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Unlocking the Therapeutic Potential of Antimicrobial Natural Products with Synthetic Biology

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Abstract:
Although once a mainstay of drug discovery efforts within the pharmaceutical industry, enthusiasm for the use of natural products as a starting point for the development of new medicines has steadily declined since the early 1990s. As a consequence, many companies have opted to jettison their natural product screening programs in favor of high-throughput synthesis and combinatorial chemistry, approaches that have ultimately failed to deliver on their early promise. Yet despite their deprioritization, > 60% of all small molecule drugs in current clinical use can trace their origins back to natural product scaffolds. There is now an increasing realization that these privileged structures represent the optimal starting point for the development of clinically viable assets. Here, we outline the current state-of-the-art in antimicrobial natural product drug discovery, with a specific focus on how the emerging field of synthetic biology is delivering the tools and technologies required to unlock the therapeutic potential of natural products. We illustrate how these approaches are circumventing many of the problems that have historically plagued conventional screening programs, enabling the expedient discovery of new molecules with novel functions, and the design and development of therapeutically optimized 'unnatural' natural products.

Introduction

Natural products have been used for therapeutic purposes for millennia. The earliest records date back to Mesopotamia, 2600 B.C., and describe ~ 1000 plant-derived extracts which were used to treat conditions as diverse as parasitic infections, skin disorders and the common cold[1]. The ancient Egyptians and Assyrians chewed on the leaves of willow trees to treat joint pain, and Hippocrates advocated the use of willow leaf extract as an analgesic for childbirth[2]. Efforts to isolate the active ingredients of natural remedies started in earnest in the 1800s, culminating in the discovery of quinine, morphine and salicylic acid; the latter being the active ingredient of willow leaves[3]. Building on these pioneering studies, Bayer successfully developed the antipyretic salicylic acid derivative aspirin, which is still widely used to this day.

Whilst the concept of natural products as a starting point for drug development began to gain traction in the 19th century, it was undoubtebly the discovery of penicillin in the 1920s by Alexander Fleming, and its subsequent manufacture at scale in the 1940s, that ushered in the golden era of natural product drug discovery[4]. The realization that microorganisms and plants represented a plentiful resource of bioactive molecules with therapeutic potential established a foundation from which the pace of natural product-based drug discovery grew exponentially during the early-to-mid 20th century. This fruitful period served to deliver many of the keystone classes of antibiotics in use today, along with a plethora of allied therapeutic agents. This contrasts starkly with equivalent success rates for antibiotic drug discovery during the past 50 years. Since 1970 only 3 antibiotics have been developed which are sufficiently chemically differentiated from known molecules to be classified as

'new' assets; the polyketide mupirocin in 1985, the oxazolidinone linezolid in 2000, and the lipopeptide daptomycin in 2003[5].

Interestingly, the degree of representation of natural products and their derivatives amongst successfully realized pharmaceuticals runs counter to the paucity of active research programs in this area, within the pharmaceutical sector. The late 20th century saw major investments by Pharma in high-throughput screening (HTS) platforms, structural biology infrastructure and combinatorial chemistry. The emergence of these methods was coupled with a changing view that natural product-based drug discovery was no longer an economically viable proposition. Screening of natural products was beset by issues of compound rediscovery and the often-intractable issue of developing efficient syntheses for what were often highly structurally complex molecules. Consequently, the time taken to discover, optimize and bring to market a natural product based drug was deemed to be prohibitively long and expensive, with the focus instead shifting to target based approaches[6]. This period did, however, see a burst in modifications of natural products, which resulted in second, third, fourth and fifth generation cephalosporins, for example – but new scaffolds were not being discovered[7].

Regrettably, it is now evident that this decision to transition away from natural products as a starting point for drug discovery has precipitated a decline in the productivity of the pharmaceutical industry, with an emerging view that natural product-based discovery was prematurely jettisoned. This is exemplified in the area of antibiotic discovery, where a failure to deliver new molecules with novel modes of action, in parallel with the emergence of anti-microbial resistance (AMR), is now driving a global healthcare crisis[8]. For compelling commercial and scientific reasons, the reengagement of Pharma with natural products is now long overdue. Fortunately, the emerging field of synthetic biology, which seeks to apply the principles and practices of engineering to the design or redesign of biological systems, has in recent years provided researchers with the tools and technologies necessary to circumvent many of the inherent problems associated with the development of medicines from natural molecules. With these game-changing advances, the complexities of natural product hit generation, lead optimization and scalable manufacture can now be readily addressed, unlocking a myriad of new opportunities. Significantly, these approaches can be readily retrofitted within established drug discovery workflows, minimizing disruption and the requirement for infrastructure reconfiguration (Figure 1).

Hit Generation

Classical target-based drug discovery hinges on the identification and validation of a suitable cellular target, which is subsequently subjected to screening, in a high throughput manner, against proprietary libraries of small molecules. This approach enables the identification of 'hit' compounds, which serve as a starting point for functional enhancement via iterative cycles of medicinal chemistry and binding studies. This approach by definition, is limited by both library composition and the sensitivity of the assay used and is contingent on an assumption that the observed *in vitro* behaviour can be realized *in vivo*.

In contrast, natural product discovery approaches rely on the identification of bioactive compounds, usually isolated from microbial culture collections or equivalent repositories of plant extracts. Historically, this process has been laborious and expensive, with no guarantee of success. When screening microbial collections, the process is further complicated by the fact that under standard laboratory growth conditions many of the biosynthetic pathways that encode the enzymatic machinery necessary for natural product assembly are inactive, or 'silent', thus significantly reducing the size of the accessible pool of bioactives. Importantly, however, the genes which encode natural product pathways, including those to the four main classes of natural products, polyketides, non-ribosomal peptides, alkaloids and terpenoids[9], are often colocalized into clusters within the producing host's genomic DNA. This subsequently opens up the possibility of 'mining' available genomic sequences for the presence of gene clusters that encode novel biosynthetic pathways, which assemble hitherto unreported chemical scaffolds. The development of Next Generation Sequencing, and the associated time and cost savings that it brings, has led over the past decade to an explosion in the

number and quality of genome sequences available for analysis. This has enabled *in silico* screening approaches to be developed and applied to the search of novel bioactive compounds using only genomic DNA sequences. This approach circumvents any requirement for wet lab based screening processes and accounts for all pathways present within a genome, whether expressed under laboratory conditions or not. This method of compound discovery has been greatly aided by the development of reliable genome mining software, e.g. antiSMASH and Pep2Path, which can be deployed to identify all the biosynthetic gene clusters within a target genome and which are also able to make predictions about the likely chemical structure of each pathway product. Consequently, this approach greatly expands the scope of the chemical space available for discovery. The mining of actinobacteria genomes, for example, has revealed that the *Streptomyces coelicolor* (*S. coelicolour*) genome harbors ~10 fold more natural product gene clusters than previously proposed based on the number of isolatable natural products from this bacterium[10].

Once a potential natural product lead compound has been identified *in silico*, it must then be produced within the laboratory in sufficient quantities to enable bioactivity screening to take place. The elaborate chemical scaffolds of natural products frequently present a significant challenge for synthesis. Thus, compound generation is often best achieved via pathway expression in the either the native host, where feasible, or more commonly via expression in a heterologous host. Genomic information and prediciton of the structure may also be used to adjust growth conditions to access the molecule of interest. Natural products impose a high metabolic cost upon the producing organism, which generally results in low expression levels. Strains of the bacterium *Escherichia coli* (*E. coli*) and the yeast *Saccharomyces cerevisiae (S. cerevisiae)* have been selectively engineered to overcome this hurdle. The use of these chassis microorganisms, in tandem with innovative gene cloning methods, e.g. Transformation Associated Recombination (TAR) cloning, allows for large stretches of DNA to be easily manipulated and transferred to heterologous hosts. This technique can be used in the cloning of entire clusters from bespoke native producers into well characterized, metabolically optimized surrogates for expression.

Together, the approaches outlined above can be applied to mitigate many of the major bottlenecks in early stage natural product drug discovery. However, the identification of a hit, and its subsequent isolation and characterization, is of little value if the compound under investigation has minimal bioactivity, or is a previously reported molecule, or close relative thereof. This issue of chemotype replication in natural product drug discovery is generally considered the primary reason as to why Pharma has shifted its focus away from natural products, as dereplication is non-trivial, laborious, and resource intensive. The development of the antibiotic resistance platform (ARP) by Wright and colleagues has, however, provided a fit-for-purpose tool, which can be used for quick, low-cost antibiotic dereplication, as well as for the discovery of antibiotic adjuvants (inhibitors of resistance). The ARP currently comprises 15 antibiotic resistance genes that have been transformed into *E. coli*. Natural product extracts, or secondary metabolite-producing microorganisms, can be tested against these resistant strains either by agar-overlay or using an agar-plug method. *E. coli* colonies will survive if they house the corresponding resistance gene for the antibiotic produced, allowing rapid, robust identification of the molecules present and prioritization of antibacterial assets. With respect to adjuvant discovery (antibiotic adjuvants are nonantibiotic compounds that improve antibiotic activity), this system has been used to identify several molecules that enhance the activity of aminoglycosides against the resistance determinant nucleotidyltransferase ANT(2”)-la, thus resensitizing strains to aminoglycoside antibiotics. This offers a starting point for the rational design of inhibitors to improve the efficacy and longevity of this class of natural product drugs[11]. The ARP platform, along with equivalent dereplication approaches, are of the utmost importance for directing effective antibiotic natural product drug discovery programs.

Hit to Lead Development and Lead Optimization

Following hit identification, hit to lead (H2L) development and lead optimization must take place. These processes are necessary to establish functionally optimized candidate scaffolds that are best

suited for clinical use, e.g., increased affinity for their cellular target and reduced off target effects. Conventional drug discovery approaches employ iterative cycles of medical chemistry coupled to compound testing to achieve this desired outcome. This is time consuming, expensive and poorly suited to automation. In contrast, a natural product focused synthetic biology approach employs genetic manipulations of the biosynthetic pathway to a given target compound, such that the resulting engineered pathway assembles functionally optimized unnatural-natural products. This requires an intricate knowledge of the biosynthetic process to the parent compound. The application of this approach is best illustrated using the example of the broad-spectrum polyketide antibiotic erythromycin, a clinically used compound first isolated from the soil bacterium *Saccharopolyspora erythraea*[12]. Similarly to other polyketide natural products, erythromycin is biosynthesized in a stepwise manner, via a series of sequential condensation reactions catalyzed by an assembly line like mega-enzyme complex, termed a type 1 polyketide synthase (PKS; Figure 2)[13,14]. The colinear gene-protein-bioactive compound relationship common to these systems makes them an ideal target for synthetic biology based combinatorial approaches, as exemplified by the work of Jiang and colleagues in 2013. In this study, the deoxysugar pathways employed for polyketide tailoring were transferred to an *E. coli* host engineered to express the erythromycin PKS. The resulting *E. coli* strain was shown capable of biosynthesizing a suite of novel erythromycin analogs with desirable characteristics[15].

Another elegant example can be seen in studies of daptomycin, a lipopeptide produced by a non-ribosomal peptide synthase (NRPS), which employs an analogous assembly line like biosynthetic process as that employed by PKSs. The primary differentiator is the use of amino acids as substrates by NRPSs, as opposed to carboxylic acids in PKSs. In this example, a heterologous condensation-adenylation di-domain was fused to the biosynthetic enzyme DptD, with the resulting engineered pathway consequently incorporating an asparagine at the C13 position of the product chain. Interestingly, this optimized lead molecule showed increased antimicrobial efficacy vs. *Staphylococcus aureus in vivo*[16].

Combinatorial biosynthesis can also be employed to generate biosynthetic chimeras, which incorporate enzymatic machinery from different natural product biosynthetic pathways, often originating from different microorganisms. Saponins are large, highly decorated polycyclic structures that comprise one or more glyconemoietes combined with a triterpoene or steroid derivative. They are produced by multiple plant species and exhibit a variety of biological activities. The sapogenin backbone is formed via multiple cytochrome P450 mediated oxidation-reduction reactions. Synthetic biology-based methods have been successfully used to incorporate a non-cognate cytochrome P450 from *Bupleurum* (CYP716Y1) into the sapogenin pathway of an unrelated plant species. Expression of this chimeric pathway in yeast results in the production of a novel non-natural saponin, which is of major industrial value[17].

A similar approach has been applied to enable the site-specific halogenation of natural products. Although more common in biosynthetic processes than previously thought, halogenation reactions are none-the-less considered highly desirable modifications, with the isolation of halogenated natural products from plants and microbes widely to be considered to be a non-trivial task. Runguphan and colleagues expressed the chlorination biosynthetic machinery from a soil bacterium in the Madagascar periwinkle, which subsequently produced chlorinated alkaloids[18]. This proved the viability of a synthetic biology approach for natural product optimization in plants, which are generally viewed as less tractable targets for combinatorial biosynthesis.

Whilst the above examples predominantly involve the substitution or augmentation of natural product pathways to alter product chemistry, one may also exploit the inherent promiscuity of a pathway by, for example, the feeding of non-cognate precursor substrates. This approach has been successfully applied to the Rhizoxin PKS from the *Rhizopus* symbiont *Burkholderia rhizoxinica*, which biosynthesizes a potent phytotoxin antibiotic. Here, a range of unnatural precursors were synthesized and their biotransformation by a reconstituted Rhizoxin PKS module monitored *in vitro*. Interestingly, the resulting products were shown to resemble the clinically relevant antibiotic cycloheximide[19]. Not

only did this study demonstrate the potential of feeding natural precursors to produce new molecules, but it sheds light on the biochemistry underpinning the production of cycloheximide.

Scale-Up

The capacity to produce clinical leads at scale is one of the most important elements of any drug development process. Even the most efficacious compounds will not transition to clinical use if they cannot be produced in sufficient quantities. Scale-up therefore represents one of the most significant challenges in natural product drug development. Given the chemical complexities of natural products they more often than not must be produced via fermentation of a suitable production host. This imparts a significant metabolic burden on the chosen host, which is often intolerable for the natural producer[20]. However, emerging advances in chassis optimization via genome engineering, along with improvements in cell culturing methods, are now being applied to overcome this challenge.

The primary consideration when developing a natural product fermentation process is the choice of production host. Biosynthetic pathway expression in a heterologous host is often tractable, but it is highly dependent on the compatibility of the pathway gene and consequent polypeptide sequence with the chosen chassis, e.g. codon usage, availability of precursor substrates and chaperones. For these reasons the optimization of natural host microorganisms has become an area of major interest.

Ribosome engineering is a well-established method for host optimization. This technique was originally applied to a strain of S*treptomyces*, a bacterial genus known to harbor numerous silent gene clusters. A mutation in the ribosomal S12 protein resulted in the production of the blue pigment antibiotic actinorhodin. It was subsequently demonstrated that the mutations introduced into the ribosome coding sequence promotes the binding of bacterial alarmone guanosine 5'-diphosphate 3'-diphospahte (pp-Gpp), produced on the ribosome, to RNA polymerase, thus increasing its affinity for promoter regions involved in secondary metabolite production[21]. A vast array of bioactive secondary metabolites have subsequently been produced at scale using this method, including daptomycin, erythromycin and vancomycin. Ribosome engineering has also been used in the discovery of new natural products with antibacterial properties[22].

Another method for increasing natural product titres in host strains is that of metabolic engineering. This approach involves making defined changes to the sequence of a producer's genome, in an effort to direct metabolic flux towards the desired product. Metabolic engineering is a particularly attractive method for yield enhancement in actinobacteria, which are amongst the most prodigious producers of microbial natural products. For example, incorporating metabolite-responsive promoters into the genome of *S. coelicolour* resulted in a 9.1-fold increase in the production of the antibiotic oxytetracycline[23]. Other examples of metabolic engineering efforts in actinobacteria include riboswitches, natural product-specific biosensors for dynamic product regulation, and multiplex site-specific genome engineering (MSGE). This latter approach enables target clusters to be amplified in the natural host and has been used successfully in actinobacteria to overproduce the antibiotic goadsporin 2.3 fold[23].

With respect to heterologous hosts, a number of different cell chassis have been explored. Commonly used examples include *E. coli* and *S. cerevisiae*, which both benefit from fast doubling times, having well characterized genomes and proteomes, and the availability of robust molecular genetic tools which enable their manipulation. Interestingly, *E. coli* and *S. cerevisiae* can be deployed in a combinatorial co-culture approach, which has been successfully used to produce oxygenated taxenes at scale. In this example, an *E. coli* host synthesizing taxadiene was grown in a coculture with *S. cerevisiae* expressing enzymes required to perform site-specific oxygenation reactions. This elegantly demonstrates the benefit of distributing metabolic pathways among a microbial consortium[24]. In terms of antibiotic natural product production *Streptomyces* strains are still considered the gold standard. For example, the repertoire of post-translational modification systems in *Streptomyces* is more extensive and sophisticated than that of *E. coli*, enabling a ready supply

precursor molecules and cofactors required for polyketide, non-ribosomal peptide and terpene biosynthesis[25].

In addition to chassis choice, one must also consider provision of the requisite enzymatic machinery required to assemble the target product. Databases such as NP.searcher[26] can be used for the prediction of gene clusters during the genome mining phase of development, and ATLAS[27] and RetroPath2.0[28] can be used to design synthetic pathways based on known biochemical reactions. There are also a wealth of transporter databases available that can be used to find a suitable candidate to enable product efflux[25]. A key emerging enabler of these methods is artificial intelligence, which may also be used to predict alternative pathways to target compounds that may be more tractable for scale-up[29]. Protein engineering and directed evolution approaches also offer a mechanisms for the enhancement of product titres, e.g. by increasing enzyme specificity for a target substrate, reducing off-target reactions, or for the development of non-natural biosynthetic pathways[30].

Once an optimized biosynthetic route has been formulated and an appropriate chassis selected, pathway reconstitution in the host must be undertaken. Modern DNA assembly methods, either *in vitro*, e.g. Golden Gate assembly, or *in vivo*, e.g. TAR cloning, in combination with CRISPR/Cas9 based methods are now enabling DNA constructs of > 1.5 MB to be routinely successfully reconstituted[31]. In tandem, the development of dCas9 (deactivated Cas9) and CRISPRi (interference CRISPR) may be used to achieve regulatory control over reconstituted pathways in a manner that is inherently more tunable than was previously possible[25].

Remaining Challenges and Future Prospects

The past decade has seen major advances in our fundamental understanding of natural product biosynthesis. These insights, coupled with the tools and technologies of synthetic biology, are now driving a resurgence of interest in the use of natural products as a starting point for drug discovery efforts. Figure 3 highlights the major areas where emerging synthetic biology tools could impact drug discovery processes. Interestingly, Pharma's deprioritization of natural scaffolds means that they are now poorly positioned to retransition into this area, with the most innovative work in this field now being undertaken in academia, or by emerging Biotech small to medum sized enterprises (SMEs).

With respect to compound discovery, genome mining is now enabling the identification of new biosynthetic pathways and the prediction of their corresponding natural products at a rate once considered improbable. The issue is no longer one of target identification, but rather one of target prioritisation. Here, artificial intelligence appears set to make major contributions, enabling autonomous screening of genome databases and the application of predictive tools that can rank candidate pathways and associated metabolites based on chemical novelty and druglike properties. Similarly, our capacity to selectively manipulate biosynthetic pathways, enabling access to bespoke non-natural natural products, is advancing rapidly. The promise of combinatorial biosynthesis is being realized, with effective tools for pathway redesign and optimization now readily accessible.

Despite these advances, issues still exist. The development of fit-for-purpose chassis organisms remains a major obstacle to success, with future efforts undoubtebly focusing on the establishment of general-purpose heterologous hosts which can be employed for compound manufacture agnostic of pathway identity and/or native producer. Ultimately, this may necessitate the development of cell-free manufacturing processes, but such systems are still very much in the development phase, particularly for compound manufacture at scale[32]. Improved genetic manipulation tools are also a priority, specifically those which can be applied in a strain independent fashion. Undoubtably, these will leverage recent game-changing progress in the development of the CRISPR-transposon system[33].

Without question, the next decade will witness the re-emergence of natural products as a favored starting point for drug discovery. This will be most keenly felt in the area of antibiotic development, where the move away from natural products in the 1990s has resulted in a catastrophic decline in the rate of asset discovery and development. Future natural product drug discovery workflows will be less dependent on physical infrastructure and access to extensive compound and

strain collections and will instead be founded on *in silico* led distributed development programs, which are inherently nimbler and can be pursued with significantly lower operating costs. When it comes to natural products drug discovery, the model very much is, back to the future.

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Conflicts of Interest

The authors declare no conflicts of interest.

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Figures:

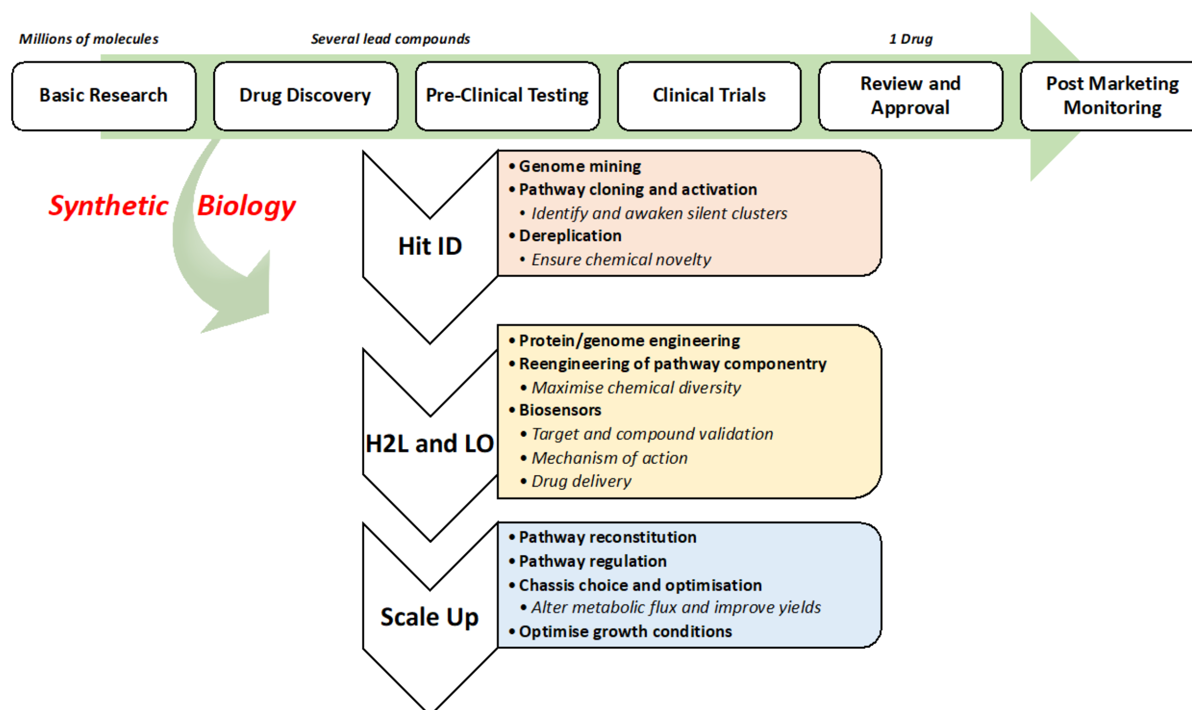

Figure 1. Generalised workflow for natural product drug discovery and development. Aspects of natural product hit identification, hit-to-lead development, lead optimization and scale up which can be expedited using synthetic biology-based methods are identified.

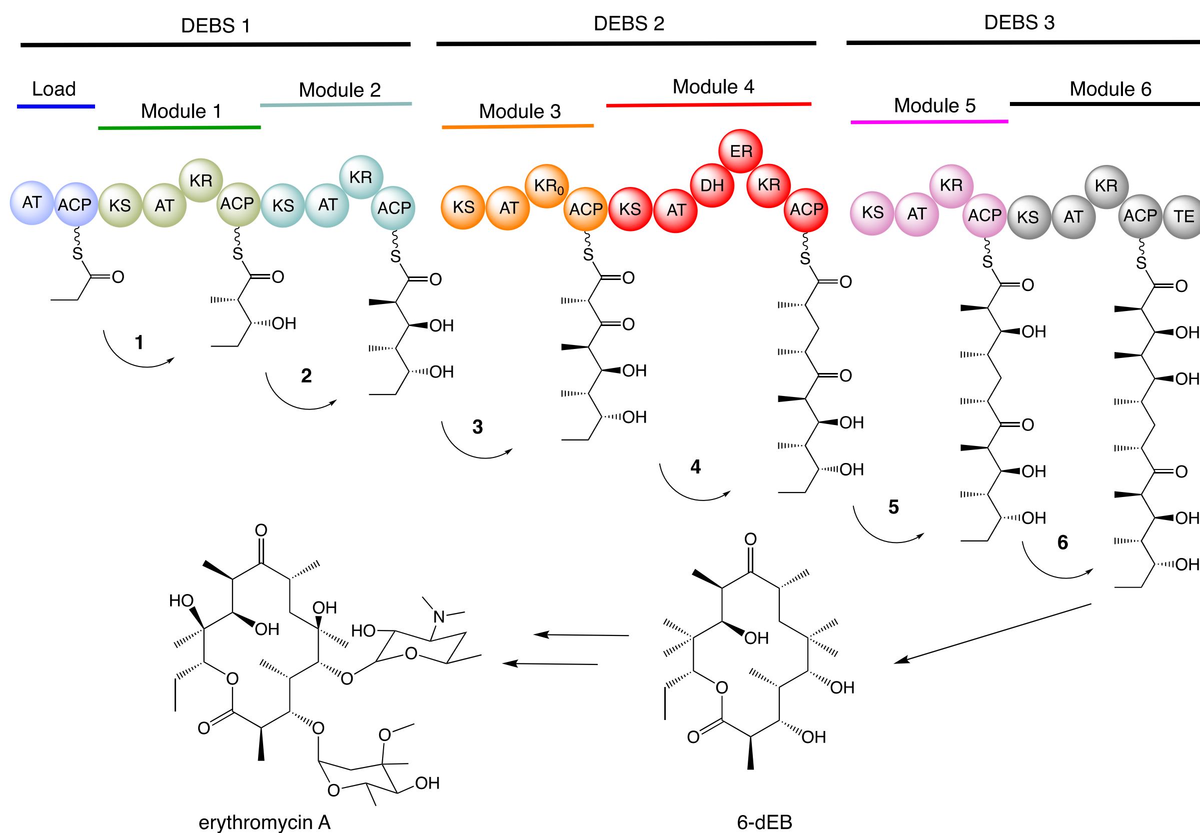

Figure 2. Biosynthesis of 6-deoxyerythronolide B synthase (DEBS) and the biosynthetic route to erythromycin A. Individual synthase domains are organised into discreate modules that catalyze single chain extension events, exemplifying the modular, assembly line-like route to polyketide natural products. Numbered arrows indicate the direction and order of product chain extension and transfer.

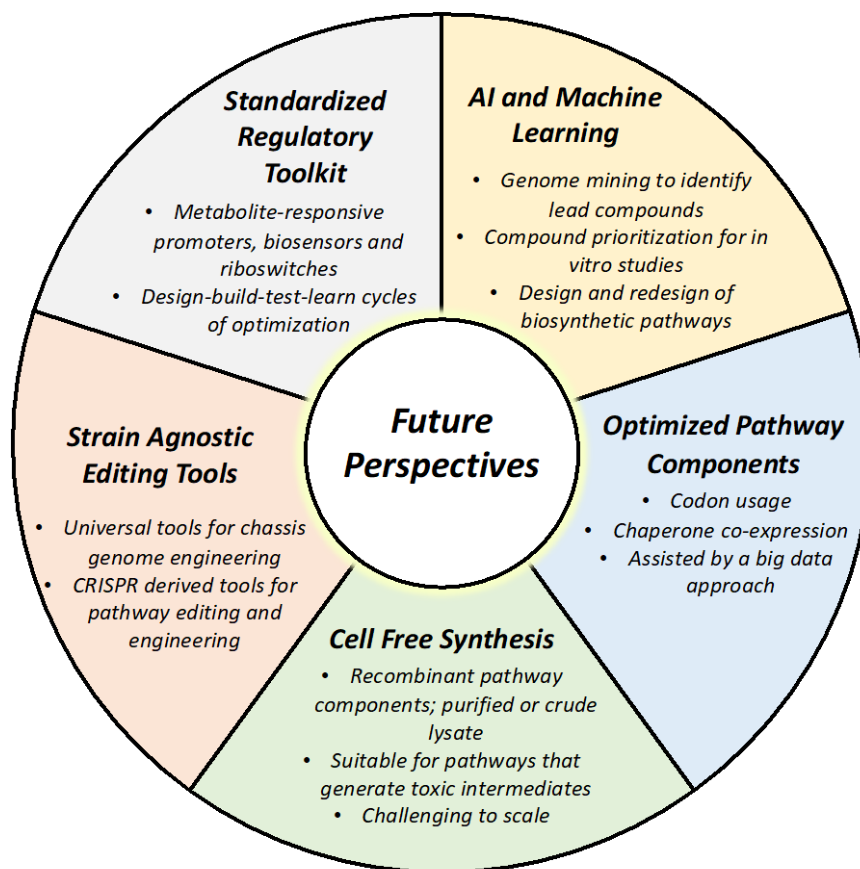

Figure 3. Emerging opportunities for the use of synthetic biology in natural product drug discovery and development. Methods are categorised based on application area and alignment with standard drug discovery workflows.

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