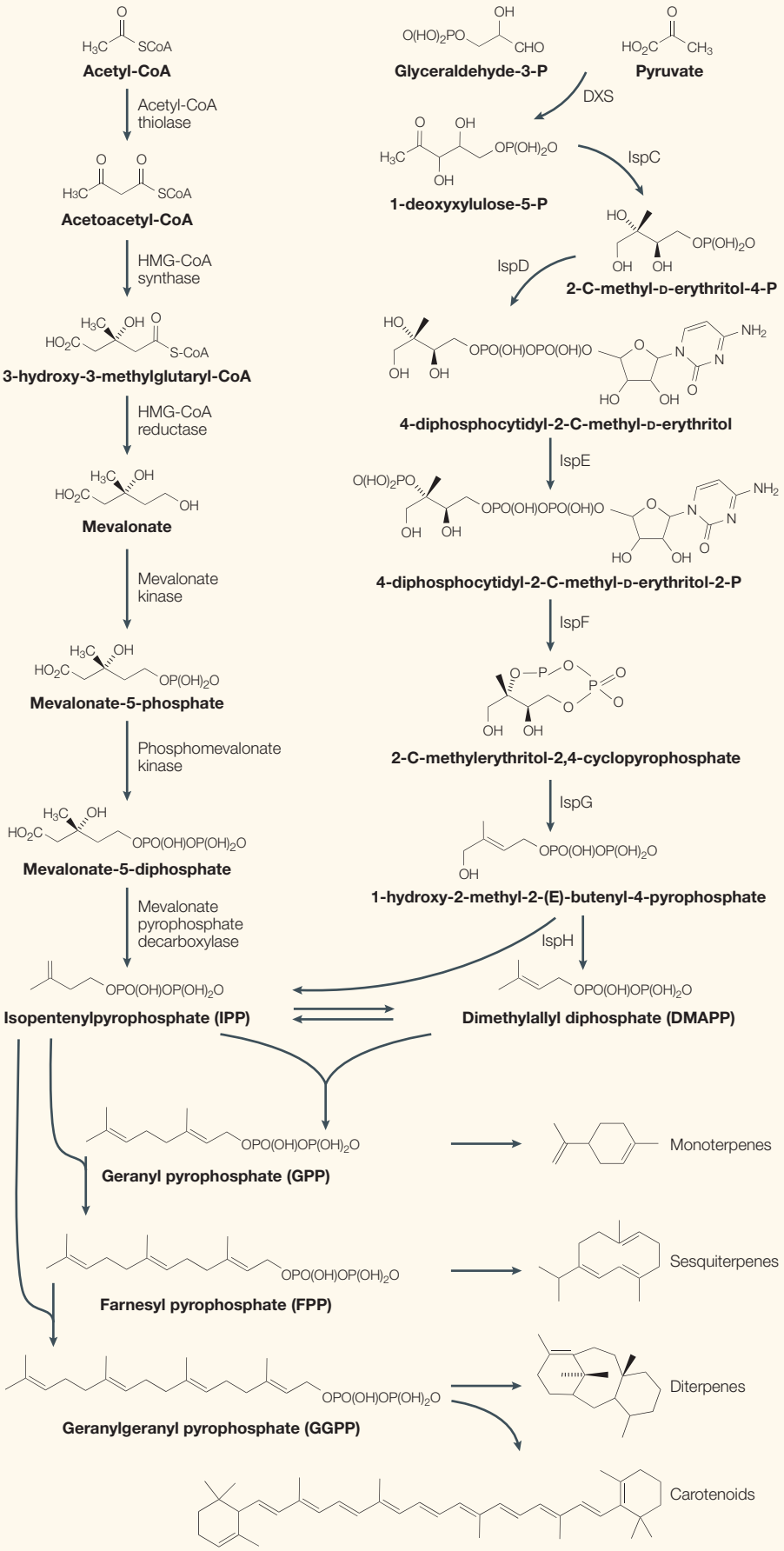


Figure 2 | **Isoprenoid biosynthesis.** Right, DXP (non-mevalonate) pathway. Left, mevalonate pathway. DXS, DXP synthase; IspC, DXP reductoisomerase; IspD, 2-C-methylerythritol-4-phosphate cytidyltransferase; IspE, 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase; IspF, 2-C-methylerythritol-2,4-cyclodiphosphate synthase; IspG, 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase; IspH, 4-hydroxy-3-methylbut-2-enyl diphosphate reductase.

the cell, or synthesized inside the cell, which will necessitate the incorporation of yet more heterologous pathways. The ability to carry out these complicated reactions in a heterologous host will open up an entire field of molecules to production in heterologous hosts. Laboratory evolution of terpene cyclases, terpene hydroxylases and a host of other terpene-functionalizing enzymes, and the combinatorial expression of these evolved enzymes inside a heterologous host, will enable the production of variants that might be more potent against human disease than the natural products that evolved for a purpose other than treating human disease.

Polyketides

Polyketides (FIG. 1) are a large family of natural products built from acyl-CoA monomers. These metabolites include many important pharmaceuticals, veterinary agents and agrochemicals. The enormous structural diversity and complexity of these biomolecules is impressive. Although the actual biological roles of each of these metabolites in the native microorganisms in which they are produced are unclear, an extraordinary variety of pharmacological properties have been associated with naturally occurring polyketides. Widely used polyketides include antibacterials (erythromycin, tetracycline and rifamycin), antifungals (amphotericin), anticancer agents (doxorubicin), immunosuppressants (FK506 and rapamycin), cholesterol-lowering agents (lovastatin and compactin), animal-health products (avermectin, tylosin and monensin) and agrochemicals (spinosyn). Newer polyketides, such as the enediyne calicheamicin, the macrolide epothilone and the marine natural product discodermolide, have also started to attract clinical interest.

Polyketides are biosynthesized by large multi-enzyme systems called polyketide synthases (PKSs; FIG. 3). These large synthases, which are modular in architecture and function, catalyse the step-wise elongation of a polyketide chain, as well as associated functional-group modifications[48]. At each step in the chain-elongation process, an acyl group monomer is recruited from the available

metabolic pool of acyl-CoA precursors. Typical precursors include metabolites such as acetyl-CoA, propionyl-CoA and other alkyl-CoAs, which are used as chain initiators, and malonyl-CoA and methylmalonyl-CoA, which are used for the elongation process. The chain length of the polyketide product is controlled by the number of repeated acyl chain-extension steps. Throughout its biosynthesis, the growing polyketide chain is covalently tethered to the protein assembly.

Since the identification of the actinorhodin gene cluster in *S. coelicolor*[49], hundreds of polyketide biosynthetic pathways (including pathways for natural products not yet physically characterized) have been genetically characterized. Robust technologies have been developed for cloning targeted gene clusters[50], and the explosive growth of DNA sequence data from diverse microbial genomes (see Further information)[51] has led to the realization that polyketide biosynthetic genes are widespread. For example, ~1% of the genomes of *S. coelicolor*[52], *Bacillus subtilis*[53] and *Mycobacterium tuberculosis*[54] are dedicated to polyketide biosynthesis. Surprisingly, the genome of *E. coli* is devoid of any genes encoding polyketide secondary metabolites.

The renewed interest in polyketide natural products has prompted the development of metabolic engineering approaches for producing these interesting but expensive materials. Broadly speaking, two strategies are emerging: directed[55] and shotgun[56,57] approaches are being developed to enhance the productivity of selected polyketides in their natural hosts; in addition, polyketide pathways are being horizontally transferred into model heterologous hosts such as *S. coelicolor*, *E. coli*, *S. cerevisiae* and *Myxococcus xanthus*[58]. For example, new macrolide antibiotics of medicinal interest are synthesized in *S. coelicolor* and *E. coli*, and the promising antitumour agent epothilone has been produced both in *S. coelicolor* and *M. xanthus*. The subsequent application of metabolic engineering principles to overproduce these metabolites in heterologous hosts is greatly facilitated by the well-developed body of fundamental knowledge, genetic technology and fermentation development capabilities associated with these organisms. For example, 6-deoxyerythronolide B production in *E. coli* requires propionyl-CoA and methylmalonyl-CoA, neither of which are ordinarily available in *E. coli* and which must therefore be synthesized *in situ*. Three approaches have been evaluated for methylmalonyl-CoA production: carboxylation of propionyl-CoA by propionyl-CoA carboxylase, isomerization of succinyl-CoA by methylmalonyl-CoA mutase, and ATP-dependent synthesis of methylmalonyl-CoA from methylmalonate. All three approaches have been shown to work, although the former yields the greatest flux of precursors for 6-deoxyerythronolide B biosynthesis.

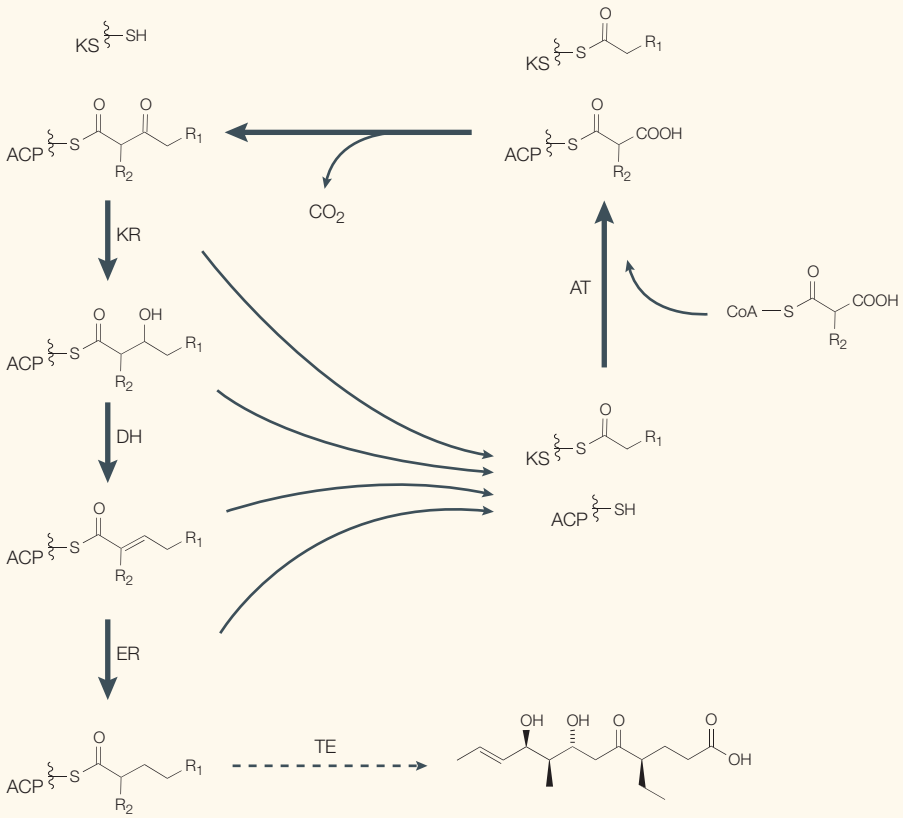


Figure 3 | **Polyketide biosynthesis.** The ketosynthase (KS) and acyl carrier protein (ACP) are the core proteins in a polyketide synthase. The acyl transferase (AT) catalyses building-block transfer from acyl-CoA metabolites onto the ACP. The ketoreductase (KR), dehydratase (DH) and enoyl reductase (ER) are optional enzymes in the polyketide catalytic cycle. The thioesterase (TE) enzyme catalyzes chain release from multienzyme system, often with concomitant macrocyclization of the product.

In addition to overproducing known polyketides of medicinal interest, a number of reports have described new methods for engineering polyketide metabolism to produce modified natural products. The unusual ease with which polyketide biosynthesis can be manipulated stems from the modular nature of both the chemistry and the enzymology of polyketide biosynthesis. For example, it is possible to delete, substitute or add catalytic domains to PKS modules, with the resulting product reflecting corresponding catalytic changes to the assembly-line biosynthetic pathway[59]. Alternatively, one can recombine intact catalytic modules from PKSs, thereby generating hybrid polyketide products[60,61]. New polyketides can also been generated by manipulating the repertoire of metabolically accessible building blocks in the host cell[62]. Last, chemo-biosynthetic approaches have been developed to incorporate non-biological building blocks into polyketide products[63,64]. In turn, the resulting functional handles also become sites for selective semi-synthetic modification of the natural product. More recently, these tools and principles have also been applied to the construction of combinatorial polyketide libraries[65]. Although the long-term impact of metabolically engineered polyketide libraries remains to be realized, given the exceptionally high frequency at which new polyketides have been developed as drugs, this represents one of the most potentially important long-term directions for metabolic engineering.

Non-ribosomal peptides

Metabolic engineering has played an important role in the recent development of new analogues of penicillins and cephalosporins, arguably the most well-known natural products. As a rational complement to classical strain improvement, the application of metabolic engineering principles to overproduce β-lactam antibiotics was one of the earliest examples of the relevance of this field to drug discovery and development[66]. More recently, metabolic engineering has been used for the development of a direct

fermentation process to produce blockbuster semi-synthetic intermediates such as 7-amino-deacetoxycephalosporanic acid (ADCA) by *Penicillium chrysogenum*[67].

Other non-ribosomal peptides, such as gramicidin, tyrocidin, cyclosporin, bacitracin and vancomycin, have also been a rich source of bioactive natural products[68]. Like polyketide synthases, non-ribosomal peptide synthetases are modular multifunctional enzymes, whose assembly-line biochemistry can be predictively modified to synthesize new drug candidates[69]. As a class of natural products, the primary advantage of non-ribosomal peptide synthetases is the enormous building-block diversity they harness into their products. Within the past decade there has been an explosive growth of sequence information for non-ribosomal peptide synthetases. In turn, this has created an enormous resource of modules representing this extraordinary range of functional groups. At the same time, deeper insights into the biochemistry of non-ribosomal peptide synthases, how they interact with other classes of biosynthetic pathways (especially polyketide synthases), and how they can be geometrically constrained through cyclization reactions, are providing the intellectual foundation to harness this genetic information to make new libraries for drug discovery.

Of particular recent interest is the isolation and characterization of macrocycle-forming enzymes from multifunctional non-ribosomal peptide synthetases[70] and, more recently, PKSs[71]. Given the difficulties associated with synthetic ring-closing procedures, this has led to the development of a fundamentally new approach for chemo-enzymatic synthesis of macrocyclic complex molecule libraries, some of which have already yielded unique bioactive leads[72].

Carbohydrates and other products

Metabolic engineering has also made contributions to the biosynthesis of essential vitamins such as folate, riboflavin and cobalamin, as well as nutraceutically important sugars such as mannitol, sorbitol, tagatose and trehalose[73]. An important step forward in the discovery of new glycosidic drug candidates has emerged from recent studies on the biosynthesis and glycosyl transfer of deoxy-sugar moieties attached to microbial natural products[74]. Many important antibiotics, such as erythromycin, vancomycin and doxorubicin, are decorated with unusual carbohydrate groups; these modifications are often crucial for the high *in vivo* activity of these drugs. The biosynthesis of these glycosidic moieties initiates with TDP-glucose, and typically involves a carefully orchestrated sequence of redox, amino-transfer and methyl-transfer events. Examples of important deoxy-sugar moieties whose biosynthesis has been elucidated include desosamine, mycarose, mycaminose, noviose, daunosamine and rhamnose. Several studies have demonstrated the feasibility of generating new deoxy-sugar moieties by mixing-and-matching enzymes from these pathways[75–77]. Perhaps more significantly, the glycosyl transferases responsible for transferring these deoxy-sugar moieties onto other (typically polyketide or non-ribosomal peptide) natural products seem to have remarkable substrate tolerance for both the nucleophilic acceptor as well as the electrophilic donor substrate[78–81]. A few of these enzymes have been structurally characterized, thereby opening the door for harnessing protein engineering to expand the repertoire of glycosylated natural products[82,83].

Conclusions

During the past decade, metabolic engineering has gained acceptance as a useful tool for the discovery and development of new natural-product drugs. Two major hurdles are encountered in the development of new natural-product leads. First, their structural complexity presents a daunting challenge for the medicinal chemist charged with the task of rapidly creating many new analogues for pharmacological evaluation. Second, the extraordinarily high cost of producing the molecule makes such a programme intrinsically risky. In the initial stages following the isolation of a novel natural product, the cost of production typically exceeds US $1,000 per gram of purified material. As summarized in this article, a variety of metabolic engineering technologies have emerged to make analogues of natural-product leads and to produce them in useful quantities. Moreover, biosynthetic engineering has also given rise to fundamentally new approaches for creating natural product-like libraries, although their long-term value remains to be realized. As was the case for genetic engineering of protein drugs, the second decade of this new science is likely to yield new marketable products at an increasing pace, thereby validating the promise of the field for the pharmaceutical industry.

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Competing interest statement

The authors declare competing financial interests: see Web version for details

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CORRECTION

COX-2 AND BEYOND: APPROACHES TO PROSTAGLANDIN INHIBITION IN HUMAN DISEASE

Garrett A. FitzGerald

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The sulphonamide groups in celecoxib and valdecoxib and the trifluromethyl group in celecoxib are now accurately depicted. An omitted chlorine group in etoricoxib is also now included.