Understanding the Diversity and Molecular Basis of Biosynthesis of Heterocycles in Natural Products Produced by Actinobacteria

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Abstract – Many of the natural products produced by actinomycetes have useful biological activities including antibiotic, antitumor and immunosuppressant activities, reflecting their structural diversity. In addition, many of them have heterocyclic rings in their structures, and these rings are considered to be important for their biological activities. Various chemical reactions and enzymes catalyzing them are used for their formation reactions. In this review, our recent examples of biosynthetic studies on the natural product with heterocycles in actinomycetes are summarized. These include indoline and tetrahydroquinoline ring formation using the nitrene-forming reaction found in the biosynthetic pathway of benzastatin, oxazoline ring formation in nonribosomal peptide synthetases and polyketide-derived piperidine alkaloid biosynthesis. These studies are expected to provide novel insights into enzyme chemistry as well as a new idea for synthetic organic chemists.

Introduction

Actinomycetes are known to produce a wide variety of secondary metabolites. Because many of them have useful biological activities, actinomycetes have long been considered a useful pharmaceutical resource. The antibiotics vancomycin and streptomycin, the immunosuppressant FK506, the antiparasitic agent avermectin, and the anticancer agent bleomycin are all secondary metabolites produced by actinomycetes. The structures of these compounds reveal that they have several heterocyclic rings, and these ring structures are thought to contribute to their biological activities. The existence of such a variety of biologically active
compounds indicates that actinomycetes utilize enzymes that catalyze a variety of chemical reactions which have been acquired during their evolution. The formation of ring structures in microbial secondary metabolism is of great interest in the field of biosynthesis of secondary metabolites because of their importance for the biological activity of compounds as well as their chemical interest. Various reaction mechanisms, such as Diels-Alder cyclization and polyether biosynthesis coupled with epoxide ring-opening, are used to form these structures. Understanding these mechanisms is important for the development of technologies to produce new compounds by engineering enzymes.

Our research group has focused on secondary metabolites produced by actinomycetes and has conducted various studies to understand their biosynthetic mechanisms, including the diazo group containing natural products and a polyene-containing natural product synthesized by type II polyketide synthase. Biosynthetic pathways of some of these compounds involve heterocyclic reactions involving enzymes with interesting catalytic mechanisms, such as nitrene transfer reaction. This paper summarizes our recent results on the biosynthesis of secondary metabolites from actinomycetes with heterocyclic rings.

INDOLINE AND TETRAHYDROQUINOLINE SYNTHESIS IN THE BIOSYNTHESIS OF BENZASTATINS

Benzastatins are secondary metabolites produced by several actinomycetes and are a group of compounds composed of \( p \)-aminobenzoic acid (PABA) and geranyl-derived moieties (Figure 1). Based on their structural characteristics, they are classified into three classes, those with linear chains, indoline rings, and tetrahydroquinoline rings (Figure 1). Several compounds with chloro groups (eg. virantmycin, 1) have also been reported (Figure 1). Some of these compounds show useful activities such as neuronal cell protection and antiviral activities. Since the heterocycle biosynthetic mechanisms remained unclear, we decided to understand the formation mechanism of indoline and tetrahydroquinoline rings (Figure 1).

Figure 1. Examples of benzastatins produced by actinobacteria. The benzastatin derivatives from \textit{Streptomyces} sp. R118 are highlighted with black boxes.
We focused on the actinomycete *Streptomyces* sp. RI18, which was isolated by Shin-ya et al. at the National Institute of Advanced Industrial Science and Technology (AIST) and reported to produce JBIR-67 (2), virantmycin (1), JBIR-73 (3), and 7-hydroxylbenzastatin D (4) (Figure 1). Genome sequencing analysis revealed a biosynthetic gene cluster encoding a p-aminobenzoic acid synthase and a UbiA-type prenyltransferase that is thought to catalyze its geranylation. The 10 genes (named *bez* genes) comprising this gene cluster were cloned and expressed in a heterologous host, *Streptomyces lividans*. The resulting recombinant strain produced benzastatin derivatives including JBIR-67 (2), virantmycin (1), and 7-hydroxylbenzastatin D (4), indicating that this gene cluster is indeed responsible for the biosynthesis of benzastatins. Interestingly, this heterologous strain also produced new benzastatin derivatives, benzastatin K (5) and methylbenzastatin K (6), which had not been previously described (Figure 2).

![Figure 2. Structures of benzastatin K (5) and methylbenzastatin K (6)](image)

Next, we generated gene disruption strains using the heterologous expression strain in which one of the *bez* genes is inactivated and analyzed the metabolic profiles of the obtained strains. From these results, the function of each gene was predicted. Importantly, the results showed that two enzymes, BezG (acetyltransferase) and BezJ (*N*-oxygenase) are essential for the biosynthesis of benzastatins with indoline and tetrahydroquinoline rings (e.g. 1, 2, 4, Figure 1). Furthermore, the production of benzastatins with indoline and tetrahydroquinoline rings (e.g. 1, 2, 4) was dramatically reduced in the *bezE* gene disruption strain, indicating that BezE, cytochrome P450, contributes to the biosynthesis of these structures in some way. This experiment also indicated that BezA (methyltransferase) and BezC (cytochrome P450) catalyze methylation and hydroxylation of the geranyl moiety.

To further understand the biosynthetic pathway, we carried out *in vitro* analysis of the recombinant enzymes. First, the enzymes responsible for the modification of the geranyl moiety were analyzed. We prepared recombinant BezA and BezC proteins, which presumably catalyze C-methylation and hydroxylation of the geranyl moiety. Initially, we expected that these enzymes should use geranyl-PABA (7, Figure 7) as a substrate. However, in contrast to our expectation, neither of the enzymes accepted geranyl-PABA (7) as a substrate. Therefore, we expected that these reactions proceed by using geranyl pyrophosphate (GPP, 8, Figure 3) as a substrate. As expected BezA and BezC catalyzed C-methylation of
GPP to synthesize methylgeranyl pyrophosphate (MGPP, 9) and hydroxymethylgeranyl pyrophosphate (HMGPP, 10), respectively, in vitro (Figure 3). The reaction mechanism of BezA was further analyzed in detail by the structural elucidation using X-ray crystallography and Quantum Mechanics/Molecular Mechanics (QM/MM) calculation in addition to biochemical experiments.²⁶ These experiments strongly indicated that the carboxylic acid of glutamate moiety in the active site act as a catalytic base to abstract a proton from the transition intermediate to complete the C-methylation and enabled to design unprecedented farnesyl pyrophosphate C6-methyltransferase.

Next, we attempted to analyze the mechanism of the five and six-membered ring structure formation. Unfortunately, we were unable to detect BezJ activity in vitro.¹⁸ Therefore, we synthesized N-hydroxy-p-aminobenzoic acid (HABA, 11, Figure 4), one of the putative products of BezJ. Supplementation of this compound to the heterologous expression strain without bezJ restored the production of benzastatin derivatives indicating that BezJ catalyzes N-hydroxylation of PABA (Figure 4). The in vitro analysis of BezG clearly showed that BezG catalyzes acetylation of the hydroxy group of HABA to synthesize N-acetoxy-p-aminobenzoic acid (AcPABA, 12) using acetyl-coenzyme A (CoA) (Figure 4).
Next, we attempted to get an insight into the function of BezE using the recombinant enzyme. Considering the analysis of BezG and BezJ, we expected that BezE should catalyze cyclization reactions using geranyl-AcPABA derivatives (13, 14, 15) to synthesize indoline (2, 16) and tetrahydroquinoline (17, 18) rings (Figure 4).¹⁸ To prove this hypothesis, AcPABA (12) enzymatically synthesized by BezG was geranylated by BezF using GPP, resulting in geranyl-AcPABA (13). When BezE was included in the reaction mixture, a benzastatin with indoline ring, JBIR-67 (2), was synthesized. In contrast, only benzastatin K (5) was synthesized in the absence of BezE. The results showed that benzastatin K (5) is mainly synthesized by nonenzymatic degradation of geranyl-AcPABA (13) and BezE is involved in the cyclization reaction to synthesize the indoline ring (Figure 4). A similar result was obtained by using enzymatically prepared methylgeranyl-AcPABA (14); methylbenzastatin K (6) was only synthesized in the absence of BezE while 7-hydroxybenzastatin F (16) was synthesized in the presence of BezE (Figure 4).

Finally, we attempted to reconstitute the tetrahydroquinoline scaffold biosynthesis in vitro.¹⁸ The hydroxymethylgeranyl-AcPABA (15) was synthesized enzymatically by using BezA, BezC, BezG and BezF and incubated in the presence and the absence of BezE. As a result, benzastatin derivatives with the
tetrahydroquinoline scaffold (17, 18) were only observed in the presence of BezE. These results clearly showed that the BezE catalyzes the cyclization of hydroxymethylgeranyl-AcPABA (15) to synthesize the tetrahydroquinoline scaffold. Hydroxymethylgeranyl-AcPABA (15) seemed to be more unstable compared to geranyl-AcPABA (13) and methylgeranyl-AcPABA (14). Therefore, specific degradation products can not be observed from this compound.

The synthesis of the indoline and tetrahydroquinoline rings in benzasatins has been predicted in the past to proceed via epoxidation of the geranyl group of geranyl-PABA derivatives (7, 19, 20) (Figure 5).27 At first glance, this pathway appears to be correct, since BezE is a member of the cytochrome P450 family and cytochrome P450 normally catalyzes monooxygenation reactions. The reduction of heme iron by ferredoxin and ferredoxin reductase is normally required for cytochrome P450 to catalyze the oxygenation reaction.28,29 However, the cyclization reaction catalyzed by BezE does not require these electron transfer systems.18 Therefore, BezE was expected to catalyze cyclization reactions without using oxidation reactions.

![Figure 5. The putative biosynthetic pathway of indoline and tetrahydroquinoline rings via epoxidation reaction](image)

How BezE catalyzes the cyclization was predicted according to the artificial cytochrome P450s catalyzing nitrene formation and insertion.30,31 These artificial enzymes catalyze reactions that form iron nitrenoid-like intermediates from azides and the insertion of the nitrene to a double bond to form aziridine rings. In many cases, azides are used as the source of nitrenes in these artificial enzymes, but there are also examples where an N-acetoxy group is used.32 Therefore, BezE, like these artificial enzymes, was expected to promote acetate elimination by coordinating the nitrogen atom of the N-acetoxy group to heme iron to form a nitrene-like intermediate (21–23, Figure 6).18 By inserting this nitrene into a nearby double bond, intermediates with aziridine rings (24–26) are synthesized (Figure 6). The nucleophilic attack of a water molecule on the 9-position is followed by the formation of an indoline ring (for 2 and 16), and the nucleophilic attack of a chloride ion on the 10-position results in the formation of a tetrahydroquinoline

$\text{R}^1=\text{R}^2=\text{H} \quad (7)$

$\text{R}^1=\text{Me}, \text{R}^2=\text{H} \quad (19)$

$\text{R}^1=\text{Me}, \text{R}^2=\text{OH} \quad (20)$

$\text{HO} \quad \text{NH}_2 \quad \text{O}$

$R^1=R^2=\text{H} \quad (7)$

$R^1=\text{Me}, R^2=\text{H} \quad (19)$

$R^1=\text{Me}, R^2=\text{OH} \quad (20)$

$\text{HO} \quad \text{O}$

$\text{NH}_2 \quad \text{R}^1 \quad \text{R}^2$

$R^1=R^2=\text{H}$

$R^1=\text{Me}, R^2=\text{H}$

$R^1=\text{Me}, R^2=\text{OH}$

$\text{HO} \quad \text{O}$

$\text{H} \quad \text{N} \quad \text{OH} \quad \text{OH}$

$R^1=\text{H}: \text{JBIR-67} \quad (2)$

$R^1=\text{Me}: 7$-hydroxylbenzastatin F \quad (16)$

$\text{HO} \quad \text{O}$

$\text{H} \quad \text{N} \quad \text{OH} \quad \text{OH}$

$\text{a}$

$\text{b}$

$9 \quad 10$

$18$

$2106$
ring (17). This mechanism provides a rational explanation for the introduction of the chloro group in virantmycin (1) and proposes a new halogenation mechanism in secondary metabolism.

Before the discovery of BezE, it was already known that artificial cytochrome P450s catalyze nitrene formation and transfer reactions, but it was not known that any natural cytochrome P450 catalyzing such a reaction. Thus, BezE is the first cytochrome P450 enzyme reported to be a naturally occurring nitrene transferase. However, it is not fully clear why BezE can catalyze nitrene formation and transfer reactions and does not catalyze the oxygenation reaction. Future X-ray crystallography and quantum chemical calculations of BezE should reveal why this enzyme catalyzes these reactions.

Interestingly, xiamenmycin A (27), which has a benzopyran structure similar to tetrahydroquinoline, has been reported to be biosynthesized through epoxidation of the prenyl side chain (Figure 7). The biosynthesis of this compound requires five enzymes. First, 4-hydroxybenzoic acid (28) is synthesized from chorismic acid (29) by XimC (chorismate lyase). Then, it is geranylated by XimB which belongs to ABBA (named form the folding of the protein, α–β–β–α) prenyltransferase family, resulting in 30. Then, the double bond of the side chain is epoxidized by XimD (flavin-dependent monooxygenase) to form 31. Nucleophilic attack of an aromatic hydroxy group to the epoxide ring catalyzed by XimE (putative cyclase) results in the ring-opening and the benzopyran structure formation of xiamenmycin B (32). Finally, XimA (ATP-dependent ligase) catalyzes the condensation of threonine with xiamenmycin B to form xiamenmycin A (27).
Nonribosomal peptides are a particularly important group of natural products. Many are known to possess useful biological activities, such as the antibacterial activity of vancomycin and the anticancer activity of bleomycin. Instead of ribosomes, nonribosomal peptides are synthesized by a group of enzymes called nonribosomal peptide synthetases (NRPSs). NRPS can synthesize a wide variety of peptides by using amino acids (nonproteinogenic amino acids) other than the 20 amino acids used in the synthesis of proteins. NRPSs are composed of modules that generally catalyze the formation of a single peptide bond. Within the module, there are functional units called domains, each of which plays a different role in peptide bond formation. The adenylation (A) domain selects an amino acid, activates it with ATP, and then loads them to a phosphopantetheinyl group attached to the carrier protein (CP) domain via a thioester bond. The CP domain is responsible for transporting substrate amino acids and peptide intermediates and delivering them to the catalytic domains. The C domain catalyzes the condensation reaction between a peptide and an amino acid bound to the CP domain and the formation of a peptide bond. In addition to these domains, NRPSs can have specialized domains, which contribute to expanding the structural diversity of nonribosomal peptides. For example, the E domain catalyzes epimerization of the alpha-position of amino acids, and the MT domain catalyzes N-methylation reactions. The C domain is sometimes replaced by a domain called the heterocyclization (Cy) domain, a variant of the C domain that can catalyze two reactions, peptide bond formation, and subsequent heterocyclization, using serine, threonine, or cysteine as substrates.

The mechanism of hetero ring formation in the Cy domain has not been clarified for a long time, but recently two groups have performed X-ray crystallographic analysis and proposed a catalytic mechanism. According to these studies, the side chains of threonine (Thr) and aspartate (Asp) near the active center are predicted to catalyze the heterocyclization (Figure 8). The detail of the mechanism of peptide bond formation is still under investigation.
formation in the Cy domain is still not clear. It may be likely that the active center of the Cy domain does not contain any amino acids directly involved in peptide bond formation, and the peptide bond formation may proceed spontaneously when the thioester and amino group of the CP-bound amino acids are properly positioned in the active center.

Among nonribosomal peptides, we focused on JBIR-34 (33) and -35 (34) isolated from *Streptomyces* sp. Sp080513GE-23 (Figure 9).\(^\text{40}\) They possess weak radical scavenging activity, a highly modified indole ring, and an oxazoline ring with a methyl group in an unusual position. JBIR-34 (33) and -35 (34) have a methyl group at position 4 of the oxazoline ring. Naturally occurring oxazoline rings are usually synthesized by cyclization of peptides and thus have the structures shown on the right side of the Figure 9 (35, 36).\(^\text{35}\) The oxazoline ring can also have a methyl group, but it is usually at the position 5 because the methyl group is derived from threonine. Therefore, we predicted that an unknown biosynthetic enzyme is involved in the formation of the oxazoline ring of JBIR-34 (33) and -35 (34).\(^\text{15}\)

Various analyses indicate that the methyl group is derived from the methyl group of \(\alpha\)-methyl-L-serine (37), a nonproteinogenic amino acid. \(\alpha\)-Methyl-L-serine (37) is synthesized by the PLP-dependent enzyme FmoH catalyzing the hydroxymethylation of D-alanine (38) with \(N^5,N^{10}\)-methylene tetrahydrofolate (mTHF, 39) as a coenzyme and concomitant formation of tetrahydrofolate (THF, 40) (Figure 10).\(^\text{15}\) In *vitro* analysis
of the reverse reaction of FmoH using the recombinant enzyme showed that this enzyme specifically hydroxymethylates D-alanine (38), and weakly uses glycine or L-alanine as substrates. This α-methyl-L-serine (37) is utilized by NRPSs harboring the Cy domain to create the methyloxazoline rings of JBIR-34 (33) and -35 (34). The overall JBIR-34 (33), -35 (34) biosynthetic pathway was predicted by bioinformatics and analysis of the substrate specificities of NRPSs (Figure 11).

Figure 10. The reaction catalyzed by FmoH to synthesize α-methyl-L-serine

Figure 11. Proposed biosynthetic pathway of JBIR-34, and -35. L-Trp, L-tryptophan; A, adenylation; Cy, heterocyclization; CP, carrier protein; TE, thioesterase; α-Me-L-Ser, α-methyl-L-serine; L-Ser, L-serine.
Only a few NRPSs utilizing amino acids with substituents at the alpha position are known. Only \( \alpha \)-methyl-L-serine (37) and 2-aminoisobutyric acid (41) are known to be used for the biosynthesis of natural products.\(^{15,41-43}\) 2-Aminoisobutyric acid is a component of several natural products, such as tryptoquialanine and peptaibols.\(^{44-46}\) The biosynthesis of 2-aminoisobutyrate was reported in 2021.\(^{41}\) It is synthesized by TqaL-catalyzed oxidation of valine (42) to form an aziridine ring (43), hydrolysis of the aziridine ring by TqaF to synthesize \( \beta \)-amino acid (44), and subsequent oxidative decarboxylation by TqaM (Figure 12). Some natural products, such as yersiniabactin, have a methyl group at the 4-position of the thiazoline ring.\(^{47,48}\) This is not synthesized from \( \alpha \)-methylcysteine, but by S-adenosylmethionine-dependent methyltransferase during the peptide assembly process catalyzed by the NRPS.

Interestingly, even though JBIR-34 (33), -35 (34) has only one oxazoline ring, there were two Cy domains in the NRPS responsible for this biosynthesis (Figure 11).\(^{15}\) Since the normal Cy domain alone catalyzes peptide bond formation and heterocyclization reactions, we were curious why two Cy domains were present in this biosynthetic pathway.\(^{38}\) To elucidate these functions, we attempted to analyze the functions of the FmoA2 and FmoA3 Cy domains by site-directed mutagenesis.\(^{49}\) As a result, we found that the Cy domain of FmoA2 only catalyzes the peptide bond formation while FmoA3 only catalyzes the heterocyclization (Figure 13). In addition to FmoA2 and A3, there are several NRPSs that have two Cy domains but only have one corresponding heterocycle in their products.\(^{38,50,51}\) In most cases, such Cy domains seemed to be used to synthesize an oxazoline ring derived from serine or threonine, while the single Cy domain seemed to be more often used to synthesize a thiazoline ring derived from cysteine.\(^{38}\) A thiol group is usually more reactive than a hydroxy group because of its higher acidity. Therefore, although a similar mechanism is utilized for oxazoline ring and thiazoline ring formation, the catalytic efficiency should be lower when the hydroxyl group is used as the substrate. To compensate for this lower reactivity, two Cy domains are expected to be acquired during the evolution, in which one of the Cy domains is specialized for heterocyclization.

![Figure 12. Biosynthesis of 2-aminoisobutyric acid](image-url)
We next attempted to use structural biology to analyze why the Cy domain of FmoA3 does not catalyze peptide bond formation reactions.\textsuperscript{49} We succeeded in analyzing the structure of FmoA3 by two methods: X-ray crystallography and cryo-EM single-particle analyses. Interestingly, FmoA3 was shown to adopt a dimer structure, even though most of the NRPS structures reported so far are monomers.\textsuperscript{52–54} This is the first demonstration of the dimer structure of NRPS and the first successful cryo-EM analysis of NRPS; there are two more examples of NRPSs with dimeric structures whose structures were solved by cryo-EM analysis to date.\textsuperscript{55,56} Based on this structural information of FmoA3, the structure of the active site was compared with the structure of the Cy domain, which has already been reported to catalyze both peptide bond formation and heterocyclization reactions.\textsuperscript{38,39} However no special features were found near the active site. Therefore, we predicted that the loss of the peptide bond formation might be due to the loss of interaction between the CP and the Cy domain of FmoA3. The C domain, which is a homolog of the Cy domain and only catalyzes the peptide bond formation, has two substrate entrances for the active site, one for the CP domain upstream (N-terminal side) and the other for that downstream (C-terminal side).\textsuperscript{37} Since the Cy domain is assumed to have a similar feature, it was predicted that if the Cy domain of FmoA3 lost its ability to interact with the upstream CP domain, it would lose its ability to form a peptide bond. The binding position of the CP domain of the Cy domain was predicted from structural comparison with the C domain of other NRPSs.\textsuperscript{57} The CP domain has a phosphate group derived from phosphopantetheinyl moiety and is expected to repel acidic amino acids. Therefore, three acidic amino acid residues present in the putative CP domain-binding site of the Cy domain of FmoA3 were expected to repel the phosphate groups of the CP domain to inhibit the binding of CP domain. The FmoA3 variant in which two of these glutamate residues were replaced with glycine resulted in a gain in peptide synthesis ability.\textsuperscript{49} These results suggest that FmoA3 potentially can synthesize peptide bonds and has evolved into a Cy domain specialized for heterocyclization catalysis by losing its ability to interact with the upstream CP domain.

BIOSYNTHESIS OF POLYKETIDE DERIVED ALKALOIDS SYNTHESIZED VIA THIOESTER REDUCTION AND TRANSAMINATION

Actinomycetes are known to produce polyketide-derived alkaloids with piperidine rings.\textsuperscript{58} Polyketides are acetate-based compounds that are biosynthesized by sequential decarboxylative Claisen condensation of
malonyl units with acyl thioesters catalyzed by polyketide synthases (PKS). These alkaloids are biosynthesized by the following common reaction pathway (Figure 14). First, polyketide chains (45) are synthesized by PKS. These polyketide chains often have a polyene structure formed by the reduction and dehydration of keto groups during the elongation process. It also has a keto group that remains unmodified in the middle of the chain and is attached to the CP domain of PKS via a thioester. When they are released from PKS, they undergo reductive cleavage by the nucleophilic attack of NAD(P)H-derived hydrides and are converted to aldehydes (46). This terminal aldehyde is converted to an amino group (47) by reductive amination by aminotransferase. Finally, the nucleophilic attack from the formed amino group to the keto group creates the dehydropiperidine ring (48) (or hemiaminal, 49), which is the core structure of these alkaloids.

Figure 14. Reactions commonly used in the biosynthesis of polyketide-derived piperidine alkaloids

These intermediates with piperidine rings are further subjected to various modification reactions to synthesize a variety of alkaloids (Figure 15). An most famous example is coelimycin P1 (50) isolated from Streptomyces coelicolor (Figure 15). They may also be converted to nitrogen-containing polycyclic structures in many cases. For example, argimycin P1 (51) has a cyclopenta[b]pyridine ring structure (Figure 15). Cyclizidine (52) has an octahydroindolizine ring, and indolizomycin (53) has a cyclopropane ring fused to octahydroindolizine (Figure 15). Thus, it is known that various polycyclic heterocycles with an nitrogen atom can be synthesized by this pathway.

Figure 15. Examples of polyketide-derived piperidine alkaloids
BIOSYNTHESIS OF STREPTAZONE E

We focused on streptazone E (54, Figure 16)\textsuperscript{17,67} and iminimycin (55, Figure 19)\textsuperscript{16,68,69} to understand the biosynthetic mechanism of the nitrogen-containing polycyclic structures of this family. First, we focused our analysis on \textit{Streptomyces} sp. MSC090213JE08, a streptazone E (54) producing strain.\textsuperscript{17} Genomic analysis of this strain revealed genes encoding aminotransferases and a PKS with a terminal thioester reductase domain, which appears to form a secondary metabolite biosynthetic gene cluster. Disruption of some of the genes encoding in the cluster resulted in the loss of streptazone E (54) production. These results indicated that this cluster is responsible for streptazone E (54) biosynthesis.\textsuperscript{17}

Several of these disrupted strains accumulated compounds that appeared to be biosynthetic intermediates. However, all of these were unstable and could not be isolated and structurally determined. These compounds were expected to have imines, and we hypothesized that these imines were responsible for the instability of the compounds. Therefore, the imine was reduced using sodium borohydride, and then the compounds were isolated and characterized by NMR. These results indicate that nigrifactin (56) is accumulated in the \textit{stzI} (dehydrogenase) and \textit{stzJ} (dehydrogenase) disrupted strains (Figure 16). The \textit{stzK} (FAD-dependent monooxygenase) disruption strain also accumulated nigrifactin (56), but in a smaller amount than \textit{stzI} and J disruption strains. Both the \textit{stzE} and \textit{stzF} disruptants accumulated a compound with epoxy ring at the dehydropiperidine ring of nigrifactin (57). Based on these analyses, streptazone E (54) was predicted to be biosynthesized by the following pathway. First, an aldehyde (58) with three double bonds and one keto group is synthesized by three PKSs, StzDCB (Figure 16). Then, an aminotransferase, StzH, converts the aldehyde to an amino group, and the following nonenzymatic cyclization results in the formation of nigrifactin (56). StzI and StzJ then cooperatively introduce a double bond into nigrifactin to synthesize 59, which is epoxidized by StzK (FAD-dependent monooxygenase), resulting in 57. This reaction is feasible because many FAD-dependent monooxygenases have been reported to catalyze the epoxidation of double bonds. Finally, StzE and StzF act cooperatively to form a five-membered ring structure with cleavage of the epoxy ring, resulting in the biosynthesis of streptazone E (54).\textsuperscript{17}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure16.png}
\caption{Putative biosynthetic pathway of streptazone E (54)}
\end{figure}
Of particular interest in this biosynthetic pathway is the formation of the five-membered ring by StzE and StzF. Unfortunately, the reaction mechanism has not yet been clarified. These enzymes belong to the NTF-2-like protein family. This enzyme family include MonBI, MonBII, and Lsd19, enzymes that catalyze epoxy ring-opening reactions and conjugated polyether synthesis (Figure 17). Lsd19 has two NTF-2-like protein family domains and is known to adopt a heterodimer-like structure. Therefore, StzE and StzF are also expected to form a heterodimer, and the loss of either of them would result in a loss of activity. However, more detailed functional analysis, including \textit{in vitro} analysis, is needed to understand the catalytic mechanism of these interesting enzymes.

Figure 17. Example of NTF2-like superfamily enzymes catalyzing polyether biosynthesis. Lsd19A and Lsd19B indicate N-terminal and C-terminal NTF2-like domain of Lsd19, respectively.

**BIOSYNTHESIS OF IMINIMYCINS**

Iminimycin (55) is a compound isolated from \textit{Streptomyces griseus} OS-3601 by a group at Kitasato University, and two derivatives are known (iminimycin A [55] and B [60], Figure 19). Both have an iminium cation and a 6-5-3 tricyclic structure. In addition to the core structure synthesized by PKSs, iminimycin B (60) has an N-acetylcysteine moiety. There are only a few natural products with iminium cations from actinomycetes, and their biosynthetic mechanisms have not been elucidated.

To understand the biosynthetic mechanism of iminimycins, we first attempted to analyze the genome of \textit{S. griseus} OS-3601, the production strain of iminimycins (55 and 60). This analysis revealed that this strain is almost identical to \textit{Streptomyces griseus} IFO13350 (NBRC13350), an actinomycete whose entire genome has already been determined. Therefore, we investigated whether this bacterium produces iminimycins (55 and 60), and found that it does indeed produce iminimycins (55 and 60).

Next, we analyzed the genomic information of this strain and found a biosynthetic gene cluster encoding a PKS with a thioester reductase domain and an aminotransferase, similar to streptazone E (54). We then disrupted the putative biosynthetic genes: \textit{imiB} (aminotransferase), \textit{imiC} (NTF2-like protein), \textit{imiD} (protein of unknown function), \textit{imiE} (protein of unknown function), \textit{imiF} (dehydrogenase), \textit{imiI} (flavin reductase), and \textit{imiM} (protein of unknown function). All strains which lack one of these genes lost iminimycin production. In addition, accumulation of new compounds was observed in some of them (Figure 18). Since these compounds were also extremely unstable and could not be isolated, they were isolated after reduction of imine with sodium borohydride and structurally analyzed by NMR. The structures of the compounds...
accumulated in each strain are shown in the Figure 18 (61–65). However, some of these compounds are expected to be degradation products rather than intermediates (64, 65). In particular, the compounds (64, 65) that accumulated in the imiE, imiF, and imiL disruption strains are expected to result from the epoxidation of the terminal double bond and the following nucleophilic addition from the amine involving double bond migration and epoxide ring-opening.

![Figure 18. The compounds accumulated in imi gene mutants](image)

The biosynthetic pathway of iminimycin was roughly predicted from the bioinformatics analysis of the biosynthetic gene cluster and the compounds that accumulated in the disruption strains (Figure 19). First, a 17-carbon aldehyde (61) is synthesized by the PKSs ImiA1, A2, A3, and A4. The aldehyde is aminated by aminotransferase, and the following non-enzymatic cyclization reaction results in a compound with hemiaminal moiety (66). The compound is thought to exist in equilibrium with the imine and hemiaminal structures. This compound can be converted to a compound with an epoxide ring (63, accumulated in the imiE disruptant strain) by ImiM, F, and L and further converted to iminimycins by ImiD, E and C. Unlike streptazone E (54), the function of many of these enzymes is difficult to predict by bioinformatic analysis.

![Figure 19. Putative biosynthetic pathway of iminimycins](image)
The biosynthesis of iminimycin A (55) has interesting features when compared to cyclizidine (52), which has a similar structure (Figure 20). Both of these compounds has hetero-polycyclic structures consist of 5 and 6-membered ring sharing a nitrogen atom. The difference is that iminimycin A (55) has an additional cyclopropane ring attached to this structure, while cyclizidine (52) has a methyl group at a similar position. Therefore, one may think at a glance that iminimycin A (55) is biosynthesized by oxidative cyclization of the methyl group of cyclizidine (52). However, based on the bioinformatic analysis of PKS and the compound (61) accumulated in the imiB (aminotransferase) disruption strain, the methyl group of cyclizidine (52) should be derived from the methyl group of methylmalonyl-CoA, while the carbon at the similar position in iminimycin A (55) should be derived from the methylene group of malonyl-CoA (Figure 20). Despite having similar backbones, these compounds are created by different carbon chain folding. Elucidation of the biosynthetic mechanism of these compounds will be an interesting research subject in the future.

Figure 20. Mechanistic differences in cyclization reactions between iminimycin A (55) and cyclizidine (52)

CONCLUSION

In this paper, studies on biosynthetic mechanisms of heterocycles found in actinomycetes are summarized. First, the mechanism of indoline and tetrahydroquinoline ring biosynthesis which is synthesized via unique cytochrome P450 is described. This cytochrome P450 BezE catalyzes nitrene formation from aromatic N-acetoxyamino groups and aziridine ring formation by its insertion into the adjacent double bond. The nucleophilic ring-opening of the aziridine ring results in indoline and tetrahydroquinoline rings. Next, the mechanism of oxazoline ring formation in nonribosomal peptides was introduced: the methylloxazoline rings of JBIR-34 and -35 are biosynthesized from α-methyl-l-serine, and by the two Cy domains from FmoA2 and FmoA3. Also, the function and molecular basis of these Cy domains are discussed. Finally, some examples of polyketide-derived alkaloid biosynthesis were presented. Although there are still many things to be understood in the biosynthetic pathways due to the instability of the intermediates, it has been suggested that some interesting enzymatic reactions are hidden in the pathways.

Thus, these examples further proved that the secondary metabolism in actinomycetes is a rich source of chemical reactions and enzymes for extending the structural diversity of natural products. Understanding
the chemical reactions in actinomycetes will contribute to the advancement of enzyme chemistry. In addition, these studies are expected to provide new ideas for biomimetic organic synthesis and trigger the development of new reactions.

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REFERENCES AND NOTES


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