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Biosynthesis-based artificial evolution of microbial natural products

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Natural products are often secondary metabolites in living organisms with a wide variety of biological activities. The diversification of their structures, aiming to the search for biologically active small molecules by expanding chemical and functional spaces, is a major area of current interest in synthetic chemistry. However, developing synthetic accessibility and efficiency often faces challenges associated with structural complexity. Synthetic biology has recently emerged and is promising to accomplish complex molecules; by contrast, the application to structural diversification of natural products relies on the understanding, development and utilization of compatible biosynthetic machinery. Here, we review the strategies primarily concerning the artificial evolution of microbial natural products whose biosynthesis features template enzymology, including ribosomally synthesized and post-translationally modified peptides as well as the assembly line-resultant polyketides, non-ribosomal peptides and hybrids. The establishment of these approaches largely facilitates the expansion of the molecular diversity and utility through bioengineering at different stages/levels of biosynthetic pathways.

biosynthesis, artificial evolution, RiPPs, NRPS, PKS, enzymatic diversity

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1 Introduction

Natural products are the compounds originated from living organisms especially microorganisms, which universally have complex structures and form the foundation for many drugs currently in commercial use or in development [1]. They are, in the wider sense, molecules that can be subdivided according to their functions (such as antibiotics, vitamins, toxins) or to their chemical compositions (such as peptides, polyketides, polysaccharides, isoprenoids) (Figure 1). The traditional routes of developing new drugs, based on natural product discovery, are often accompanied with difficulties and risks, due to high rates of rediscovery [2]. Therefore, researchers' attention has been attracted to the artificial evolution of the existing natural products, with the purpose of exploiting biological activities or optimizing physical properties [3].

The artificial evolution of natural products has provided great quantities of new drugs with less manpower and financial investment; for example, ampicillin [4], clarithromycin [5], artemether [6], and spinetoram [7] have been developed, which in turn inspires researchers to exploit more effective methods and tools for the rapid production of chemical libraries. Early efforts were primarily focused on the use of synthetic chemistry; however, the difficulties associated with chemical synthesis limited its application as drug leads for further development. Nevertheless, the elucidation of biosynthetic mechanisms in recent years has been dramatically promoted by the advances in genome sequencing and data anal-

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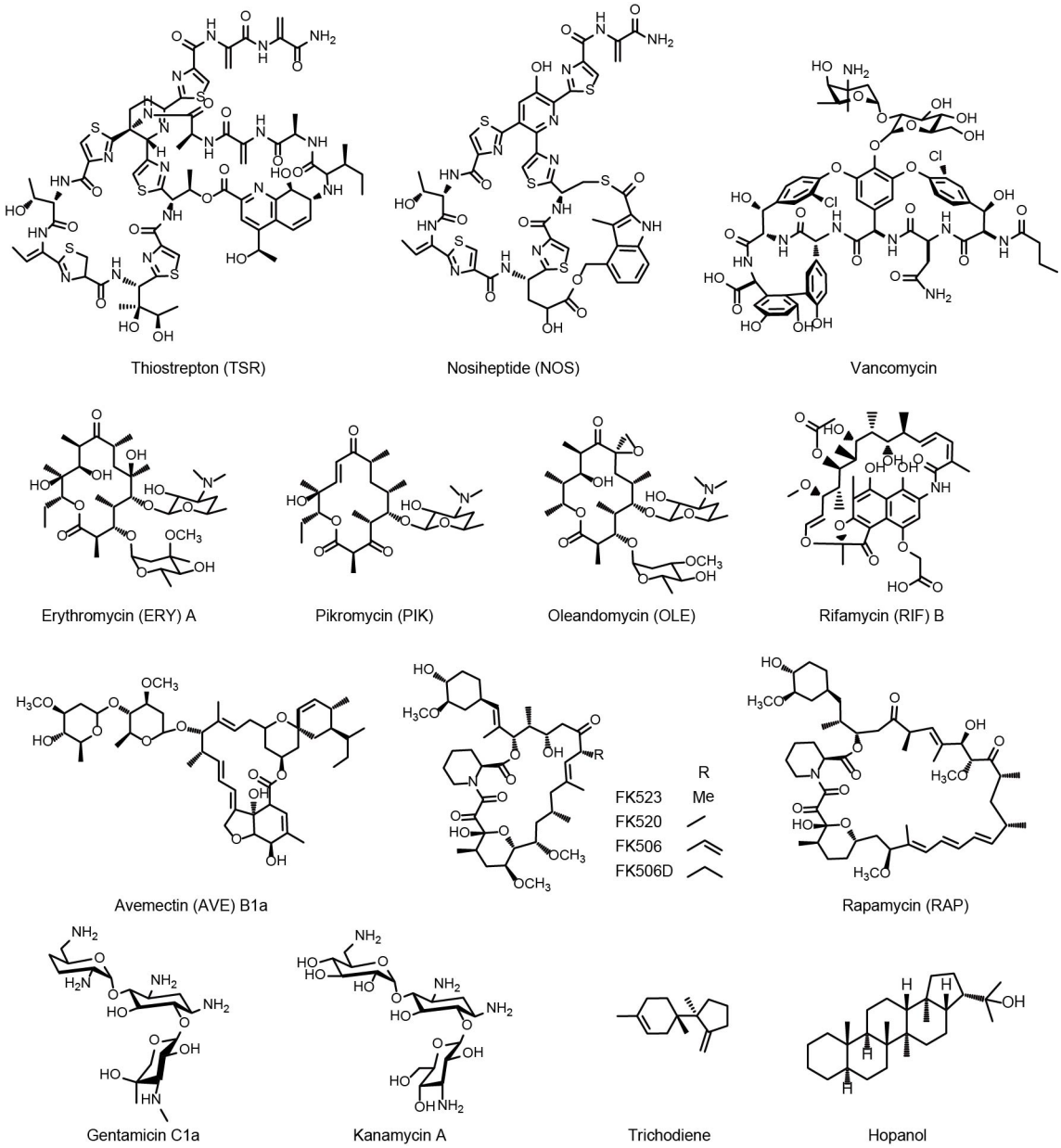

Figure 1 Structures of several representative natural products. The compounds exhibit diverse chemical features and potent biological activities, accounting for a large proportion of the valuable chemicals currently in use or in development.

ysis. In combination with the progress in biotechnology and methodology, bioengineering is widely accepted at present as a complement to chemical methods for the artificial evolution of natural products [8].

Over billions of years, nature's way for the evolution of natural products under environmental pressure has been driven by mutation and recombination, which is also the bioengineering principle for the development of novel derivatives. Based on the concept of "learning from nature", "unnatural" natural products are yielded by the manipulation of genes and correlative enzymes, or even by the reprogramming of organisms, which exploits a novel field known as synthetic biology. Notably, the process of artificial evolution has been markedly accelerated by the application of powerful strategies [8]. Herein, we would like to summarize the main strategies that have been applied within this field in accordance with the different biosynthetic origins of microbial natural products, which may provide convenience for the follow-up developments.

2 Ribosomally synthesized and post-translationally modified peptides

Ribosomally synthesized and post-translationally modified peptides (RiPPs), consisting of thiopeptides, lantipeptides, lasso peptides, microcins, and others, are a rapidly growing

class of natural products with diverse structures and activities. As a newly excavated wealth of pharmaceutically important molecules, the biosynthetic pathway to RiPPs, which is called post-ribosomal peptide synthesis (PRPS), has been explored deeply. Nearly all the compounds produced by PRPS are initially synthesized as longer precursor peptides encoded by structural genes, typically 20–110 residues in length, which are then processed by post-translational modifications (PTMs) to yield mature structures (Figure 2) [9]. The precursor peptides can be divided into two sections, namely core peptides and leader peptides, on the basis of whether they are modified by tailoring enzymes. In most RiPPs, the leader peptide is appended to the N-terminus of the core peptide, but in some rare examples, such as bottromycins, it is appended to the C-terminus termed as "follower peptide" [10]. Before the generation of mature compounds, the leader peptides are mostly removed.

With the current progress in understanding PRPS, a common feature has been uncovered that the biosynthesis of RiPPs depends on a leader peptide-guided mechanism. Most PTM enzymes specially recognize the leader peptide, and they are highly tolerant of mutations in the core peptide. Recently, the discovery of similar leader peptide-binding domains in unrelated PTM enzymes effectively supported the correctness of the leader peptide-guided mechanism [11]. The features of PRPS indicate that the strategies applied to the evolution of RiPPs have their own characteristics. Herein, we choose some representative examples, especially those related to the manipulation of thiopeptides [12], to exemplify the universal strategies used for the artificial evolution of RiPPs.

As a major class of RiPPs, thiopeptide antibiotics feature sulfur-rich, highly modified macrocyclic peptide bearing several azoles (or azolines) and often possess multiple dehydrated amino acid residues. A defining feature of the thiopeptide macrocycle is a six-membered nitrogenous ring

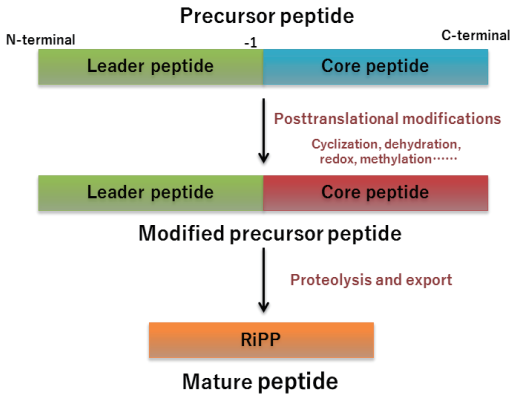

Figure 2 General biosynthetic pathway for RiPPs. The precursor peptide contains a core region that is transformed into the mature product (color online).

that can be presented in one of three oxidation states: piperidine, dehydropiperidine, or pyridine [12]. Many members in this family exhibit potent activities against various drug-resistant bacterial pathogens. In the past years, more and more thiopeptide biosynthetic gene clusters have been identified, including thiocillin, thiostrepton (TSR), nosiheptide (NOS), thiomuracin, cyclothiazomycin, GE37468, and others [13]. In addition to the precursor peptide, at least six proteins are found within each cluster, which provides the minimal set of post-translational modifications required to construct the defining thiopeptide scaffold. Those proteins are [4+2] cycloaddition enzyme, dehydratase, cyclodehydratases and dehydrogenases, responsible for the formation of the central pyridine/dehydropiperidine/piperidine, dehydroalanine (Dha, or dehydrobutyrine, Dhb) residues and azoles (or azolines), respectively. The development in understanding the biosynthetic mechanism of thiopeptide guides the rational application of biotechnology for pathway engineering, which have facilitated the development of a number of new thiopeptide analogs [12,14].

2.1 General strategies: editing core peptide-encoding genes and engineering PTM enzymes

The structural gene-encoded nature of the precursor peptides coupled with the high-level promiscuity of PTM enzymes make the artificial evolution of RiPPs possible, which mainly focused on the manipulation of precursor peptides and the engineering of PTM enzymes.

2.1.1 Editing core peptide-encoding genes

The leader peptide-guided biosynthetic mechanism of RiPPs facilitates the point mutation study of core peptide-encoding genes as a major strategy in the evolution of RiPPs. For instance, the research groups of Walsh and Kelly, as well as our own have developed thiopeptide libraries by introducing single-residue mutations into the core peptides of thiocillins [15,16], GE37468 [17], and TSR [18–20] (Figure 3(a), (b) and (d)). Moreover, the same strategy was applied to engineer lasso peptides, leading to the discovery of a number of analogs with improved activities and physical properties [21]. In addition, a technique was developed to explore the chemical space of GE37468 rapidly through codon randomization, which may be readily adapted to other RiPPs. This methodology applied high-throughput matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry to sample mutants that permitted full maturation of the antibiotic, resulting in a much more rapid evaluation of the analogs produced by the colonies in solid media [22].

Another common strategy based on the characteristics of PRPS is to expand or reduce the core peptide-encoding genes to obtain macrocyclic peptide analogs of various sizes. These studies, including the deletion of Thr3 and the introduction of

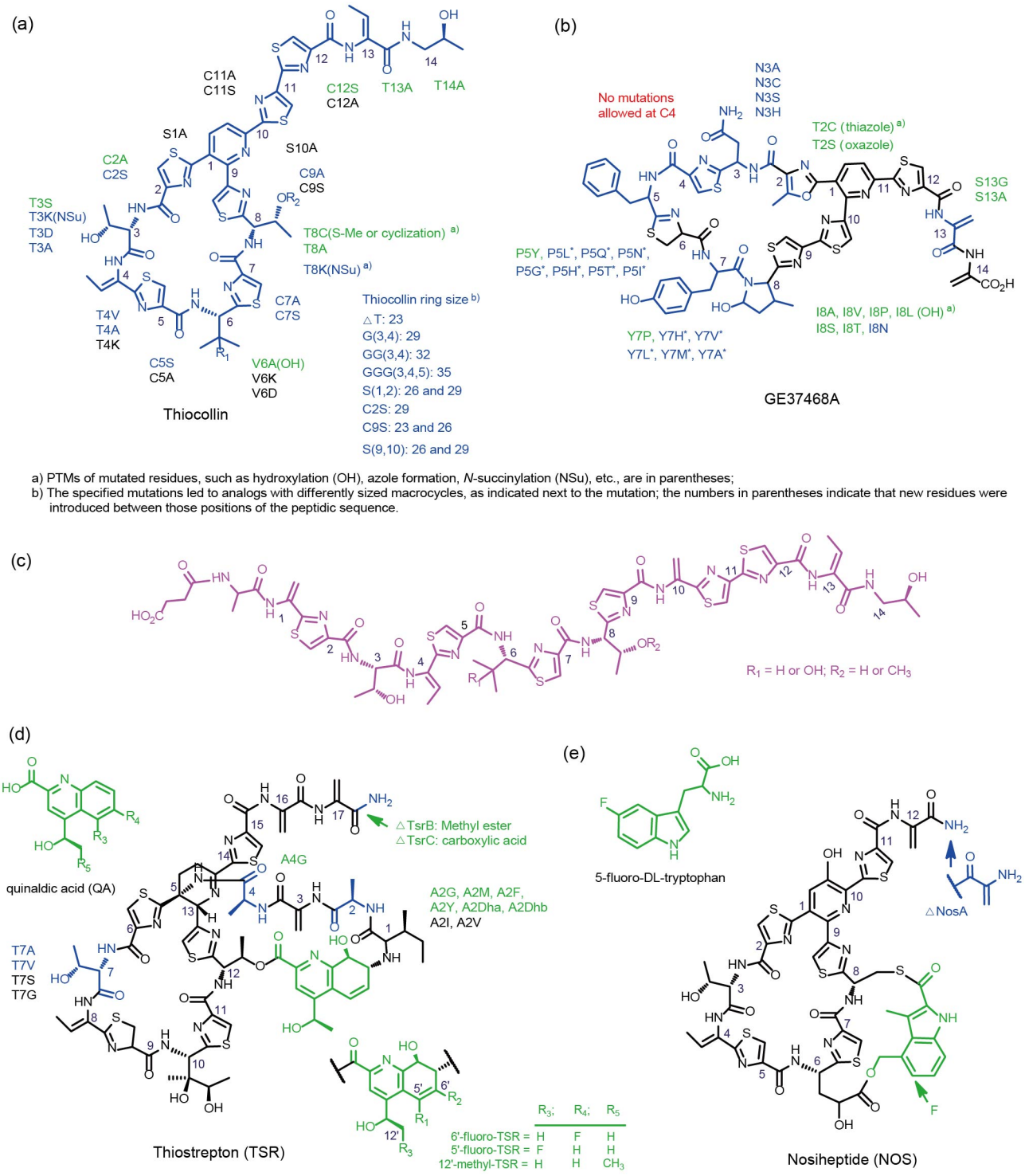

Figure 3 Production of analogs by pre-peptide mutagenesis, by PTM-encoding gene deletion, or by feeding with modified building blocks. (a) Thiocillins; (b) GE37469; (c) the linear thiocillin precursors isolated from fermentations of the *tclM* knock-out strain; (d) TSR; (e) NOS. Modifications that produced active analogs are indicated in green. Residue replacements that resulted in non-active mature products are shown in blue, and those that did not produce the mature analog or did not provide high enough yields for testing are shown in black. A star highlights the mutations that resulted in altered downstream processing; Δ is equal to deletion; QA and 5-fluro-*DL*-tryptophan were used for feeding experiments; Dha and Dhb refer to dehydroalanine and dehydrobutyrine (color online).

up to three extra glycine residues between the Thr3 and Thr4 of thiocillin, gave rise to analogs containing 23, 29, 32, and 35-atom macrocycles. Notably, the 23 and 32-membered rings are not found in any naturally occurring members of the thiopeptide family. Besides, the incorporation of a new serine residue, which might be transformed into Dha, could also produce new compounds with different ring sizes [23] (Figure 3(a)). The strategies of engineering the

core peptide-encoding genes mentioned above could be universally applicable to the artificial evolution of RiPPs, as inspired by their biosynthetic features [21].

2.1.2 Engineering PTM enzymes

With the discovery of more and more PRPS machinery, the importance of PTM enzymes to the structural diversity of RiPPs has attracted wide attention. It is also an effective strategy for generating RiPP analogs by purposefully knocking out the correlative encoding genes. For example, our group has obtained the terminal methyl ester derivative of TSR through the inactivation of TsrB, which is one order of magnitude more potent than the natural amide. We have also gained the terminal carboxylic acid derivative of TSR with improved solubility through the inactivation of TsrC [24]. This strategy also succeeded in NOS, the C-terminal amide of which was formed after the cleavage of the last Dha residue of the structural peptide by NosA. The derivative of NOS with an extra Dha amino acid was yielded by knocking out *nosA* [25] (Figure 3(d) and (e)). Moreover, the inactivation of the methyltransferase NosN resulted in the production of an analog displaying the same extended tail, but without the second macrocycle. This finding demonstrated that NosN was responsible for the 4′-methylation of the indolic acid moiety, which was required for subsequent cyclization [26]. The deletion of *tclM* produced linear thiocillin precursors with no biological activity, but it demonstrated that TclM was the enzyme responsible for the cycloaddition step [27] (Figure 3(c)). With these inactivation experiments, both the biosynthetic pathway and the *in vivo* production of analogs can be explored synergistically. It is worth mentioning that in most cases, the inactivation of the PTM enzymes responsible for the late-stage modification during the maturation process is likely to produce mature analogs, while the inactivation of the PTM enzymes responsible for the early-stage modification may fail, because of the substrate specificity of the follow-up enzymes. Therefore, reforming PTM enzymes to fit more unnatural substrates is necessary for the evolution of natural products, which will be discussed in the fourth section.

2.2 Special strategy: feeding with modified building blocks

In some RiPPs, a few special moieties whose formation is independent of the precursor peptides are generated by incorporating the corresponding building blocks into the core system, which means that feeding experiments with the analogs of such building blocks are a possible strategy for modifying these RiPPs. One representative group of these RiPPs is the bimacrocyclic thiopeptides, with TSR and NOS as examples [28]. Both compounds contain a side ring system whose formation shares *L*-tryptophan as a common substrate but can proceed in completely different ways to afford variable groups, as exemplified by the quinaldic acid (QA) and indolic acid (IA) moieties. The formation of the QA moiety in TSR biosynthesis involves the methyl transfer onto *L*-tryptophan and the subsequent rearrangement to produce a quinoline ketone as the key intermediate. Meanwhile, the IA moiety formation in NOS biosynthesis requires a radical-mediated enzymatic reaction, resulting in a typical 3-methyl-2-indolic acid intermediate. Analogs of thiopeptides that contain modified QA and IA moieties have been obtained by incorporating the correlative modified building blocks into the biosynthetic pathways. The modified precursor 5-fluoro-*DL*-tryptophan was fed to wild-type *Streptomyces actuosus*, resulting in the generation of a fluorinated NOS derivative [29]. Feeding 6-fluoro-QA to a mutant strain of *Streptomyces laurentii*, in which the *in vivo* conversion of *L*-tryptophan was blocked with the disabled methyltransferase TsrT, yielded a 6′-fluoro-TSR analog [28]. The use of this strategy completely prevented the competition from the native precursors and only the desired analog was obtained. In both cases, fluorination resulted in increased *in vitro* potencies, thereby highlighting the huge potential of this feeding method. Recently, our group [30–32] designed and obtained more TSR derivatives that varied in terms of the QA moiety by using this strategy. Some of the derivatives, namely 5′-fluoro-TSR and 12′-methyl-TSR, showed improved pharmaceutical properties (Figure 3(d) and (e)). All these successful examples demonstrated the feasibility of mutasynthesis for the purposeful engineering of the RiPPs that contained independent building blocks.

3 Polyketides and non-ribosomal peptides

Polyketides and non-ribosomal peptides represent a great variety of natural products that exhibit enormous structural diversity and biological activity. Nowadays, the optimization of the existing polyketides or non-ribosomal peptides by specifically chosen structural modifications is preferred, which requires a profound understanding of their biosynthetic mechanisms [33]. The biosynthetic machinery of polyketides frequently relies on a typical multienzyme megacomplex named modular polyketide synthase (PKS), in which simple acyl-coenzyme A (CoA) monomers are programmed into complex macrocyclic or linear polyketides [34]. Independent of the ribosomal pathway, the biosynthesis of non-ribosomal peptides requires another multimodular enzyme called non-ribosomal peptide synthetase (NRPS), which builds amino acids into polypeptides in a mode similar to that of modular PKS [35]. The enzymatic activities of modular PKSs and NRPSs are carried out by independent domains, which are grouped into basic working “modules” [36]. A PKS extension module should minimally contain the

following three domains: an acyl transferase (AT) domain introducing a building block into the synthase, a ketosynthase (KS) domain catalyzing a Claisen-like condensation, and an acyl carrier protein (ACP) domain carrying the intermediates with a covalent bond. Meanwhile, the PKS extension modules optionally include ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER) domains, which reduce the corresponding β-keto, β-hydroxy, and double bond in an orderly fashion, resulting in varied reduction degree and stereochemistry. Similarly, an NRPS extension module contains at least adenylation (A), condensation (C), and thiolation (T) domains for one-round amino acid transpeptidation and amide bond formation. Additionally, there are extra domains for the modifications of aminoacyl substrates or peptidyl intermediates. At the end of the modular PKS and NRPS assembly lines, a thioesterase (TE) domain frees the processing chain from the multienzyme through a hydrolysis or cyclization reaction. The released backbones may undergo further post-PKS or post-NRPS modifications to obtain mature products [34,35]. It is remarkable that the compatible biosynthetic logic leads the modular PKS, NRPS, and hybrid PKS-NRPS systems to share common manipulation strategies [36].

3.1 General strategies: genetic engineering of the multimodular enzymes

The clear correspondence between the components of the multimodular enzymes and the structures of the final metabolites makes the manipulation of polyketides and non-ribosomal peptides amenable [33]. Based on the understanding of the biosynthetic mechanism, particular structural changes in the skeleton are feasible by rational genetic engineering: the only requirement is to distinguish the responsible domain, module, or even enzyme, and then modify it by the versatile methods of gene deletion, insertion, or replacement.

To illustrate these strategies, we will discuss the successful genetic engineering of 6-deoxyerythronolide B synthases (DEBSs), the first PKSs that were identified and characterized in the 1990s [37]. DEBSs assemble one propionyl-CoA (as a starter unit) and six methylmalonyl-CoAs (as extender units) into the 14-membered skeleton, namely 6-deoxyerythronolide B (6-dEB), which will go through sequential post-PKS modifications to form the final product known as erythromycin (ERY) A (Figure 4) [38]. It should be mentioned that the general strategies used for manipulating DEBSs are also suitable to illustrate the principles for engineering NRPSs, because of the similarity in the structures and functions of the two multimodular enzymes.

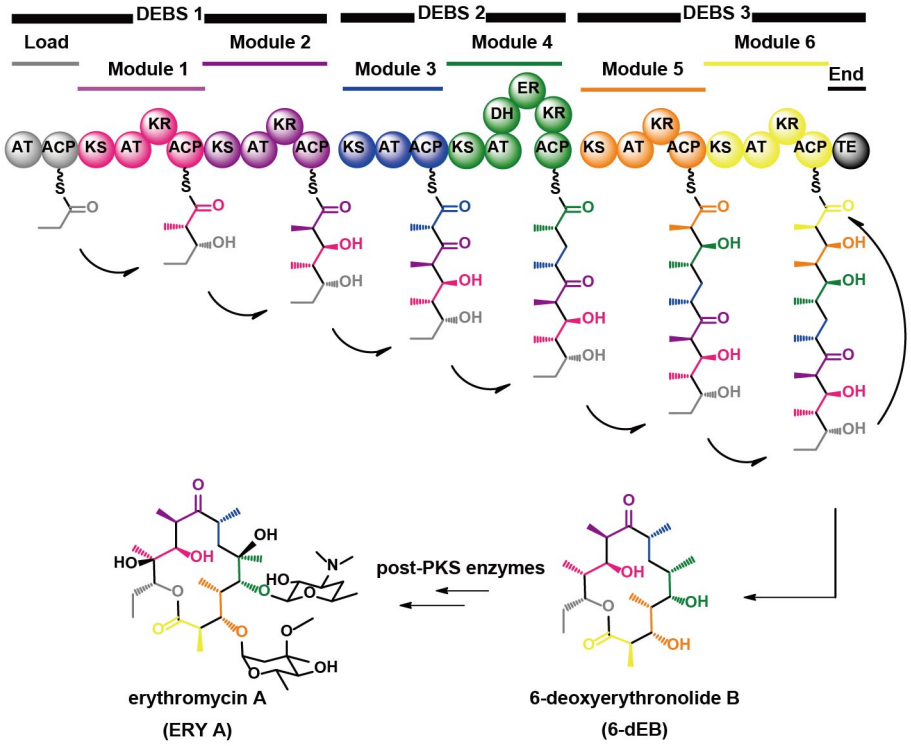

Figure 4 The ERY PKSs (DEBSs) responsible for the biosynthesis of 6-dEB. The PKSs contain 3 gigantic subunits, namely DEBS1, DEBS2, and DEBS3, each of which comprises several modules. The loading module initiates the 6-dEB biosynthesis by introducing the starter unit propionyl-CoA. The extension modules subsequently incorporate 6 methylmalonyl-CoAs as the extender units, and reductively process the growing polyketide chain until TE terminates the assembly. Each module is composed of multiple functional domains separated by linker regions (not shown). The released intermediate 6-dEB will go through further post-PKS modifications to yield the final metabolite, ERY A [38,39] (color online).

3.1.1 Genetic engineering of DEBSs on the domain level

The manipulation of β-keto processing activities in PKS systems could be accomplished through the inactivation, deletion or insertion of the KR, DH, and ER domains. Given the minimal invasion of the PKSs, domain inactivation via site-directed mutation is likely to lead to ideal effects. Disabling KR6 (the KR domain in module 6) and ER4 by mutating the key amino acids resulted in 3-keto-substituted and 6,7-unsaturated 6-dEBs, respectively (Figure 5(a)) [39,40]. However, the attempt to generate the full-length derivative failed, even though just a single active histidine in DH4 was changed [41]. The deletion of the KR, DH and ER domains is an alternative strategy for remolding the reductivity and stereospecificity accordingly, despite the unexpected byproducts that might emerge as a consequence of the perturbed protein spatial structure. For instance, apart from the desired 3-keto-substituted 6-dEB, an unexpected product, 2-demethylated 6-dEB, also emerged when the entire KR6 domain was deleted, probably due to the affected specificity of the adjacent AT domain (Figure 5(a)) [40]. Moreover, the insertion of the heterologous KR, DH, and ER domains is a typical gain-of-function strategy that produces new analogs. The substitution of KR2 and KR6 with the DH-KR domains from module 4 of rapamycin (RAP) PKSs resulted in 10,11-dehydrated and 2,3-dehydrated 6-dEBs, respectively. Meanwhile, the 11-dehydroxylated and 5-dehydroxylated 6-dEBs were found when KR2 and KR5 were replaced with the DH-ER-KR domains from module 1 of RAP PKSs, respectively. However, no predicted 3-dehydroxyl product was detected in the case of the KR6 replacement, but the 3-keto-substituted and 2,3-dehydrated 6-dEBs were found instead (Figure 5(a)) [42]. Therefore, even though the manipulation of the KR, DH, and ER domains has been fruitful, there is still a risk that altered protein structures probably cause failures in obtaining the target derivatives.

In DEBSs, the AT domains of extension modules are specific for methylmalonyl-CoA, while the AT domain in the loading module utilizes propionyl-CoA. The replacement of the AT domains is a promising strategy for introducing distinct side chains to the skeleton. For instance, the substitution of AT1-AT6 with the AT domains specific for malonyl-CoA did create a series of demethylated 6-dEBs at the designed sites [42–45]. Similarly, with an ethylmalonate-specific AT5 of the niddamycin PKSs replacing AT4, a new erythronolide exhibiting a 6-ethyl group was generated (Figure 5(b)) [46]. Notably, unproductive AT swapping occurred frequently. On the basis of numerous experiments, the choice of domain boundaries was suggested as a critical determinant as to whether reasonable titers of novel polyketides could be produced [47]. By targeting the catalytic residues within ATs, the substrate specificity could also be altered. Comparative sequence alignments and crystal structure analyses have revealed the putative catalytic residues for different ATs. Point mutations within AT4 produced 6-demethylated 6-dEB and 6-dEB as well, due to the relaxed substrate specificity [48]. Recently, a point mutation of a valine in AT6 magnificently expanded its substrate pool by allowing the introduction of a chemo-synthesized extender unit, 2-propargylmalonate (Figure 5(b)) [49]. More sophisticated methodology for AT domain engineering is required to make larger polyketide libraries realistic.

The relocation of the TE domains could advance the release of premature polyketide chains, which significantly influences structural identities. By moving TE from its native site to the C-terminus of DEBS1 where it was fused to ACP2, a triketide lactone was generated (Figure 5(c)) [50]. The resulting DEBS1-TE system has frequently served as a model for researching the biosynthetic mechanisms of PKSs. The heterologous expression of DEBS3 in *Streptomyces coelicolor* CH999 produced another triketide lactone (Figure 5(c)). In this case, the extender unit methylmalonyl-CoA was turned into the starter unit propionyl-CoA via decarboxylation, and KR5 instead of KR1 determined the stereospecificity of the 5-ethyl group [51]. Notably, the TE domains showed great tolerance to the length of different polyketides. The fusion of TE to ACP5 yielded a novel 12-membered lactone (Figure 5(c)) [52]. TE could also accept recombinant multienzyme systems and offload hybrid intermediates. In comparison with other domain-level manipulation strategies, TE relocation is more likely to obtain the expected products at acceptable titers.

3.1.2 Genetic engineering of DEBSs on the module level

In modular PKSs and NRPSs, each catalytic cycle is proceeded by one module. Accordingly, the manipulation of a given module is thought to alter the corresponding catalytic activity. The exchange of the two loading modules of DEBS1 and the avermectin (AVE) PKS in the ERY-producing *Saccharopolyspora erythraea* resulted in novel erythronolide derivatives, including the 2-*iso*-propyl and 2-*sec*-butyl 6-dEBs, which were derived from the incorporation of the branched-chain starter units characteristic of AVE (Figure 5(d)) [53]. Profited from the relaxed substrate specificity of the AVE loading module, a total of 12 derivatives were harvested by supplying the recombinant strain with various short-chain fatty acids [54]. In addition to the loading module swapping, the disabling of the loading module is also functional in structural evolution. Mutasynthesis occurs when unnatural starter units are fed to the mutant strains in which the generation or incorporation of the starter units are disabled, and allows for the production of more structurally modified derivatives. This strategy permits the efficient incorporation of unnatural starter units because the preference in the choice of natural ones has vanished.

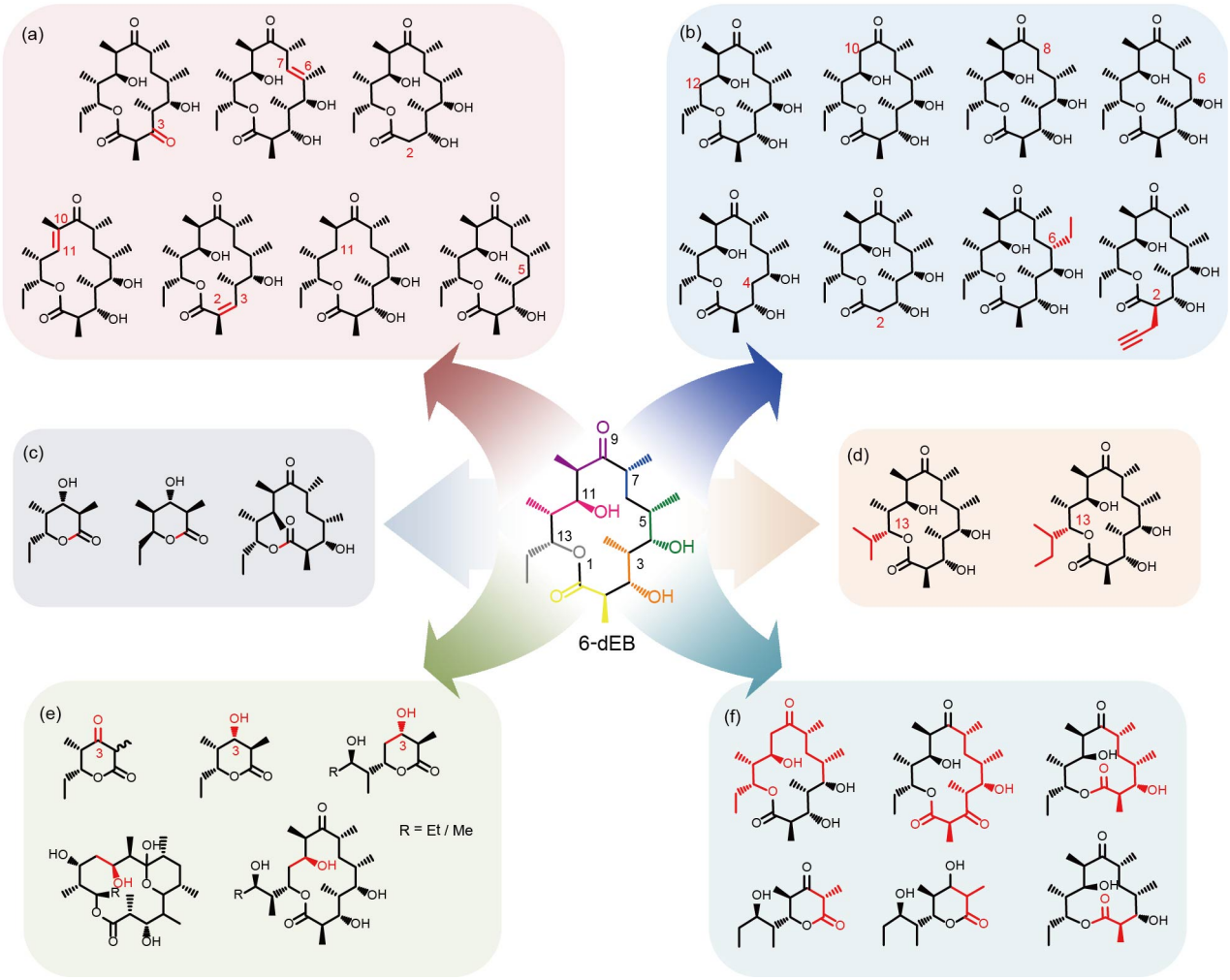

Figure 5 The 6-dEB analogs generated by manipulating DEBSs, including the inactivation, deletion or insertion of the KR, DH, and ER domains (a), the replacement or mutation of the AT domains (b), the relocation of the TE domains (c), the substitution of heterologous loading modules (d), the substitution or insertion of heterologous extension modules (e), and the reconstruction of subunit interactions (f). The modified and introduced moieties are color-coded in red (color online).

The manipulation of extension modules to modify DEBSs has been proved approachable. By using the DEBS1-TE mini-PKS system, new triketide lactones were yielded when the original module **2** was substituted by downstream module **3** and module **6**, as well as module **5** from the rifamycin (RIF) PKSs (Figure 5(e)) [55]. The importance of intermodular linkers has aroused a great deal of attention, specifically the essential contact between ACP_n and KS_{n+1} responsible for the transference of processing polyketide chains [56]. An attempt was made to insert modules **2** or **5** from the RAP PKSs into modules **1** and **2** in the DEBS1-TE system, with the aim of adding an extra round of chain extension. However, the major product was not the proposed tetraketide, but the unexpected triketide. Meanwhile, the insertion of modules **2** or **5** from the RAP PKSs into modules **1** and **2** in DEBSs produced the original heptaketide erythronolide as the most abundant product, instead of the octaketide macrolactones in the 14-membered or 16-membered form (Figure 5(e)) [57]. An explanation given on these phenomena was that in skipping of the interpolated modules, the polyketide chain was handed off directly from ACP1 (DEBS) to ACP2 (RAP) instead of to KS2 (RAP), due to the poor substrate recognition by KS2 (RAP) [58]. These examples suggested that the native ACP_n-KS_{n+1} interface should be maintained to ensure the accessible transfer of polyketide chains.

3.1.3 Genetic engineering of DEBSs on the subunit level

The transference of the processing intermediates between different polypeptides is mediated by the C- and N-termini of the subunits. These independent-folding regions, termed docking domains, play important roles in the communication that occurs between pairs of multimodular enzymes [59]. Hybrid PKSs were constructed in *Streptomyces lividans*, in which the first two subunits from pikromycin (PIK) PKSs are combined with DEBS3 or the third subunit of the oleandomycin (OLE) assembly line. The chimeric PKSs produced the ideal 14-membered lactones, showing the inherent ability of evo-

lution-related subunits to interact productively (Figure 5(f)) [60]. With regards to less-related proteins, the engineering of docking domains is required to enable recognition. To facilitate the chain transfer from DEBS1 to the foreign acceptor module, the N-terminal docking domain of DEBS2 was incorporated at the N-terminus of the PIK and RIF modules [61,62]. Similarly, the interactions between DEBS2 and two non-native modules, namely module **6** in DEBS3 and module **5** from the RIF PKSs, were constructed by using the N-terminal docking domain of DEBS3, resulting in the expected hexaketide (Figure 5(f)) [62]. The suitable transplantation of docking domains is necessary to create novel hybrid metabolites by directing the correct chain delivery.

3.2 Special strategies: engineering unusual precursors and tailoring modifications

The structural diversity of polyketides and non-ribosomal peptides can be attributed to many affecting elements. In addition to the above-mentioned chain length, reduction degree and stereochemistry, the introduction of unusual precursors as well as modifications by tailoring enzymes are also pivotal factors. For the former, unusual starter and extender units have been elucidated to add chemical complexity to the skeleton; for the latter, particular tailoring enzymes have been highlighted to manage structural diversity at the specific sites [63].

3.2.1 Engineering unusual precursors

Recently, the discovery of unusual precursors has accelerated exploitations of the promiscuity that occurs in PKS and NRPS biosynthetic machinery. In modular PKS systems, rare moieties such as alkynes, branched-alkyl chains, halogenated pyrroles, and aromatic chains are incorporated into the thiotemplate-driven assembly lines [64]. AT, the gatekeeper domain in modular PKSs that controls the utilization of extender units, is responsible for introducing most of these unusual PKS building blocks. In NRPS systems, nonproteinogenic amino acids present diverse structural features [65]. The entry of these amino acid substrates is normally governed by the A domains. During the rational design of modular PKS and NRPS manipulation, precursor engineering is a complementary strategy to gain more complex side chains.

To explain this strategy, we would like to emphasize the precursor engineering of FK506, also known as tacrolimus. FK506 as well as its native analogs, FK520 and RAP, are famous for their immunosuppressant and antifungal activities, whose biosyntheses are performed by hybrid PKS-NRPS systems [66–68]. The PKSs in FK506 biosynthesis feature the incorporation of four unusual precursors known as 4,5-dihydroxycyclohex-1-enecarboxylic acid (DHCHC) [69], allylmalonyl-CoA [70], methoxymalonyl-ACP [71], and *L*-pipecolate [72], whose biosyntheses are considered pathway-specific (Figure 6).

FK506, FK520 and RAP possess common cyclohexane and pipecolate moieties, arising from the incorporation of DHCHC and *L*-pipecolate, respectively [73]. DHCHC, derived from chorismic acid, is generated by FkbO (or RapK) and incorporated into the assembly lines as a PKS starter unit [69]. Mutations via the in-frame deletion of *fkbO* and *rapK* disabled the production of FK506, FK520 and RAP. Synthetic analogs of DHCHC were fed to the mutant strains, resulting in numerous derivatives with varied cyclohexane moieties [74–77]. In comparison, *L*-pipecolate functions as the only NRPS extender unit, which is catalyzed by FkbL (or RapL) through the cyclodeamination of *L*-lysine [72]. Supp-

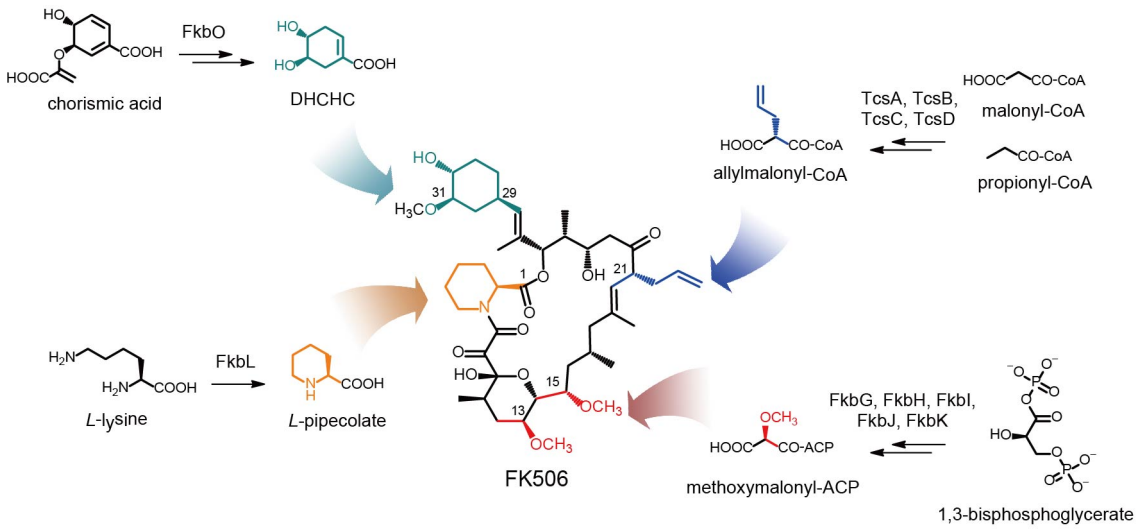

Figure 6 FK506 biosynthesis incorporating 4 pathway-specific precursors. DHCHC, the PKS starter unit, is generated by FkbO with chorismic acid as the substrate. Allylmalonyl-CoA and methoxymalonyl-ACP, the PKS extender units, are generated by two sets of enzymes encoded by the sub-gene clusters. *L*-pipecolate, the NRPS extender unit, is generated by FkbL by using *L*-lysine as the substrate (color online).

lying the *rapL*-deleted mutant strain with synthetic *L*-pipecolate analogs generated various RAP derivatives [78]. These successful examples of precursor-directed mutasynthesis demonstrated that the removal of competition from the natural precursors allows for the more efficient incorporation of mutasynthons.

The structural characteristics of FK506 relay greatly on the 21-allyl group, which is derived from a unique PKS extender unit called allylmalonyl-CoA. Its biosynthesis depends on a non-canonical discrete PKS system composed of four enzymes, TcsABCD [70]. AT4 from the FK506 PKSs is in charge of allylmalonyl-CoA introduction, which also naturally accepts propylmalonyl-, ethylmalonyl-, and methylmalonyl-CoAs, resulting in the generation of FK506D, FK520, and FK523, respectively [79]. The in-frame deletion of *tcsB* could totally block the production of FK506 and FK506D, but not FK520 or FK523, whose intracellular precursors had not been knocked out [80]. This mutasynthesis created a relatively clean background not capable of producing the initial major products. Under these circumstances, feeding experiments with unnatural acyl-CoAs efficaciously generated FK506 derivatives with modified C-21 side chains. A recombinant biosynthetic pathway was constructed by biologically complementing the *tcsB*-deleted mutant strain with heterologous genes that encoded the biosynthesis of isobutenylmalonyl-CoA, yielding a new metabolite, 36-methyl-FK506 [81]. Currently, the significant impact of crotonyl-CoA carboxylase/reductase (CCR) has attracted extensive attention for the reductive carboxylation of α,β-unsaturated acyl precursors could purposefully provide unusual PKS building blocks [82]. We have previously demonstrated the prevalence of a functional CCR named AntE in the mixed NRPS-PKS assembly line for antimycin (ANT) biosynthesis. AntE exhibited remarkable tolerance towards a variety of enoylated CoAs, showing its potential as a useful tool in synthetic biology by complementing various polyketide pathways [83]. As a promising material for extending the diversity of polyketides, AntE is now under careful protein engineering [84,85].

3.2.2 Engineering tailoring modifications

Tailoring modifications, involving enzymatic conversions in the backbones of polyketides and non-ribosomal peptides, contribute considerably to the structural diversity and biological activity of these natural products. Functionally, post-PKS and post-NRPS enzymes are classified into oxidoreductases, methyltransferases, aminotransferases, glycosyltransferases, acyltransferases, prenyltransferases, halogenases, and others [65,86]. Great effort has been made to reprogram tailoring enzymes, generating plentiful unnatural metabolites not likely accessible by chemical modifications.

Vancomycin is a famous glycopeptide antibiotic that has been subjected to systematic manipulation, leading to numerous derivatives from hybrid tailoring steps [87]. A representative example of the engineering of its tailoring modifications has focused on two glycosyltransferases, namely GtfE and GtfD, which catalyzed the glycosylation reactions in a reversible manner. The *L*-vancosamine shed from vancomycin could serve as a donor for aglycone exchange when the equilibrium of the GtfD-catalyzed reaction was altered by other preferred glycoside acceptors (Figure 7(a)) [88]. In a dual-glycosyltransferase system, a variant of OleD (a glycosyltransferase from OLE biosynthesis) was coupled to GtfE. By using 2-chloro-4-nitrophenyl glycosides as the donors, a total of 11 vancomycin-type derivatives were formed (Figure 7(b)) [89]. Glycosyltransferases exhibit significant substrate promiscuity, making them ideal materials for protein engineering.

4 Modern strategies focused on protein engineering

Based on the individual biosynthetic characteristics, the successful strategies used for the artificial evolution of the natural products with the template enzymology included the above-mentioned RiPPs, and the assembly line-resultant polyketides, non-ribosomal peptides and hybrids have their own features. For other biosynthetic natural products, researchers have also developed a number of successful approaches to obtain the designed compounds, including saccharides [90], terpenoids [91] and alkaloids [92], which largely depend on the substrate promiscuity of the corresponding biosynthetic machinery, but limitations still remain. These limitations depend on the specificity of enzymes that exist not only in the non-templated biosynthetic compounds but also in the biosyntheses of RiPPs, polyketides and non-ribosomal peptides, which results in a huge area of the chemical space that cannot be studied by using the normal strategies discussed above. Thus, expanding enzymatic diversity to fit the altered substrates has become a necessary complement that is universally applicable for nature product divarication.

The biosynthetic community specializes in characterizing new enzymes and altering the properties of known enzymes to expand their diversity. In the case of modular PKSs, the discovery of the newly characterized CCR family provided opportunities to create diverse polyketides with altered side chains [83]. For aminoglycosides, the newly characterized aminoglycoside *N*-acetyltransferases were found to be tolerant towards a variety of side chain donors and aminoglycoside acceptors, proved to be a useful tool for the preparation of a number of regioselectively *N*-acylated aminoglycosides with improved activities, such as arbekacin, amikacin, and sporaricin A [93,94]. In addition, progress has also been made in characterizing other enzymes, such as terpenoid cyclases [95], glycosyl transferases [86], oxidoreductases [96], halogenases [86], offering an exciting direction for protein en-

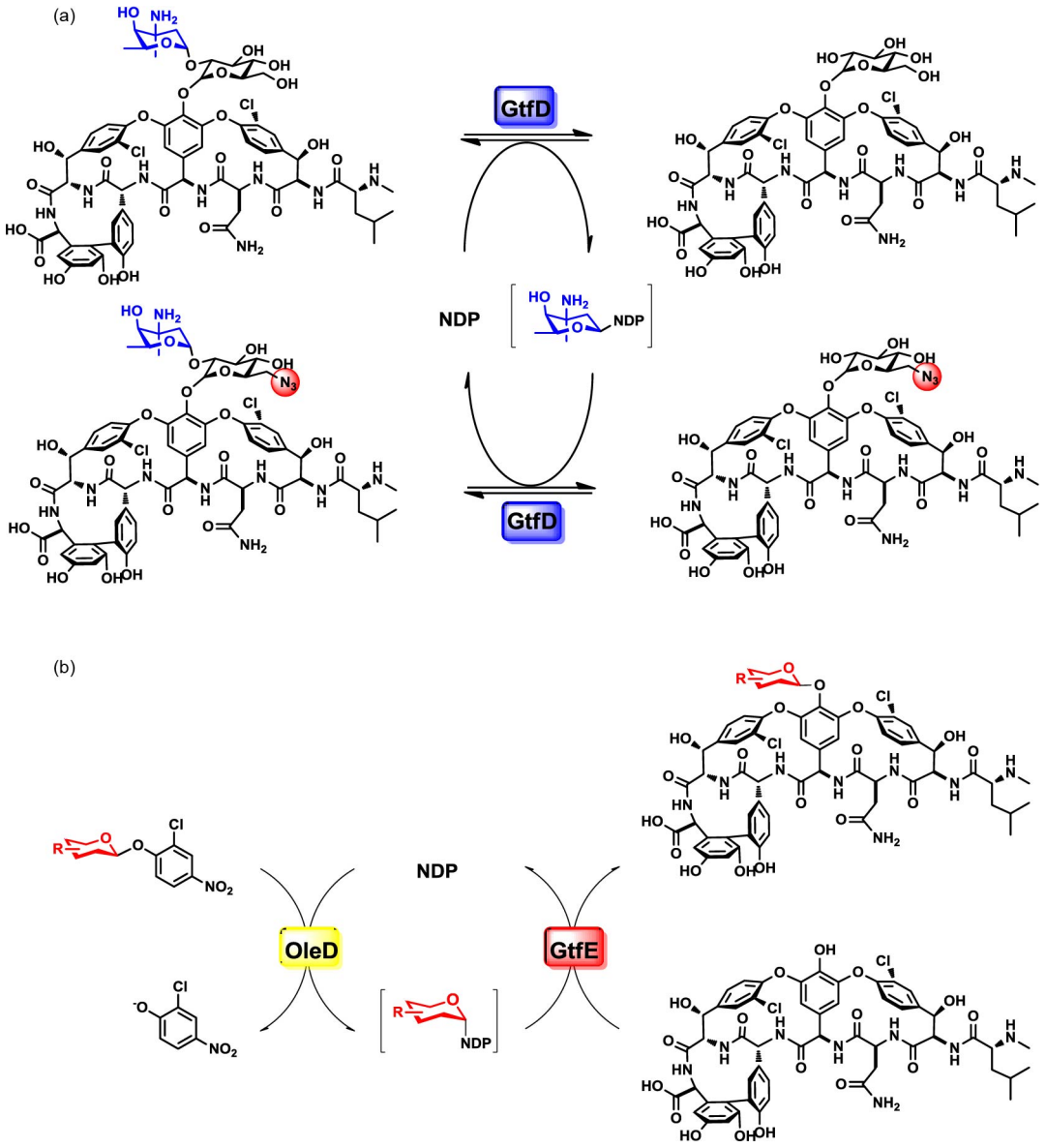

Figure 7 The utilization of GtfD and GtfE in generating vancomycin-type analogs. (a) The GtfD-catalyzed aglycon exchange reaction. The *L*-vancosamine for this reaction was generated *in situ* by a GtfD-catalyzed reverse glycosyltransfer and subsequently transferred to the unnatural 6-azidoglucose-containing derivative. (b) A two-component (GtfE and OleD) glycosyltransferase system with vancomycin aglycon as the final acceptor. The NDP-sugars were generated *in situ* by an OleD-catalyzed reverse glycosyltransfer and subsequently transferred to the vancomycin aglycon [88,89] (color online).

gineering.

For other known enzymes, modifications can be made through rational design, directed evolution (by error-prone polymerase chain reaction (PCR) or gene shuffling in combination with high-throughput screening and iterative cycles of improvement), or other engineering methods for the alteration of substrate specificity. The rational design method is mostly based on the knowledge derived from bio-information and structural data. For instance, according to the reported crystal structures of terpenoid cyclases, the active sites have a contour that is predominantly product-like, providing a promising approach for obtaining novel products by altering the contour permissiveness as product-like templates [95]. Therefore, plentiful terpenoid analogs were produced by designing the mutagenesis studies. For instance, the site-directed mutagenesis of the metal-binding residues of trichodiene synthase from *Fusarium sporotrichioides* generated 11% alternative sesquiterpene products [97,98]. Recently, the unique protonation machinery of a squalene hopene cyclase was engineered to act as a general Brønsted acid catalyst in water, enabling the highly stereoselective syntheses of various cyclohexanoids [99]. In addition to the rational design, the directed evolution method has also been successfully applied to the reprogramming of known bio-enzymes. For example, three of the most highly variant residues in the NRPS AdmK were selected for saturation mutagenesis, which generated over 14000 clones in the native producer, *Pantoea agglomerans*. The resulting library

was screened to isolate four clones that produced andrimid analogs, three of which showed increased bioactivity against *Staphylococcus aureus* [100].

5 Conclusions

Synthetic biology, as an active interdisciplinary field that combines knowledge of biology and techniques in bioengineering, holds the expectation to obtain desired natural product analogs [101]. The successful application of synthetic biology depends largely on the understanding of the biosynthetic machinery, which provides numerous productive strategies to expand the structural diversity of natural products [102]. In this context, the template-directed biosyntheses of RiPPs, polyketides, and non-ribosomal peptides are discussed. These types of compounds exhibit some generality and specificity in their biosynthetic features; on this basis, the strategies applied during their bioengineering can be classified into general and special ones. The summarization and classification of the major strategies will provide a guideline for subsequent studies. Moreover, the strategies critical to the artificial evolution of natural products are also analyzed. In the future, there will be more and more advanced approaches, allowing for the rational construction of novel compounds, not only based on the growing understanding of nature's secrets in generating valuable chemicals but also relied on the application of powerful modern technologies as useful assists.

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