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MINI-REVIEW

Genetic regulation and manipulation for natural product discovery

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Abstract Natural products are an important source of modern medical development, e.g., antibiotics, anticancers, immune modulators, etc. and will continue to be a powerful driving force for the discovery of novel potential drugs. In the heterologous hosts, natural products are biosynthesized using dedicated metabolic networks. By gene engineering, pathway reconstructing, and enzyme engineering, metabolic networks can be modified to synthesize novel compounds containing enhanced structural feature or produce a large quantity of known valuable bioactive compounds. The review introduces some important technical platforms and relevant examples of genetic regulation and manipulation to improve natural product titers or drive novel secondary metabolite discoveries.

Keywords Natural product · Regulation and manipulation · Discovery

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Introduction

Natural products are a rich source of bioactive compounds with hopefully therapeutic potential. They include compounds from plants, bacteria and fungi, as well as synthetic or semi-synthetic compounds based on natural products. This is widely accepted to be true that natural products had become a dominated drug discovery and development pathway since broadly use and research of penicillin after the Second World War (Li and Vederas 2009). According to statistics, by 1990, more than 80 % of the drugs on offer were either natural products or relative analogs or derivatives (Harvey 2008). It is, however, still true that the figure has been on the wane in more recent years due to the use of combinational chemistry for synthesizing some new drug entities/leads or novel structures and skeletons with therapeutic potential (Newman 2008). Natural products based drugs in the clinical studies fell by about 30 % from 2001 to 2008 (Li and Vederas 2009). Only 13 drugs from natural products and derivatives were on worldwide sales between 2005 and 2007 (Hong 2011). Nevertheless, this is likely to be temporary, as natural products from living organisms have complex structures with a large number of oxygen-containing substituents and an abundance of centers of stereochemistry, the potential for new drug discoveries in the longer term is enormous (Chillar et al. 2011; Chen et al. 2015b). All natural products, also referred to be secondary metabolites, appear to have no explicit role for the organism's normal growth, development, and reproduction (Vaishnav and Demain 2011). However, they are important sources of modern medical development. For instance, antibiotics had been successfully used in the treatment of tumors, cardiovascular diseases, infectious diseases, and immune diseases ((Davies and Davies 2010).

The research of natural products is a labor and resource intensive work. Genetic regulation and manipulation has

emerged in the past decade as a single field aiming to ramp up production of desirable natural products or produce valuable substances by continuous mutation and screening (Stephanopoulos 2012). The method has made the discovery of natural products win a new vitality. The most typical example is penicillin, the first anti-infective. By intensive screening and mutation using fungus, the level of production of penicillin used in industry is estimated to 100,000-fold higher yield, compared to its earliest discovered strain (Rokem et al. 2007). However, with the advent and application of new experimental tools and biotechnologies, metabolic networks, including some native and nonnatural biosynthetic routes, can be directly modified to synthesize various products (Stephanopoulos 2012). The progress of genetic engineering and enzyme engineering, development of synthetic biology, and more and more knowledge of metabolic engineering have greatly improved our ability to modify various complex metabolic networks. The dramatically reduced cost of genome sequencing technology, especially high-throughput sequencing and DNA microarrays, has made tens of thousands of available genomic data and generated a massive number of gene annotations involved in whole natural product pathways. Synthetic biology and genetic engineering have been developed for modifying and assembling some important living organisms and can be also used to guide metabolic network control, such as blocking competing pathways, increasing precursor and cofactor supply (Pickens et al. 2011), activating silent gene cluster (Rutledge and Challis 2015), or overexpression of pathway-specific regulator (Otero et al. 2010; Chen et al. 2015a). Furthermore, a dazzling array of biosynthetic enzymes is also very important for making a wide array of valuable natural products. These enzymes can be used to catalyze biological active molecules, aided by technological development in enzyme engineering. Researchers may freely construct enzymes with higher activities, stabilities, and specificities as ideal biocatalysts to produce massive numbers of the natural products and its derivatives (Zabala et al. 2012). By the use of these new tools and techniques, the metabolic networks of natural products can be used to synthesize structurally diverse metabolites, facilitate the yield improvement of the target compounds, and generate bioactive compounds with enhanced biological features, which are impractical using traditional organic synthetic methods. The mini review will highlight some concrete strategies to realize these goals.

Gene disruption and pathway reconstruction in heterologous hosts

One of the most efficient production methods for structural diversification is to delete an active gene that usually acts downstream of the whole metabolic pathway. A classic example (Fig. 1) is that the first designer novel polyene, 4, 5-deepoxypimaricin, was synthesized in *Streptomyces natalensis* by disruption of the downstream targeted gene *pimD*, which was responsible for encoding a cytochrome P450 epoxidase to convert 4, 5-deepoxypimaricin into pimaricin (Mendes et al. 2001). The inactivation of a ketoreductase (KR) *mtmW* gene located at the downstream of the whole mithramycin PKS genes led to the generation of three new mithramycin analogs, including mithramycin SK (MTM-SK) (Fig. 4), which showed better therapeutic index in the human tumor cell lines than its parent drug, mithramycin (Remsing et al. 2003). Some recent examples have also demonstrated that the downstream targeted gene disruption can generate new "unnatural" compounds. The inactivation of *ttmM* in tautomycin biosynthesis and *ptmQ* in pactamycin biosynthesis generates three tautomycin analogs (Ju et al. 2009) and two pactamycin analogs (Ito et al. 2009), respectively (Fig. 4). According to the understanding of the quinone biosynthetic pathway for ansamycin polyketides, Zhang MQ et al. (Zhang et al. 2008) used this approach to inactivate the gene of monooxygenase responsible, the mutant was created to produce a novel nonquinone macbecin compound (Fig. 4), which had 80-fold improved binding affinity to Hsp90 and significantly reduced toxicity.

Another major strategy is to create genome-minimized hosts for heterologous expression of secondary metabolism. The strategy is that by making systematically deletion for nonessential genes, one can utilize more primary metabolic building blocks, reducing equivalents as well as energy to produce desired secondary metabolites. Komatsu M et al. (Komatsu et al. 2010) recently reported that a wild-type *Streptomyces avermitilis* was systematically deleted to remove 83 % of nonessential genes, and upon introduction of the streptomycin biosynthesis gene cluster, the streptomycin productivity of the genome-minimized *Streptomyces* host was higher than that of the wild-type streptomycin producer *S. griseus* and the parent *S. avermitilis* carrying the streptomycin biosynthesis gene cluster. Furthermore, these genome-minimized mutants have also been confirmed to produce more than 20 novel bioactive compounds including polyketides (PKs), nonribosomal peptides (NRPs), and terpenes by heterologous expression (Ikeda et al. 2014). Except for directly act on *S. avermitilis* hosts, gene deletion has been also widely used to make succedaneous cell networks more high-efficient for the production of primary secondary metabolites. Chemler JA et al. (Chemler et al. 2010) constructed a genotype specifically engineered for improving the overall NADPH level and [NADPH]/[$NADP^+$] ratio for the purpose of increasing the biosynthesis of natural products in *Escherichia coli*. Through experimentally validated model and stoichiometric-based model, the researchers found *Δpgi*, *Δppc*, and *ΔpldA* gene deletion can increasingly improve NADPH yield and availability and [NADPH]/[$NADP^+$] ratio. Both of the flavonoid leucocyanidin and (+) catechin yields

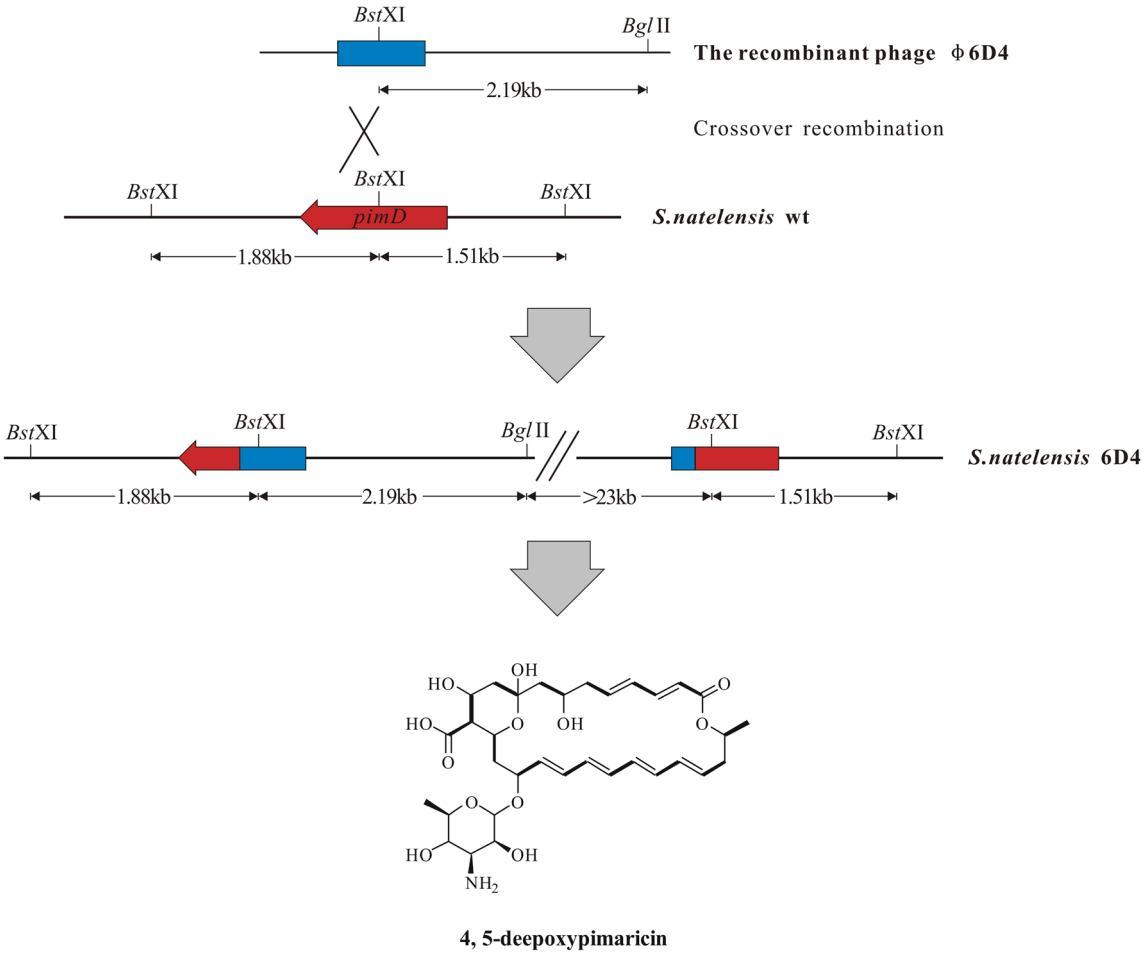

Fig. 1 Disruption of the targeted gene *pimD* in *S. natalensis* was applied to produce a novel polyene, 4,5-deepoxypimaricin. The recombinant phage Ø6D4 was used to infect *S. natalensis* wt in order to inactivate the *pimD* gene. *Red arrows* indicate the direction of transcription. *BstXI* *Bst*XI restriction endonuclease, *BglII* *Bgl*II restriction endonuclease

were also increased by a 4-fold and 2-fold, respectively, compared to the original strain. Although a mass of engineered mutants constructed here show enormous success not only for yield improvement of bioactive natural products but also for the biosynthesis of unnatural compounds, there are still a lot of knowledge about genes and cells that we are not familiar or cannot understand, and gene disruption may result in unintended results.

Rationally designing and assembling genes from metabolic networks in the succedaneous hosts

In many organisms, the original producing hosts of natural products are not optimal hosts because of their commercially unfeasible or genetically intractable. Hence, the development of succedaneous hosts, implemented for the rationally genetic manipulations and assembling of metabolic pathways, is of high interest (Pickens et al. 2011). This plug and play system will assemble biosynthetic genes from different pathways to reconstruct a functional pathway, assisting in analysis studies of biosynthesis, improving the yield of objective compounds, or facilitating manipulation of metabolic networks to synthesize novel, more bioactive or less toxic products. *E. coli* is widely used succedaneous host due to much simpler metabolic networks and easier for performing gene reconstruction. 6-Deoxyerythronolide B (6-dEB), the precursor of the antibiotic erythromycin, is a very successful example, which was synthesized in *E. coli* after plasmids were reengineered to incorporate *DEBS1*, *DEBS2*, *DEBS3*, *sfp PPTase*, *pccA*, and pcc*B* genes from *Streptomyces coelicolor* (Fig. 2). The resulting strain produced 0.1 mmol of 6-dEB in the 1 g cellular protein per day (Pfeifer et al. 2001). Subsequently, the potent antibiotic erythromycin C (Ery C) was completely synthesized in *E. coli* by constructing two artificial operons using the megalomicin gene clusters, L-mycarose, and D-desosamine, to convert 6-dEB to Ery C (Peirú et al. 2005). Taxol is an important anticancer drug only isolated from the Taxus. Hence, the production of Taxol and its analogs is extremely limited. Ajikumar et al. (Ajikumar et al. 2010) used

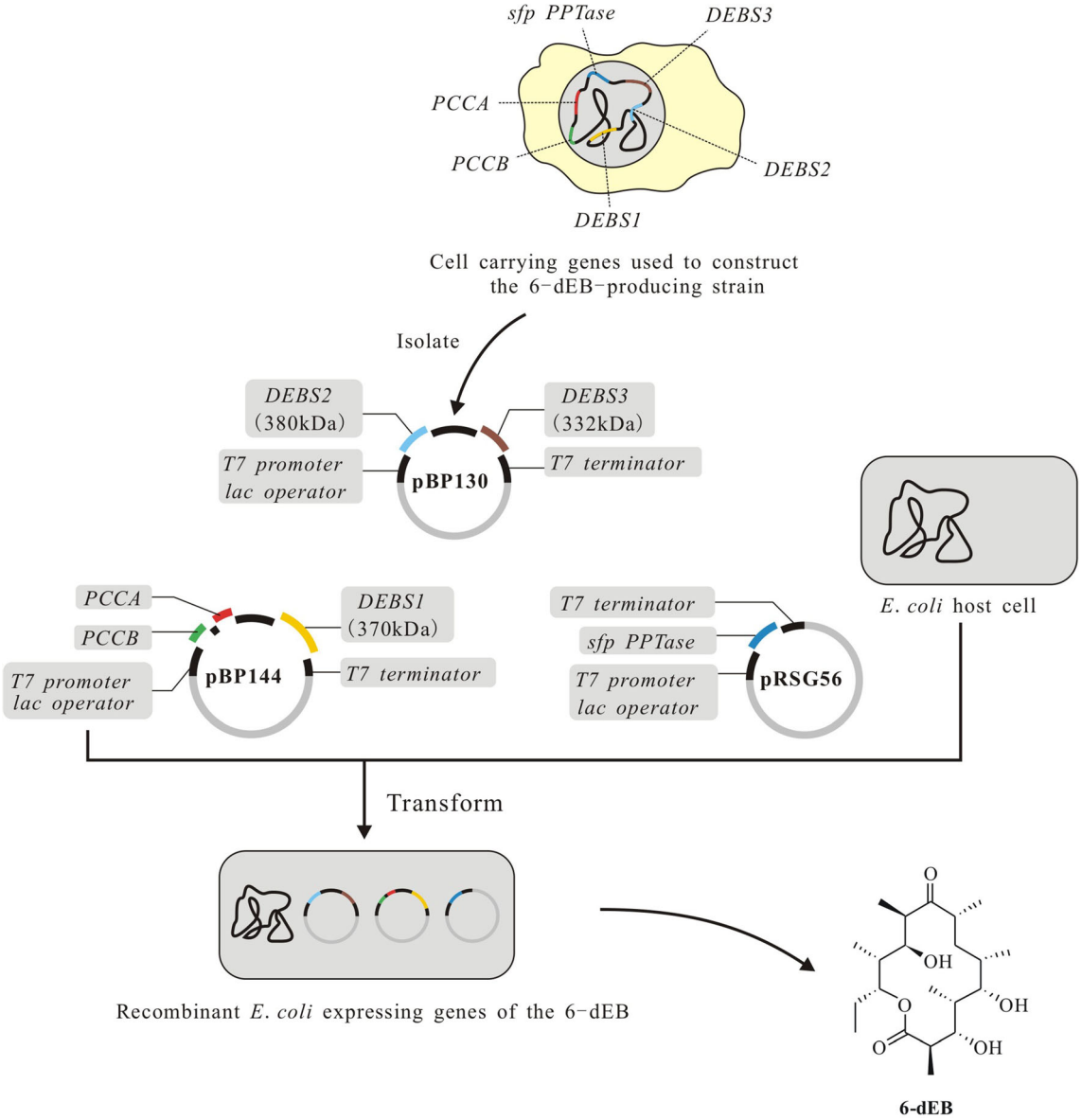

Fig. 2 Recombinant *E. coli* strain for producing the 6-dEB

multivariate-modular approach to construct a metabolic network in *E. coli* and succeeded in increasing titers of taxadiene, a taxol intermediate—approximately 15,000-fold. The success helped us to unlock a new more efficient route to terpenoid biosynthesis for commercial production of microbially derived terpenoids. Using *E. coli* as a flexible platform, the echinomycin pathway was rationally assembled to produce an unnatural nonribosomal peptide, TANDEM **(3)** (Fig. 4) in *E. coli*, which was a synthetic triostin A analog and showed a more potent antibiotic activity than its parent compound (Watanabe et al. 2009a). Furthermore, some other pharmaceutically important compounds had been also successfully biosynthesized in *E. coli*, such as the potential anticancer agents epothilones C and D (Mutka et al. 2006), a new quinomycin antibiotic SW-163s (Watanabe et al. 2009b), the ginkgolide precursor levopimaradiene (Leonard et al. 2010), the chondroitin sulfates precursor chondroitin (He et al. 2014), and the gossypol precursor 8-hydroxycadinene (Chang et al. 2007).

Although *E. coli* is often used as a powerful host for heterologous expression and pathway reconstruction, especially for reconstructing polyketides of different types (Gao et al. 2010), alternative hosts are also very necessary if the metabolites are toxic or cannot be successfully expressed in *E. coli*. *Streptomyces* is a good candidate and have enormous advantages, due to owning to the metabolic networks necessary for some precursor molecules and resistant mechanisms, and successfully biosynthesized antibiotics. In 1985, researchers first transferred the genes related to the actinorhodin metabolic network in *S. coelicolor* into the medermycin and the granaticin

synthesizing *Streptomyces* species. Two novel "hybrid" natural products, mederrhodin A and dihydrogranatirhodin (Fig. 4), were synthesized (Hopwood et al. 1985; Floss 1987). Similarly, genetically engineered *Streptomyces* strains (Li and Heide 2005) synthesized a mass of new hybrid aminocoumarins, novclobiocin 114 and 102, which would provide valuable insights into their inhibitory activity on gyrase in vitro and their antibacterial activity (Fig. 3). More recent examples showed the expression of modified synthetic gene clusters in *Streptomyces roseosporus* and *Streptomyces fradiae* generated novel analogs (Miao et al. 2005; Nguyen et al. 2006; Alexander et al. 2010). One of these compounds, CB-182561 (Fig. 4), had higher activity in the presence of bovine surfactant, lower toxicity, and could inhibit pulmonary infection of *S. pneumoniae* in the mouse model (Baltz 2010; Nguyen et al. 2010). *Streptomyces* was also used to unravel the biosynthetic network of metabolites and for the in vivo synthesis of relevant valuable antibiotics. Kanamycin is one of the most widely used aminoglycoside antibiotics, by the heterologous expression of integration of kanamycin biosynthetic gene cluster from *Streptomyces kanamyceticus* in *Streptomyces venezuelae*, Park et al. (Park et al. 2011) detailedly elucidated the biosynthetic network of kanamycins and applied the knowledge to directly synthesize 1-N-[S-4-amino-2-hydroxybutyric acid]-conjugated kanamycins in vivo. With the exception of expressing gene clusters coding for hybrid antibiotics, coumarins, nonribosomal peptides, aminoglycosides, and polyketides (Pfeifer and Khosla 2001), some peptides had been successfully expressed in *Streptomyces*, where the elongation factor thiazolyl peptide GE37468 and variants $GE37468_{mhP8I}$ and $GE37468_{mhP8A}$ (Fig. 4) had been produced in the engineered *Streptomyces lividans* (Young and Walsh 2011). Although genetic expression of heterologous *Streptomyces* is still in the early stages of development, these examples suggested that heterologous expression in *Streptomyces* is quite successful in dissecting biosynthetic steps and generating significant amounts of natural products and their analogs.

Furthermore, other alternative hosts were also being studied. *Pichia* was very excellent as a potential host for fungal polyketide biosynthesis. By introducing phosphopantetheinyl transferase gene *npgA* from *Aspergillus nidulans* and 6-methylsalicylic acid synthase gene *atX* from *Aspergillus terrus*, the recombinant *Pichia pastoris* GS115-NpgA-ATX had been successfully constructed to produce objective compound 6-methylsalicylic acid (6-MSA, Fig. 4). In the 5 L bioreactor, the recombinant can produce 2.2 g/L of 6-MSA by methanol induction for 20 h (Gao et al. 2013).

Activation of silent gene clusters

Over the past decade, the continuously increasing secondary metabolism gene clusters have been uncovered, indicating that they may become immense reservoirs of natural product discovery. However, most gene clusters are silent under laboratory conditions, presenting a huge bottleneck for natural product discovery. Thus, the activation of silent gene clusters, which is possibly responsible for natural products biosynthesis, will become a new direction of natural product discovery. To date, several methods based on genetic regulation and manipulation have been developed for the activation of the silent gene clusters. Zhou et al. (Zhou et al. 2015) successfully triggered the expression of a silent angucycline biosynthetic gene

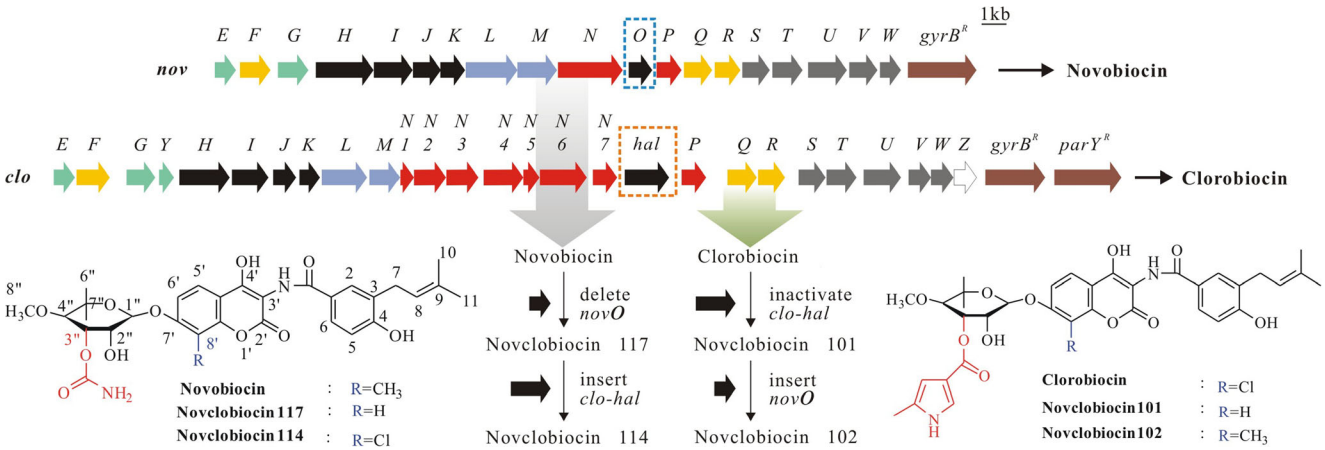

Fig. 3 Production of novobiocin/clorobiocin analogs using heterologous *Streptomyces* hosts. The aminocoumarin antibiotics novobiocin and clorobiocin have the same structural core but only difference in two substitution positions: a chlorine or a methyl group located at 8′-position of aminocoumarin ring and a 5-methylpyrrole-2-carboxyl moiety or a carbamoyl group located at 3″-OH of noviose. Using the halogenase gene *clo-hal* from the clorobiocin metabolic network of *Streptomyces roseochromogenes* to replace the methyltransferase gene *novO* from the novobiocin metabolic network of *Streptomyces spheroides* and the reverse is also true, the transformants produced the derivatives, novclobiocin 114 and 102. *Nov* and *clo*: aminocoumarin biosynthesis genes

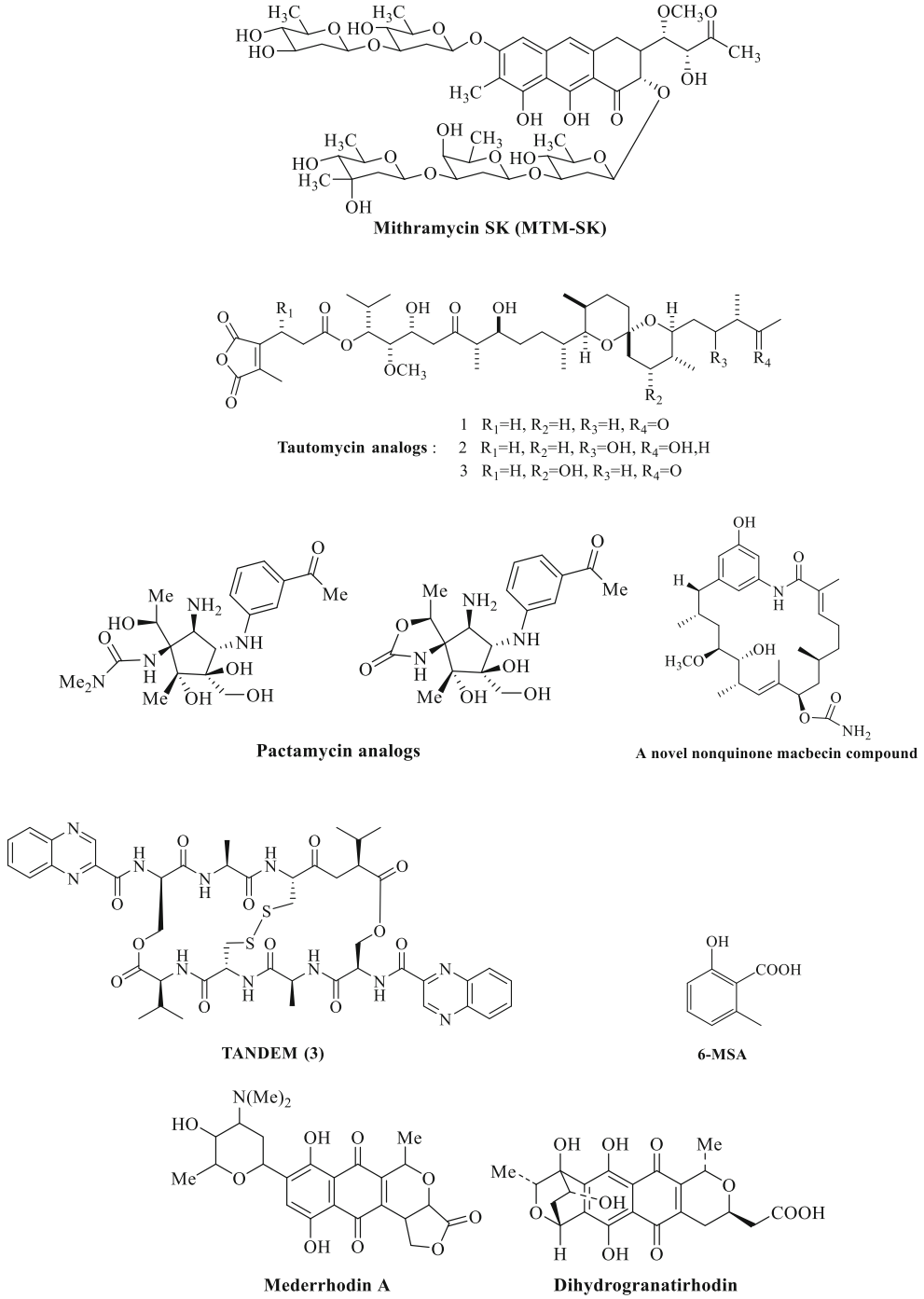

Fig. 4 Examples of novel natural products created by metabolic network reconstructions

cluster (*cha*) by overexpressing a gene *chal*, encoding a putative pathway-specific activator in *Streptomyces chattanoogensis* L10. Two novel angucycline antibiotics with good anticancer activities, chattamycins A and B (Fig. 4), were obtained. In order to activate a putatively silent glycopeptide cluster which was not expressed under laboratory test conditions, a gene (*bbr*) encoding the transcriptional activator of balhimycin biosynthesis from *Amycolatopsis balhimycina* was introduced into *Amycolatopsis japonicum* (Spohn et al. 2014). This led to the production of an antibiotically active compound, ristomycin A (Fig. 4). A reporter-guided mutant selection was used to select for activated *pga* gene cluster, leading to the production of two novel anthraquinone aminoglycosides (Fig. 4), gaudimycin D and E (Guo et al. 2015). These findings show that activation of silent biosynthetic clusters is not only a feasible method to facilitate natural product discovery but also elaborates part of biosynthetic mechanism that is otherwise not easy to deduce.

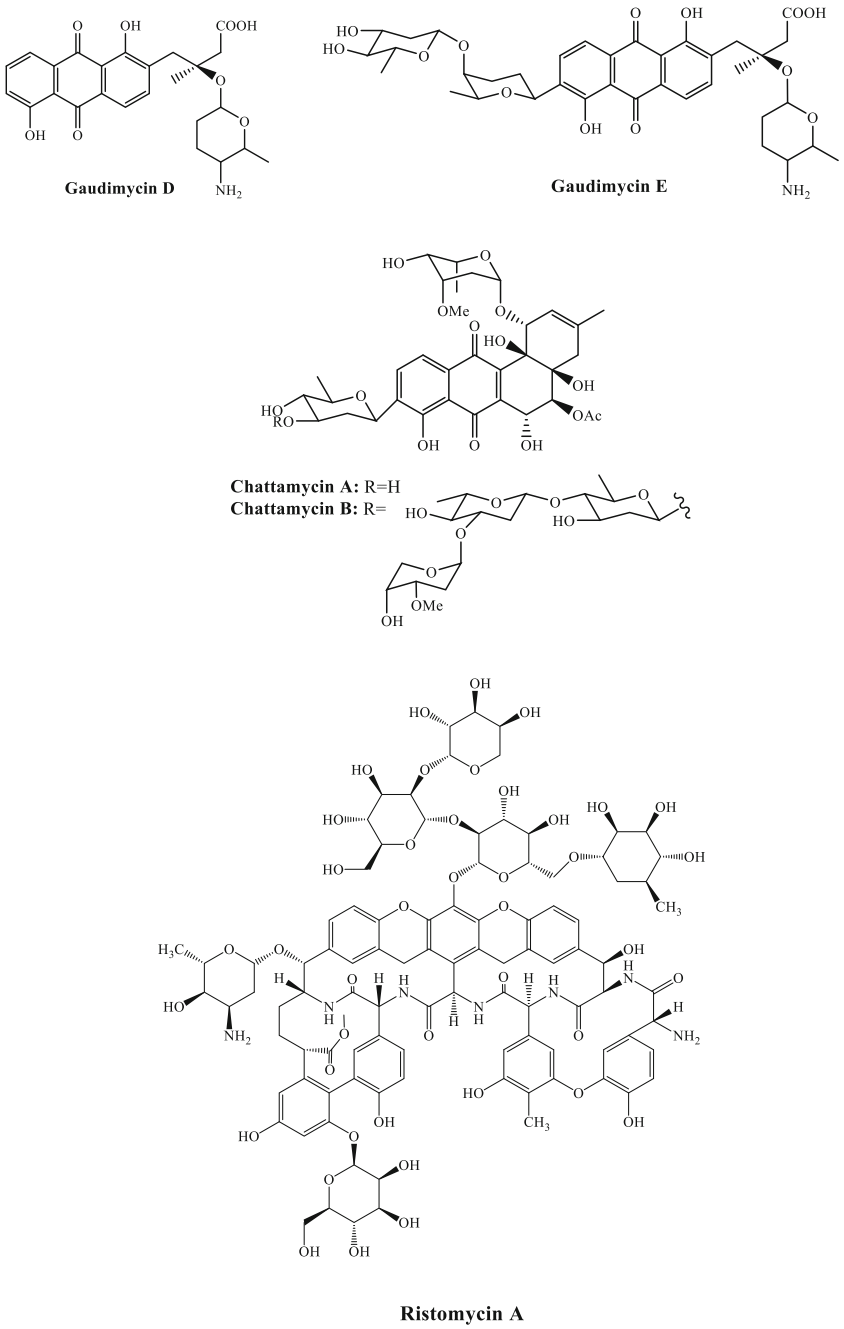

Fig. 4 (continued)

It was also possible to manipulate inducible promoters (Rodríguez-García et al. 2005; Dangel et al. 2010) or wide-domain regulators (Rutledge and Challis 2015) to activate silent genes and influence the expression of secondary metabolites. Regretfully, these mechanisms underlying the activation were still unclear. Saleh et al. (Saleh et al. 2012) introduced a constitutive promoter *ermE* upstream of the phenazine biosynthesis genes into cosmid CB15 in recombinants; the silent phenazine biosynthetic gene cluster triggered the formation of a novel phenazine derivative. Deletion of a gene encoding N-acetyltransferase in *A. nidulans* led to the formation of unparalleled fungal metabolites, pheofungins (Scherlach et al. 2011). It may be because the missing N-acetylation of proteins causes fungal stress, leading to the activation of the pheofungin biosynthesis. Moreover, some regulatory mechanisms of biosynthesis are quite complicated and have not been also investigated at all. In order to determine which silent gene clusters are the most important for the production of natural products, bioinformatics analysis of the completely sequenced genome need be also investigated in advance (Zhou et al. 2015).

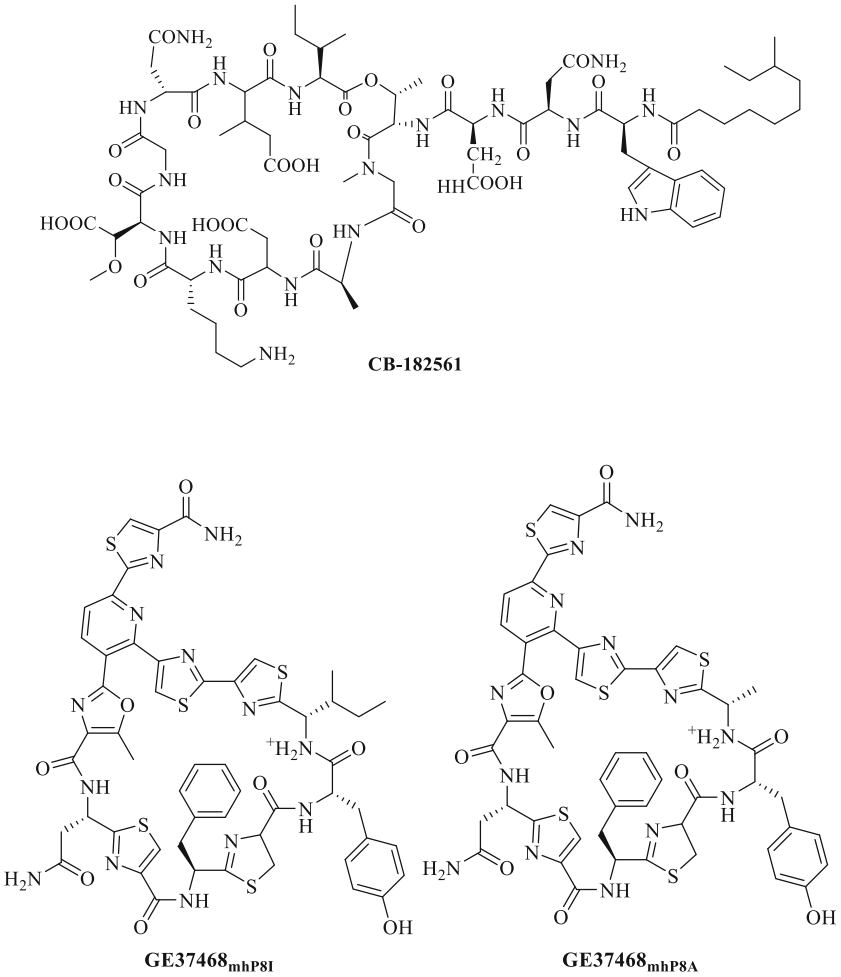

Fig. 4 (continued)

Rational design and directed evolution of biosynthetic enzymes

For over 20 years, the biosynthetic enzymes show a large potential in the biosynthesis of natural products (Bernhardt and O'connor 2009). However, few natural biosynthetic enzymes have high turnover and good expression. Many efforts are currently devoted to significantly alter substrate selectivity and specificity of biosynthetic enzymes (Zhang et al. 2013; Cacho and Tang 2013), enhance biosynthetic enzyme activity and stability (Urlacher and Girhard 2012), or redesign biosynthetic enzyme within scaffolds of known structure for titer improvement and natural product diversification (Huang et al. 2011). Wang et al. (Wang et al. 2011) used the avermectin reprogramming polyketide synthases (PKSs) in *S. avermitilis* M1 to produce the veterinary antiparasitic drug doramectin. In order to construct a strain that produced a mass of doramectin, isobutyryl-coenzyme A (CoA) loading module of the avermectin PKSs located in *S. avermitilis* M1 was replaced by the cyclohexanecarboxylic (CHC) unit from *Spirulina platensis* phoslactomycin PKSs (Fig. 5a). Doramectin production ability was increased about 6-fold, and the ratio of doramectin to avermectin was enhanced 300-folds compared to the original strain. In another case of rational design, recombined glycerol dehydratase appeared 3–5-folds more stable than the original strains by swapping the subunits of three GDHts in silico and was experimentally proved (Qi et al. 2006). The recent technological advances made biosynthetic enzyme functions become more clear and construction of complex biosynthetic enzymes from individual domains was becoming attainable. Liu et al. (Liu et al. 2011) reported that the unnatural product, 1, 4-naphthoquinone derivative of asperfuranone in *A. nidulans* was created by replacing NR-PKS AfoE asperfuranone biosynthesis SAT (starter unit ACP transacylase) domain with the NR-PKS StcA sterigmatocystin biosynthesis SAT domain (Fig. 5b) (Alekseeva et al. 2012; Wei et al. 2010). Fisch et al. (Fisch et al. 2011) also conducted rational domain swaps between the polyketide synthases and desmethylbassianin, leading to the regeneration of an extinct metabolite bassianin.

Directed evolution of biosynthetic enzymes has also enjoyed considerable success in the recent years; they are

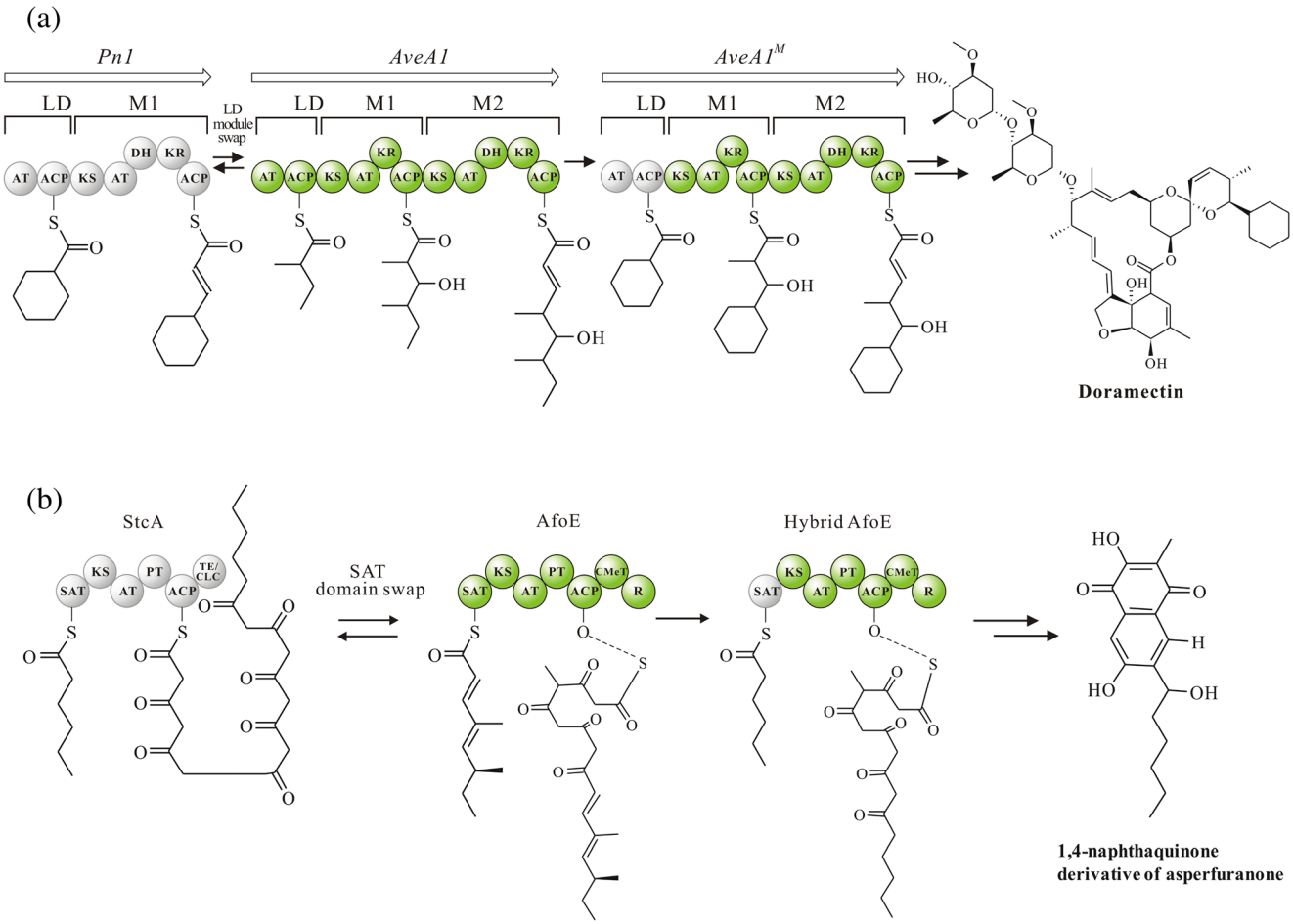

Fig. 5 Rational enzyme design through domain swapping. **a** Formation of doramectins in *S. avermitilis* M1 by PKSs loading module swap. **b** Biosynthesis of 1,4-naphthoquinone derivative of asperfuranone from hybrid AfoE in *Aspergillus nidulans*

widely used in the discovery and development of natural products (Farinas et al. 2001). In recent work, using the pigmented carotenoid lycopene as a screening tool, directed evolution of biosynthetic enzymes was used for amplifying terpene precursor flux in an engineered strain to improve the production of terpenoid (Leonard et al. 2010; Wang et al. 2000). For natural product diversification, researchers performed a rational modulation at the STR (strictosidine synthase) binding pocket and a central enzyme of the alkaloid metabolic network, to selectively accommodate secologanin substrate analogs for alkaloid biosynthesis (Chen et al. 2006). The engineered enzymes were then transferred into *Catharanthus roseus*. The transgenic *C. roseus* plant root cell cultures incubate massive novel unnatural alkaloid compounds when feeding precursors. This also demonstrated the validity of directed enzyme evolution in mutasynthesis (Runguphan and O'Connor 2009). However, for modular PKSs and nonribosomal peptide synthases (NRPSs), simple functional domain swapping often leads to attenuated productivity (Nguyen et al. 2006; Hans et al. 2003). The problem can be 2-fold by (1) simple swapping of the functional domains in the hybrid module results in proteolytic susceptibility in vivo and/or intrinsic kinetic defects in the multidomain catalyst (Hans et al. 2003); (2) the downstream domains show absolute discrimination against the modified substrates, as exemplified via mutasynthesis to change the fourth module of rifamycin polyketide synthase (Hunziker et al. 1998). However, Fischbach and coworkers (Fischbach et al. 2007) demonstrated that multiple directed evolution and in vivo screens have restored the function of chimeric assembly line enzymes NRPSs replaced with a foreign A domain, the activity of the chimeric NRPSs product yield was increased by 10-fold, as well as the potent NRP/PK inhibitor andrimid analogs were also produced (Fig. 6). Some studies also suggest that semi-directed approaches: the combination of elements of directed enzyme design and some other methods is likely to yield positive results in the structure-based enzyme redesign (Lutz 2010). For example, mutation, modeling, the substrate walking approach in combine with rounds of directed enzyme evolution was used to reassemble Ω-transaminase from *Arthrobacter* sp. for the large scale manufacture of the antidiabetic compound sitagliptin (Savile et al. 2010).

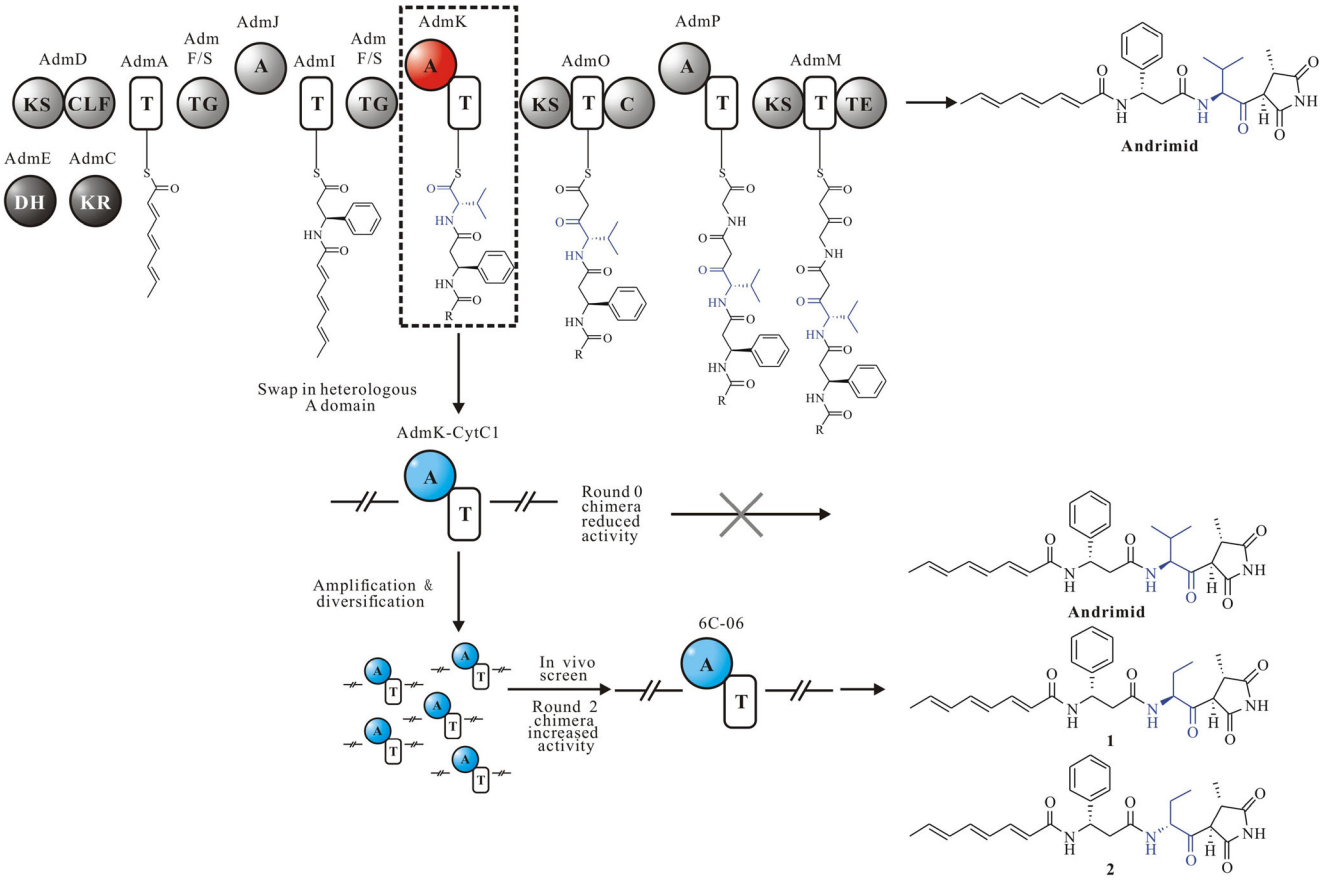

Fig. 6 Directed enzyme evolution for improving the activity of chimeric NRPS enzymes and produce new andrimid derivatives (*1* and *2*). The AdmK-A domain was swapped with AdmK-CytC1 domain, obviously leading to the ineffective activity of NRPS synthetase. The chimeric NRPS synthetase was then subjected to 2-round screen in vivo to finally express "unnatural" andrimid derivatives, *1* and *2*

Conclusions

As demonstrated by the authors in this review, the exploitation of metabolic networks often plays indispensable roles in the discovery and development of natural products. Significantly advances in genome mining, novel heterologous expression systems and enzyme engineering have allowed us to produce needed target compounds or unnatural compounds with enhanced biological features. Through genetic regulation and manipulation, some rarely structurally diverse metabolites from plants, bacteria, and fungi can also be synthesized in large qualities in the different hosts of genetic recombination. Although many experimental approaches that facilitate the improvement of metabolic networks, the production of new biologically active compounds and intermediates from natural products still face a variety of engineering challenges become of purification, identification, and biosynthesis issues. Therefore, we need to overcome these limitations by combining other tools, such as nuclear magnetic resonance (NMR) spectroscopy, fermentation optimization (Chen et al. 2009), redesign of metabolic flux, and some bioinformatic predicting tools.

Redesign of metabolic flux can divert flux into an appropriate pathway to accumulate former minor or unknown metabolites. There are several prominent examples describing the application, such as mevastatin and lovastatin (Manzoni and Rollini 2002), cyclosporine A (Survase et al. 2011), and a novel carbamoylated cephalosporin (Harris et al. 2009). Besides, some novel software tools are also being developed to support metabolic network research and redesign. GrowMatch method is often used to identify new genome-scale metabolic networks for getting more accurate and reliable reconstruction data (Kumar and Maranas 2009). The web-accessible tool of BioMet Toolbox is used to predict metabolic production rates, substrate consumption rates, and growth rates (Cvijovic et al. 2010). MeltDB and metaP-server software are applied to analyze metabolic flux based on metabolomics data during the network optimization in effort to solve toxic intermediates (Neuweger et al. 2008; Kastenmüller et al. 2010). In short, these approaches will be perhaps help us to produce a variety of useful active compound classes in the metabolic networks for meeting ever-increasing needs in human health and overall-being in the future.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical standards This article does not contain any studies with human participants or animals performed by any of the authors.

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