Engineered Biosynthesis of Pharmaceutically Important Compounds

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Received January 12, 2021

Natural products are an important source of medicinal seeds. The discovery of novel biosynthetic enzymes from nature is important for their use as biocatalysts for the enzymatic synthesis of useful natural products. In addition, genetics and structural biology developments have enabled the engineering of enzymes for the production of unnatural analogs of bioactive natural products. In this review, I describe the recent research on these two topics, the exploitation of a novel secondary metabolite enzyme involved in the biosynthesis of the sulfonamide natural product antibiotic SB-203208, and the production of unnatural bioactive depsipeptides by reconstruction of the modular enzyme assembly lines in the microbial host.

Key words natural product; enzyme; biosynthesis; microbial production; pharmaceutical compound

1. Introduction

Natural products still remain as important sources of medicines, as natural products, derivatized natural products, and chemically synthesized compounds modeled from natural products represent more than 50% of the medicines described in the literature.\textsuperscript{11} However, the rate of discovery of novel compounds based on the traditional methods, such as extraction from a natural source or activity-guided investigation, has been decreasing recently. Thus, new methodologies must be developed to prepare pharmaceutically important products. Given the increasing amounts of genomic data of living organisms available nowadays, the discovery of new enzymes and the engineering for unnatural reactions based on alterations of the gene sequences are now feasible. With the information and techniques in hand, we can proceed to the creation of pharmaceutically important compounds by enzyme engineering methods. In this review, I describe our recent efforts towards enzyme discovery and engineering for unnatural compound production.

2. Discovery of Novel Enzymes from Sulfonamide Natural Product Biosynthesis

Mining organisms for novel biocatalysts is important for their utilization as tools for the production of useful natural products and for engineering to produce unnatural compounds. Microorganisms such as actinomycetes and fungi are prolific secondary metabolite producers. We have discovered many novel enzymes, including those catalyzing the formation of heteroatom–heteroatom bonds,\textsuperscript{2,3} to yield characteristic motifs, including diazo,\textsuperscript{4,5} hydrazine,\textsuperscript{6–8} N-nitroso,\textsuperscript{9,10} triazole,\textsuperscript{11} and sulfonamide structures.\textsuperscript{12} The sulfonamide and sulfamate antibiotics are widely distributed in nature, as exemplified by nucleocidin, dealanylascamycin, alteinmecidin, ascamycin, sulphostin, SB-203207, SB-203208, and sulfadixiamycin A\textsuperscript{13} (Fig. 1). We focused on the biosyntheses of altemicidin, SB-203207, and SB-203208, which were isolated from Streptomyces.\textsuperscript{14–17} Alteinmecidin exhibits antitumor activity,\textsuperscript{14} and SB-203207 exhibits antibacterial activity by inhibiting the bacterial isoleucyl-tRNA synthetase (IleRS).\textsuperscript{16} Interestingly, SB-203207 does not inhibit the IleRS from its producer Streptomyces sp. NCIMB40513,\textsuperscript{16} implying that the IleRS in this strain might adopt a different protein structure that does not bind to SB-203207 as a self-resistance mechanism. Thus, we focused on the putative self-resistant IleRS gene to find the gene cluster of SB-203208 in the genome sequence, rather than relying on the enzyme similarity.\textsuperscript{18} As a result, we found two copies of Ile-RS genes, and one of them (sbzA) was clustered with non-ribosomal peptide synthetase (NRPS) and sugar metabolite genes.\textsuperscript{19} We named this gene cluster the sbz cluster, and expressed the genes in two operons (sbz-1 and -2) in Streptomyces lividans using two vectors with different phage-integration sites.\textsuperscript{20} The strain harboring sbz-1 yielded altemicidin, and the other strain harboring both sbz-1 and -2 produced SB-203207, SB-203208, and its new derivative. These results clearly indicated that sbz-1 and -2 are responsible for the production of these compounds. We assayed ShzI (GNAT family enzyme) with compound 6 and 2-sulfamoylacetyl-SbzG (acyl carrier protein, ACP) as substrates, ShzA with altemicidin and isoleucyl-tRNA, and ShzC with SB-203207 and (2S, 3R)-\beta-methylphenylalanyl-SbzG, and the expected products, altemicidin, SB-203207, and SB-203208 were detected in each...
reaction, respectively (Fig. 2A). SbzA is a rare tRNA-dependent aminoacyltransferase, and its structure resembles that of the multidomain isoleucyl-tRNA synthetase (IleRS) from *Thermus thermophilus*.[21] SbzA also catalyzes the canonical isoleucyl-tRNA synthesis reaction, but with lower efficiency than the other IleRS from *Streptomyces* sp. NCIMB40513. The function of SbzA as a self-resistance gene still needs to be investigated. The other unique point of this biosynthesis is 2-sulfamoylactic acid synthesis. By gene inactivation in a heterologous expression system, *sbzM* (cupin dioxygenase) and

**Biography**

Takayoshi Awakawa received his Ph.D. at Graduate School of Agricultural and Life Sciences, the University of Tokyo (2011). He has worked as an assistant professor (2011–2019) and an associate professor (2019–now) at Graduate School of Pharmaceutical Sciences, the University of Tokyo. He also worked in Prof. Bradley Moore’s laboratory as a visiting scholar between 2014 and 2016.
sbzJ (nicotinamide adenine dinucleotide (NAD)-dependent oxygenase) were identified as the biosynthetic enzymes. In an in vitro assay with L-Cys as a substrate and 4-bromophenacyl bromide as a labeling reagent for carboxylic acid, we detected 4-bromophenacyl-2-sulfamoylacetic acid as a product of the SbzM and SbzJ reactions. In the SbzM reaction with L-Cys as a substrate and 2,4-dinitrophenylhydrazine hydrochloride as a labeling reagent for the aldehyde, we detected 2,4-dinitrophenylhydrazine-2-sulfamoylacetic aldehyde. Based on the structures of the substrate and product, we propose the sulfonamide assembly from L-cysteine as a multistep reaction that involves the oxidative decarboxylation of L-cysteine to produce (Z)-2-aminovinyl sulfanolate, the oxygenation to yield (Z)-2-aminoethanolone-1-sulfonate, the intramolecular nucleophilic substitution, and the imine hydrolysis leading to 2-sulfamoylacetic aldehyde, which is oxidized by SbzJ to produce the final compound, 2-sulfamoylacetic acid (Fig. 2B). This is an impressive example of multifunctional oxidative enzyme reactions with a simple amino acid to generate highly potent secondary metabolites in nature. Efforts toward the X-ray crystal analysis of SbzM are on-going in our laboratory, to clarify the catalytic differences between SbzM and cysteine dioxygenase.

3. Production of Novel Depsipeptides by Re-programming Neoantimycin NRPS-Polyketide Synthase (PKS) Module Structures

Modular enzymes consisting of multiple catalytic domains are responsible for the biosyntheses of pharmaceutically important polyketides and non-ribosomal peptides. Among
them, we focused on the enzymes that produce hybrid molecules of polyketide-nonribosomal peptide natural products, such as the anti-cancer agents bleomycin, epothilone, and calyculin24–27) (Fig. 3). PKS consists of ketosynthase (KS), acyltransferase (AT), and acyl carrier protein (ACP) domains. KS catalyzes the decarboxylative condensation between the starter unit and malonyl- or 2-substituted malonyl-S-ACP, which is produced by AT through the thioesterification of the phosphopantetheine arm of ACP from malonyl-CoA or 2-substituted malonyl-CoA. NRPS consists of condensation (C), adenylation (A), and thiolation (T) domains. The C domain catalyzes amide formation between two acyl-S-T substrates. In the NRPS-PKS fusion systems, the peptide thioesterified on the T domain produced by the NRPS system is transferred to the KS domain in the PKS system. Due to the strict substrate specificities of the KS and C domains, the order of chain transfer is rigidly maintained in most cases. When the structure of the product of each module is modified by bioengineering, the rates of chain transfer and catalysis dramatically decrease, leading to a significant loss of the productivity by artificial module enzymes.

Antimycin, JBIR-06, and neoantimycin are a group of bioactive depsipeptides isolated from Streptomyces28–30) (Fig. 4). Antimycin exhibits strong anti-fungal activity through the inhibition of the mitochondrial electron transport chain, and JBIR-06 and neoantimycin exhibit anti-cancer activities through the down-regulation of GRP-78. These compounds share the common starter substrate 3-formamide salicylate (3-FSA) and the second extender substrate L-threonine. The antimycin biosynthetic modules (AntCD) incorporate pyruvate as the third substrate and alkylmalonyl-CoAs, which are biosynthesized by crotonyl-CoA reductase/carboxylase (CCR) AntE,31) as the fourth substrate. The JBIR-06 biosynthetic modules (SmlBC) incorporate isoleucic acid as the third substrate, leucic acid as the fourth substrate, and a dimethylmalonyl group as the fifth substrate. The neoantimycin biosynthetic modules (NatBCD) incorporate valic acid as the third substrate, phenylpyruvic acid as the fourth substrate, a dimethylmalonyl group as the fifth substrate, and isoleucic acid as the sixth substrate (Fig. 4). Each linear intermediate is lactonized by the thioesterase (TE) domain to yield the di-lactone antimycin, tri-lactone JBIR-06, and tetra-lactone neoantimycin, respectively. The amino acid sequence similarity of PKS and NRPS enzymes among these three antimycin systems is high.

![Fig. 5. Production of Unnatural Depsipeptides through the Reconstruction of Module Structures](image-url)
(e.g., PKS modules AntD and SmlC 51%, and AntD and NatC 53%). Thus, the assignment of a functional domain boundary can be reliably annotated, in the comparison of the amino acid sequences of the enzymes from these three systems.

Firstly, we aimed at the ring contraction of the tetra-lactone neoantimycin A, by deleting the NatD NRPS module and attaching the linker-TE_{SmlC} domain instead. The genetic manipulation was performed with the Streptomyces-Escherichia coli shuttle BAC vector pKU518\(^{\text{32,33}}\) in E. coli with the Red-ET system.\(^{\text{34}}\) As a result, TE_{SmlC} efficiently catalyzed lactonization between the thioester of a polyketide and the hydroxyl group of threonine in the linear intermediate, and produced a tri-lactone generated via the loss of isoleucic acid from neoantimycin A (compound-4\(^{\text{35}}\)) (Fig. 5A). The yield of this compound was 3.9 mg/L, which is 3-fold lower than that of neoantimycin A isolated from the heterologous expression strain, but was not significantly decreased, as also seen in the other module engineering studies.

We next expanded the lactone size of the tri-lactone JBIR-06 by attaching the NatD NRPS module to the ACP of SmlC via the docking domain of NatC, to retain the module interaction for the creation of a novel tetra-lactone compound. The NatD expression vector was introduced into the S. lividans expression host harboring the engineered BAC vector (pKU51806AsmICTE) for the ease of genetic manipulation. The engineered S. lividans produced the novel tetra-lactone compound-5, which was generated through the addition of isoleucic acid on the C-terminus of the JBIR-06 chain and subsequent lactonization (Fig. 5B). The yield of compound-5 (5.9 mg/L) was almost identical to that of neoantimycin A, indicating that the engineering was also successful in this case.

Finally, we substituted the dimethyl group on the polyketide moiety of JBIR-06 with diverse C4–C6 alkyl chains, by swapping the AT domain of SmlC with that of AntD and additionally expressing the engineered AntE (AntEV350G). As a result, the four major JBIR-06 type products with a butyl, 3-methylbutyl, pentyl, or hexyl group (compound-7a, 7c, 7e, and 7f, respectively) were produced in the engineered system (Fig. 5C). The sum of the yields of these products (3.8 mg/L) is comparable with that of JBIR-06 (5.0 mg/L), indicating the high efficiency of this production system. This study paves the way for the rational engineering of modular enzymes by emulating the enzyme diversification in nature. Because the alkyl chain in antimycin compounds is reported important for the Bcl2/Bcl-xL inhibitory activity for apoptosis,\(^{\text{36}}\) compound-7a, 7c, 7e, and 7f are expected to be novel anti-cancer agents.

4. Conclusion
The engineering of natural product biosynthetic pathways is a useful method to produce novel pharmaceutical seeds, in the same manner as the numerous natural products utilized as medicinal seeds. To address the continuing demands to exploit novel biocatalysts for these applications, we need to develop methods to isolate new enzyme families. For this purpose, it is beneficial to focus on the bioresources that have not been overexploited, such as plants and uncultured microorganisms. The machine learning from the substrate recognition or reactivity information will facilitate the discoveries from the genome information of various living organisms. The redesign of biosynthetic enzymes is effective to create unnatural analogs. The current trial-and-error engineering will be aided by directed evolution and computer simulations. It is also important to understand the enzyme structure–function relationships, based on the information from X-ray crystallographic and cryo-electron microscopic analyses and in silico studies, such as molecular dynamics simulations.

Acknowledgements This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan (JSPS KAKENHI Grant Number JP17H04763, JP19H04641, and JP21H02636), Mochida Memorial Foundation for Medical and Pharmaceutical Research and Japan Foundation for Applied Enzymology.

Conflict of Interest The author declares no conflict of interest.

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