Combinatorial Biosynthesis of Natural Products

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Microbial and plant natural products are among the most important pharmaceutical compounds known to mankind. These compounds are produced by dedicated metabolic pathways using enzymes that possess exquisite catalytic power. Genome sequencing of many microbial and plant species has revealed tremendous diversity among natural product biosynthetic pathways. Enzymes discovered from these pathways collectively constitute a versatile molecular toolbox that can be utilized toward the synthesis of unnatural natural products.[1]

Combinatorial biosynthesis is the modification of these biosynthetic pathways toward the production of "unnatural" natural products. The ultimate goal of combinatorial biosynthesis is to construct de novo pathways by assembling enzymes from the toolbox and to produce novel chemical entities that possess improved biological activities.[2] The new compounds may be antibiotics that can overcome current modes of bacterial resistance, anticancer compounds with decreased cytotoxicity, or cholesterol lowering drugs that decrease the risks of cardiovascular diseases. The concept of combinatorial biosynthesis has been generally recognized as a powerful method of diversifying natural product structures and affording synthetically inaccessible compounds.[3] Despite its vast potential, there are several technical bottlenecks that have impeded the technical advancement. First, there is a lack of general programming rules for recombining enzymes from different sources. Simple mix-and-match experiments generally fail because they do not account for the extensive and orthogonal protein–protein interactions required for substrate channeling and catalysis in a complex pathway. Furthermore, the narrow substrate specificity of many of the biosynthetic enzymes renders them inactive toward compounds with subtle structural variations. In addition, the lack of a universal expression host obstructs the engineering of some of the most interesting metabolic pathways, as many of them are found in hosts that are genetically intractable. As a result, high-efficient DNA transformation and large scale pathway shuffling, which are key tools of combinatorial biosynthesis, cannot be performed.

In the past 3 years, we have seen a rapid advance in our abilities to overcome these challenges. The recent expansion of the molecular toolbox, unveiling of key protein crystal structures, understanding of the interactions among the enzymatic components, and development of improved tools for genetic engineering and heterologous expression have pushed the state of the art for combinatorial biosynthesis beyond the rudimentary stage, where individual enzymes were treated as jigsaw puzzle pieces. As a result, combinatorial biosynthesis presents unprecedented opportunities as an integrated platform for drug discovery.

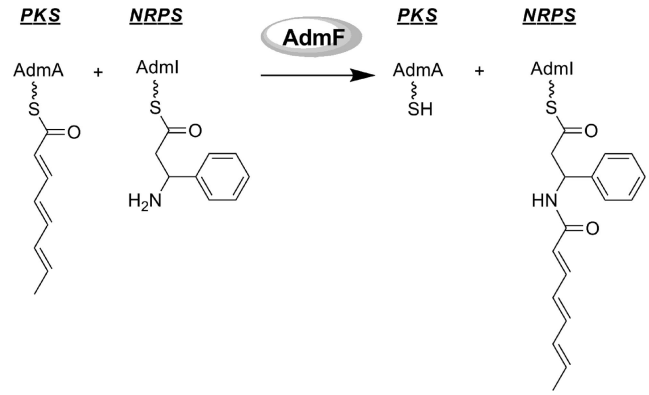

Figure 1. Formation of an isopeptide bond between the PKS module (AdmA) octatrienoyl donor and the NRPS module (AdmI) β-phenylalanine acceptor. The reaction is catalyzed by AdmF, a transglutaminase homologue in andrimid biosynthetic pathway.[6]

Expansion of Molecular Toolbox

Nature has evolved a tremendous variety of enzymes that employ novel mechanisms to synthesize structurally complex metabolites. Identification of new enzymatic tools through genome mining and gene cluster characterization will therefore undoubtedly enrich our molecular toolbox toward combinatorial biosynthesis and expand the chemical diversity seen among "unnatural" natural products.

For instance, a new bioengineering strategy for integrating fatty acid and polyketide biosynthesis has been discovered in a hybrid type I fatty acid−type III polyketide synthase (PKS[a]), which is responsible for the biosynthesis of *Dictyotelium discoideum* differentiation-inducing factors.[4] The C-terminus type III PKS in this modular system replaces the thioesterase domain common found at the end of enzymatic assembly lines. Interestingly, the type III PKS elongates the acyl chain through repeated Claisen condensations to generate hybrid products. Recently, the andrimid (*adm*) biosynthetic gene cluster has been identified[5] and a transglutaminase homologue AdmF has been characterized as a stand-alone condensation catalyst forming amide bonds between an upstream PKS module and a downstream nonribosomal peptide synthetase (NRPS) module during *adm* biosynthesis (Figure 1).[6] This free-standing and promiscuous enzyme adds another dimension to the modular assembly paradigm of NRP and polyketide natural products, allowing heterologous modules from different pathways to be functionally combined. During the iterative biosynthesis of bacterial aromatic polyketide oxytetracycline (*oxy*), a new nitrogen-inserting enzyme, OxyD, has been shown to synthesize an amidated polyketide backbone, in collaboration with the minimal *oxy* PKS enzymes.[7] The rare, polar amide starter unit in the poly-β-ketone backbone also introduces new cyclization chemistry for aromatic

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[a] Abbreviations: PKS, polyketide synthase; NRPS, nonribosomal peptide synthetase; DEBS, 6-deoxyerythronolide synthase; FAS, fatty acid synthase; ACP, acyl carrier protein; GT, glycosyltransferase; KS, ketosynthase; STR, strictosidine synthase; IPP, isopentenyl pyrophosphate.

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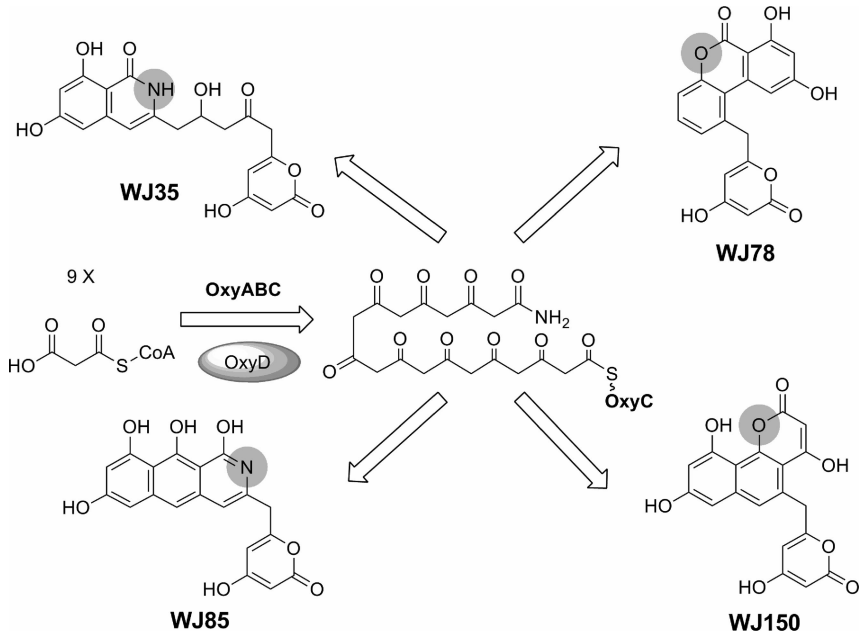


Figure 2. OxyD, an amidotransferase homologue in *oxy* biosynthetic pathway, catalyzes the formation of the amidated polyketide backbone in collaboration with the minimal PKS enzymes. The amide functionality serves either as a nucleophile or as an electrophile, leading to the biosynthesis of various products in the presence of different tailoring enzymes.[8]

polyketides, in which the amide functionality can serve either as a nucleophile in the formation of isoquinolones or as an electrophilic leaving group facilitating the biosynthesis of benzopyrones (Figure 2).[8] Equally as useful, BenQ has been uncovered to select the rare hexanoate start unit in the benastatin biosynthetic pathway.[9] Inactivation of the gate-keeping BenQ led to the synthesis of numerous novel pentacyclic polyketide primed with various straight and branched fatty acids.

New Engineering Approaches to Natural Products Biosynthesis

The reprogramming of modular NRPS and PKS assembly lines has been a primary focus of combinatorial biosynthesis.[10] This is primarily facilitated by the colinearity feature of bacterial megasynthases in which product outcome can be easily predicted on the basis of the linear arrangement of catalytic domains. This is further aided by bioinformatics analysis in which the substrate specificities of each module can be predicted based on protein sequence directly. Recent progress in understanding the underlying biochemistry of these enzymes, as well as development of directed evolution tools, has provided immense new opportunities for constructing productive and combinatorial assembly lines.

NRPS. Domain and module swapping between NRPS systems have been explored over a decade. Recently this approach has been used in the combinatorial biosynthesis of acidic lipopeptides of therapeutic value.[11] When combined with inactivation of the tailoring enzymes and natural variations in the lipid side chain, this approach was used to produce novel peptide antibiotics for drug development. Point mutations and module skipping by hybrid PKS/NRPS have also brought chemical diversity in lipopeptides products.[12] Despite these success stories, swapping heterologous modules toward producing unnatural nonribosomal peptides has achieved limited success, and the resulting chimera was usually nonfunctional or heavily impaired. The molecular basis for the selective interactions between adjacent modules has been partially resolved by discovering the critical role of communication-mediating (COM) domains.[13] It was revealed that the interaction of compatible sets of COM domains is essential for the crosstalk between partner NRPSs, leading to the ordered assembly of modules. These communication domains are analogous to the linker regions established for modular PKSs in the late 1990s.[14]

One approach to optimize heterologous NRPS interactions is to use random mutagenesis. Protein engineering, which revolutionarized antibody refinement and biocatalysis,[15] has not been adopted widely to advance combinatorial biosynthesis beyond the trial and error stage. This is largely because the products of most of these pathways are not subjects of the high-throughput screening and selection techniques that have proven to be extraordinarily useful for directed enzyme evolution. Most of the products do not complement any auxotroph that can be linked to cell viability and cannot be identified visually without using chromatographic tools. Therefore, to effectively utilize protein engineering toward functional pathway synthesis, the product of interest must be able to generate a cellular response that can be readily detected. Recently, mutagenesis has been employed in Walsh's laboratory to explore the protein–protein interaction within modular assembly lines. A cell viability assay in the presence of ultralow iron concentration was used to improve the catalytic efficiency of an engineered enterobactin (*ent*) NRPS pathway in *Escherichia coli* (*E. coli*). The interactions between heterologous carrier proteins and other catalytic domains within the *ent* assembly line were optimized, and critical surface residues that interact with various domain partners were identified on the carrier proteins.[16] In another example, direct evolution was used toward adenylation domains in both *ent* and *adm* biosynthetic assembly lines.[17] The activity losses due to domain swapping were successfully restored by random mutagenesis with high hit rates in a few rounds. Interestingly, key residues involved in efficient interdomain communication do not map to one region of the domain and are usually "randomly" distributed. These successful stories are very encouraging and suggest that as long as a suitable selection scheme is in place, direct evolution can be a straightforward

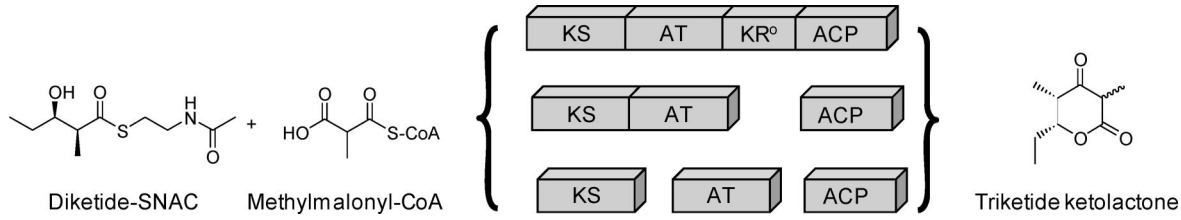


Figure 3. Chain elongation cycle catalyzed by intact and dissociated DEBS module 3.

and efficient technique to evolve and optimize the activities of chimeric megasynthases toward combinatorial biosynthesis.

PKS. Among different types of PKS, the multimodular enzymes (type I PKS) have proven to be very amenable to engineering and the model system 6-deoxyerythronolide synthase (DEBS) has been on the center stage of combinatorial biosynthesis. By use of DEBS as template, the underlying rules of module assembly and communication have been elucidated and led to the combinatorial biosynthesis of 6-DEB analogues through domain deletion, substitution, and insertion.[18] However, an unresolved bottleneck is the structural understanding of domain arrangement on the assembly line, which is critical for defining the precise domain boundaries for protein engineering. In this respect, the recent collection of high-resolution X-ray protein structures of PKS domains[19] and eukaryotic fatty acid synthases (FASs)[20] has offered a superior understanding of the molecular details of these extraordinary machineries.[21] It is now evident that domain−domain and domain−linker interactions play critical roles in the structure and function of modular PKSs. Acyl carrier protein (ACP) domain may be a key element here, which has been demonstrated to be able to collaborate with various partner domains during polyketide chain extension, shuttle significant distances with great flexibility between catalytic domains within same or adjacent modules. The structural basis of how these dynamics are achieved in PKSs is still unknown. On the basis of X-ray crystal structure of DEBS domains, intact PKS modules have been successfully dissected to stand-alone monodomains. The chain elongation activities of dissociated domains were readily reconstituted in vitro (Figure 3), allowing detailed examination of domain−domain specificity and optimization of heterologous interactions.[22,23]

Development of automated cloning strategies for high-throughput combinatorial biosynthesis is a shotgun approach to overcome incompatibilities between fused heterologous domains.[24] With employment of the universal linker technology, this approach allows many combinations of PKS modules from different organisms to be screened rapidly for acceptable catalytic efficiency. Totally synthetic preparations of entire genes enable codon optimization and facile introduction of desired restriction sites. The efficiency of this technology has been validated with 154 bimodular combinations assembled from 14 synthesized modules. Impressively, nearly half of the combinations yielded the expected triketide lactone products. Furthermore, many silent bimodular combinations could be rescued by rationally replacing the ketosynthase (KS) of the acceptor with the KS normally linked with the ACP of the donor module.[25] This strategy was further extended in the rational design and assembly of synthetic trimodular PKSs.[26]

Developing combinatorial approaches with tailoring enzymes is also an important method of diversifying natural product structures and functions. Post-PKS decoration of polyketides by dedicated enzymes is generally indispensible for "activating" the nascent polyketide and provides the warheads responsible for the observed biological activities. Recently, glycosyltransferases (GTs) have been engineered in vivo and in vitro toward expanding their substrate tolerance toward noncognate sugars and aglycons. The versatility of GTs to act in a reversible manner in vitro has been reported, demonstrating their potential as a powerful tool in the structural diversification of glycosylated natural products.[27] Most GTs examined to date are more selective toward the aglycon acceptor and are more relaxed toward the sugar donor. Site-directed mutagenesis and directed evolution approaches have been employed to expand the substrate specificities of GTs.[28] A simple high-throughput screen based on a fluorescent surrogate acceptor substrate has been used to significantly expand the capabilities of GTs.[29]

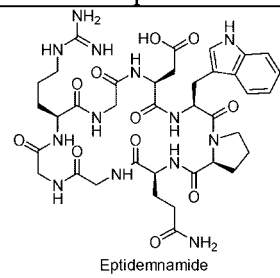
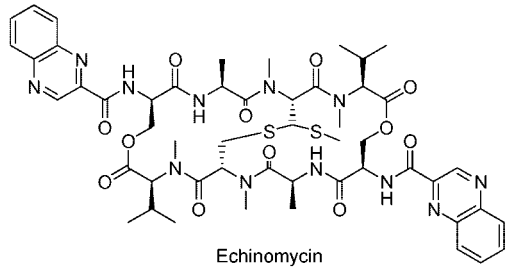
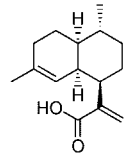
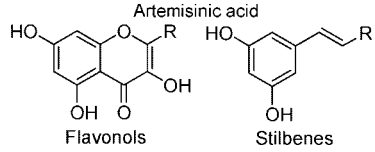
Terpenes and Alkaloids. In addition to NRPS and PKS, terpenoids and alkaloids are important natural product families with unique structural features and important pharmaceutical properties. Most of these natural products are produced by plants, which complicates efforts in the characterization and engineering of these biosynthetic pathways. Terpene synthases have been rationally engineered to achieve relaxed specificity and improved activity.[30–32] Recently, the strictosidine synthase (STR), which catalyzes the enantioselective condensation of tryptamine and secologanin, the first committed step in the biosynthesis of over 2000 terpene indole alkaloids in plants, has been successfully engineered. STR was shown to accept tryptamine analogues to produce novel alkaloid structures in both seedlings and hairy root cultures of *Catharanthus roseus*.[33] This was followed by rational redesigning the STR to selectively accommodate secologanin substrate analogues.[34,35]

Combinatorial Biosynthesis in *E. coli*

Many natural product hosts are slow-growing and/or genetically intractable; thus, it is desirable to transfer portions or an entire biosynthetic pathways to a more suitable host. Various bacteria (both Gram-positive and -negative), fungi, yeasts, and even plants have served as hosts for studying and engineering biosynthesis of natural products. The ultimate host for heterologous biosynthesis is invariably *E. coli*. The fast growth, exceptionally straightforward genetic protocols, and well-understood primary metabolism make *E. coli* an ideal heterologous host for producing natural products of all types. Extensive engineering of *E. coli* is required for the host to be useful in natural product biosynthesis. For example, post-translational modification of carrier proteins (phosphopantetheinylation) has been introduced into *E. coli* to activate PKS and NRPS assembly lines.[36] New metabolic pathways must also be introduced into *E. coli* to supply the precursors needed for PKS and NRPS assembly lines, such as acyl-CoA building blocks, unnatural amino acids, deoxysugars for post-PKS glycosylation, and essential cofactors for other tailoring enzymes. All these efforts have led to improved *E. coli* strains for the combinatorial biosynthesis of natural and "unnatural" natural products.

Total biosynthesis of macrolides such as erythromycin in *E. coli* has been well-established,[36] and different metabolic engineering strategies to improve the yield of the target compounds

Table 1. Selected Recent Examples of Natural Products Biosynthesized by Engineered *E. coli*.

Type	Compounds	Natural hosts	Ref
Peptides	 Eptidemnamide	cyanobacteria	39
NRPs	 Echinomycin	Various bacteria including *Streptomyces lasaliensis*	40
Terpenoids	 Artemisinic acid	Plants	41, 42
Polyketides	 Flavonols Stilbenes	Plants	43, 44

have been documented.[37] A recent example is the biosynthesis of cyanobacterial secondary metabolites, which have attracted increasing interest because of their large structural diversity and potent bioactivity in various bioassays.[38] However, the main obstacle to harvest these chemically diverse bioactive metabolites is the limited supply, as many cyanobacteria have so far resisted all cultivation attempts. Recently, *E. coli* has been demonstrated as a good host for functionally expressing the biosynthetic genes of these bioactive compounds (Table 1). Insights into the pathway evolution of natural combinatorial peptide libraries in cyanobacterial symbionts of marine ascidians have led to engineered biosynthesis of new compounds in *E. coli*.[39] Another example is the recent de novo total biosynthesis of echinomycin in *E. coli*, which provided a powerful platform for detailed characterizations and rapid engineering of NRPS biosynthetic pathways.[40] *E. coli* has also been successfully engineered to produce terpenoids, flavonoids, and stilbenes using plant-derived biosynthetic genes. Terpene building block isopentenyl pyrophosphate (IPP) biosynthetic genes and synthetic terpene cyclase gene have been inserted into *E. coli*, which led to the synthesis of artemisinin precursor amorphadiene in high titers.[41] This work was followed by engineered expression of plant P450 oxidases in *E. coli* toward the biosynthesis of the advanced intermediate artemisinic acid in *E. coli.* The functional reconstitution of nonnative, heme-dependent P450s significantly expands the biosynthetic capabilities of *E. coli*.[42] In the case of flavonoids and stilbenes biosynthesis, three separate pathways, including those for CoA substrate synthesis, polyketide synthesis, and post-PKS tailoring have been reconstituted in *E. coli*, leading to production of many natural and unnatural flavonoids and stilbenes in this simple microorganism.[43,44]

In conclusion, many technological advances in the past several years have significantly increased the capabilities of combinatorial biosynthesis. The combination of biochemical, structural, protein engineering, and metabolic engineering tools have pushed the state of the art to a new level. These examples also illustrate the multidisciplinary nature of research activities in this field, and this trend is likely to continue in the next decade and beyond.

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Biographies

Wenjun Zhang received her B.Sc. in Biochemistry from Nanjing University in 2002 and obtained her M.S. in Biochemistry and Molecular Biology from Nanjing University in 2004. She is currently a doctorial candidate in the Chemical and Biomolecular Engineering Department at UCLA.

Yi Tang received his Ph.D. in Chemical Engineering from California Institute of Technology under the mentoring of Prof. David Tirrell. As a postdoctoral fellow, he worked with Prof. Chaitan Khosla at Stanford University in polyketide biosynthesis. He is currently an Assistant Professor at the Department of Chemical and Biomolecular Engineering at UCLA (since 2004), and his research laboratory is focused on natural product biosynthesis and biocatalysis.

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