MICROBIAL BIOSYNTHESIS OF BENZODIAZEPINES AND NITROSUGAR

CONTAINING NATURAL PRODUCTS

By

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MICROMONOSPORA AFRICANA

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Apr	apramycin resistance
ATP	adenosine triphosphate
CPK	Corey-Pauling-Koltun
DNA	deoxyribonucleic acid
EPS	extracellular polysaccharides
FIGE	field inversion gel electrophoresis
FWHM	full width at half maximum
GDP	guanosine 5′-diphosphate
GSTs	genomic sequence tags
HPLC	high-performance liquid chromatography
Kan	kanamycin resistance
kb	kilo base pairs
LB	Lysogeny broth
LPS	lipopolysaccharides
MIC	Minimum Inhibitory Concentration
MS	mass spectrometry
NCBI	National Center for Biotechnology Information
NDP	nucleoside diphosphate
NMR	Nuclear magnetic resonance
NOS	nitric oxide synthase
NRPS	non-ribosomal peptide synthase

NRRL	Northern Regional Research Laboratories
orf	open reading frame
oriT	origin of transfer
pABA	para-aminobenzoic acid
PBDs	Pyrrolo[2,1-c] benzodiazepine
PCR	polymerase chain reaction
PEG	Polyethylene glycol
PKS	Polyketide synthase
pNBA	para-nitrobenzoic acid
RNA	ribonucleic acid
TCEP	*tris*(2-carboxyethyl)phosphine
TDP	thallium dihydrogen phosphate
TLC	thin layer chromatography

CHAPTER I

INTRODUCTION

1. Study of pyrrolo[2,1-c] benzodiazepine natural products

1.1 Overview of pyrrolo[2,1-c] benzodiazepine natural products

Pyrrolo[2,1-c] benzodiazepine (PBD) natural products are produced by various actinomycetes and exhibit potent and broad-spectrum biological activities. Representative examples of PBDs are anthramycin, tomaymycin, sibiromycin, neothramycins and DC-81 which are structurally and biosynthetically related microbial natural products (figure 1-01).[1-4] The biological activities of PBDs are due to their unique sequence-selective DNA-binding ability and consequently the inhibition of both DNA and RNA synthesis.[5] Two other classes of natural products identified to be able to interact with DNA are: (1) berenil and netropsin which can form noncovalent bonds at AT rich region in the minor groove of DNA[6, 7] and (2) daunomycin which can intercalate into base pairs of DNA using its aromatic ring.[8]
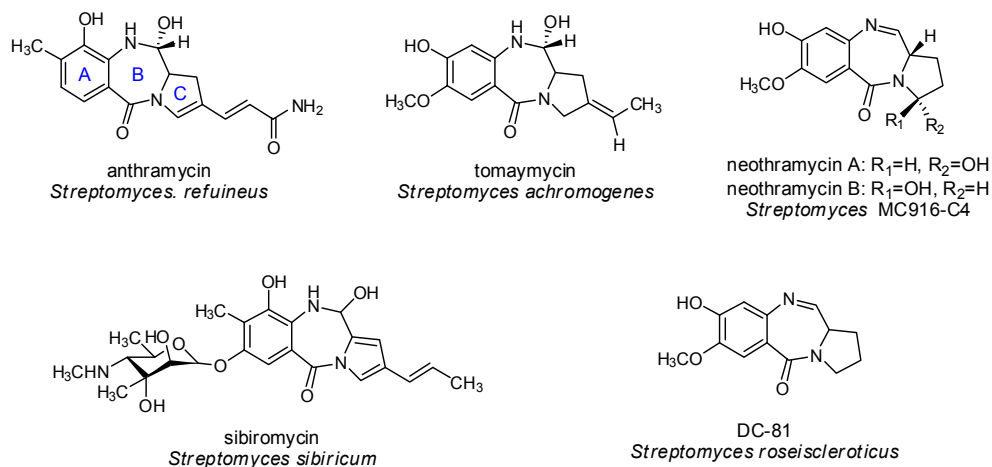
Figure 1-01. Representative examples of Pyrrolo[2,1-c] benzodiazepine.

PBDs are structurally constituted by a tricyclic system with an aromatic A-ring, a pyrrolidine C-ring and a common structural feature of a seven-membered B ring: pyrrolo(1,4)benzodiazepine nucleus. This pyrrolo(1,4)benzodiazepine nucleus possessing a N10-C11 imine-carbinolamine moiety is the key constitutional element for their biological activities because PBDs can bind with the minor groove of DNA by forming a covalent bond between their C11 position and exocyclic N2 amine of the guanine residue from double-stranded DNA (figure 1-02).[5, 9, 10] PBDs can lead to distortion of double-stranded structure by fitting into the minor groove of B-form DNA.[11] In terms of the DNA sequence preference, footprinting experiments have demonstrated that the adducts of PBDs and double-stranded DNA span 3 base pairs with different sequence specificities, although these PBDs share similar mechanisms for the covalent bond formation and inhibition of DNA synthesis. The different sequence specificities of PBDs have the potential to play very important roles in their biological activities.
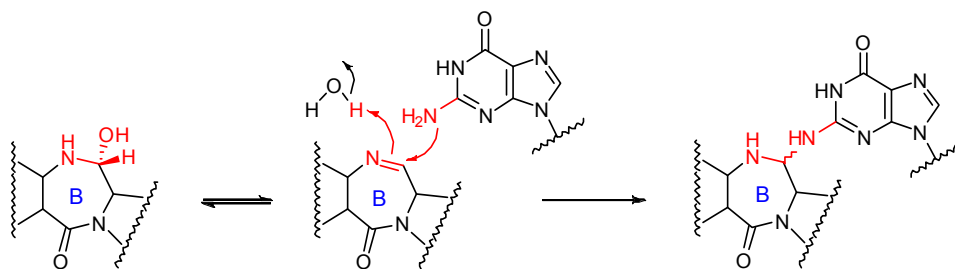
Figure 1-02. Proposed mechanism for the reaction of PBDs with DNA.

Despite the structural similarities among PBDs, there are still dramatic differences in their structures which have been demonstrated to have great effects on their biological activities. The most commonly seen structural variations of PBDs are: (1) the substitution pattern on the left aromatic A-ring, (2) the length difference of the side chain at C-2 position on pyrrolidine C-ring and (3) the degree of unsaturation between C-2 and C-3 on pyrolidien C-ring (figure 1-03).
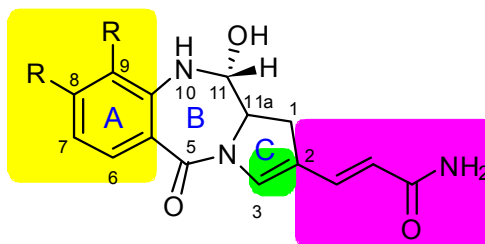
Figure 1-03. The structures of PBDs are mainly different in three parts.

The common carbinolamine N10-C11 group is the key for PBDs potent activity and can be reversibly converted to two C-11 isomers with sibiromycin as an exception because a C1-C11-C11a-N10 conjugated imine is formed in the dehydration product of sibiromycin.[3] This resulting imine structure from

sibiromycin is less susceptible to nucleophilic attack for adduct formation with DNA and makes sibiromycin less reactive.

Anthramycin is the first example of PBDs and was isolated in 1963 from the fermentation broth of a thermophilic actinomycete *Streptomyces refuineus* var. *thermotolerans* found in a compost heap.[12] Anthramycin was originally named "refuin" because it was believed to be a protein. Subsequently this biologically active compound was called "anthramycin" after the elucidation of its chemical structure because of the anthranilic acid derived A-ring of anthramycin. Total synthesis of anthramycin as well as X-ray crystallography have been performed to further confirm the structure and stereochemistry of anthramycin.[13, 14]

Sibiromycin is the second example of PBDs and was produced by an actinomyces strain *Streptosporangium sibiricum* in Russia. Sibiromycin is the only PBD natural product that contains an amino sugar sibirosamine. The stereochemistry at C-11 position of sibiromycin has not been solved yet. Similar to anthramycin, sibiromycin exhibits both antitumor and antibacterial activities. Sibiromycin is very sensitive to acid and easily converts to an inactive product after an acid treatment.[15]

Tomaymycin is another example of PBDs and was reported in 1972. Tomaymycin is produced by *Streptomyces achromogenes* var. *tomaymyceticus* isolated from a soil sample in Musashikoganei-city from Japan. The chemical structure of tomaymycin was confirmed by complete chemical synthesis.[16]

Neothramycins were isolated from *Streptomyces* No. MC916-C4 and exhibit both antitumor and antibacterial activities. There are two stereoisomers of

neothramycin: neothramycin A and neothramycin B in equal amount from their producer. Neothramycin A and neothramycin B are different in the stereochemistry at C-3 position and interchangable in aqueous solution.[17, 18]

DC-81 is a Pyrrolo[2,1-c] benzodiazepine natural product produced by *Streptomyces roseiscleroticus* with potent activity for the inhibition of nucleic acid biosynthesis. Recently there have been attempts made to design and synthesize DC-81 analogs, affording enhanced biological activities.[19-21]

Other identified PBDs include RK1441A and RK144B from *Streptomyces* sp. RK1441.[22] Both RK1441A and RK1441B show antibacterial activity, but only RK1441A exhibits antitumor activity.[23] Chicamycin is another example isolated from *Streptomyces* sp. (J576-99) and can bind to double-stranded DNA more tightly than neothramycin. Chicamycin with an α hydroxyl group at C-2 position shows more potent biological activity than its synthesized isomer with a β hydroxyl group at the same position.[24] Two other isolated anthramycin-like natural products are mazethramycin from *Streptomyces thioluteus* ME561-L4[25] and porothramycin from *Streptomyces albus.*[26] The simplest PBD identified so far probably is abbeymycin produced by an actinomycete AB-999F-52 isolated from a soil sample in Oregon, US. Abbeymycin exhibits no modification on the aromatic A-ring and no side chain at C-2 position of proline C-ring. Abbeymycin most likely exhibits weak antibacterial activity due to the above simple structural features(figure 1-04).[27]
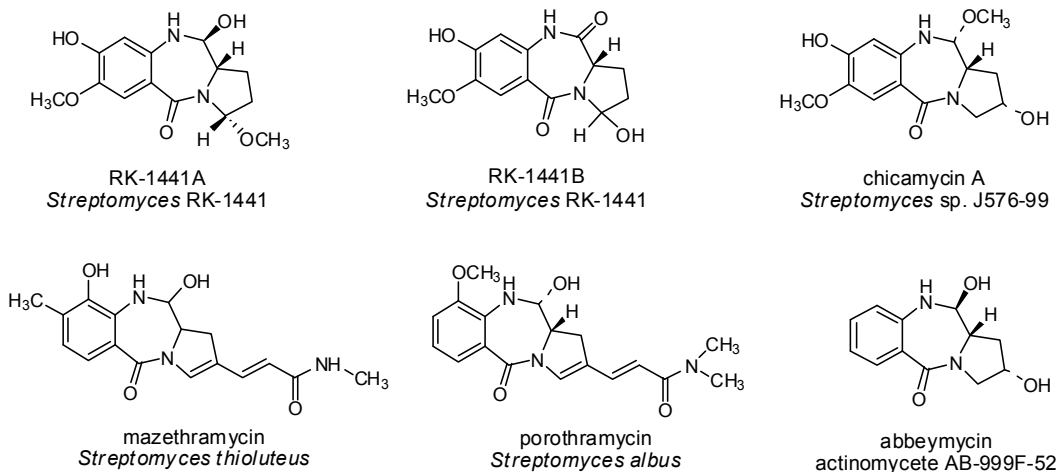
Figure 1-04. Other examples of Pyrrolo[2,1-c] benzodiazepine (PBD) natural products.

Anthramcin as well as other PBDs are usually isolated in the form of chemically stable C11-methyl ether PBDs if the isolation of PBDs is through recrystallization from hot methanol-water.[28] Methyl ether derivatives of anthramycin and tomaymycin can be rapidly hydrolyzed to anhydromycin and yield corresponding PBDs with epimerization at C-11 position, whereas sibiromycin methyl ether can not be easily converted to sibiromycin from anhydrosibiromycin.[15] The biological activities of PBDs methyl ether derivatives are basically equivalent to those of their parent PBDs.[1, 29]

1.2 Biosynthetic study of benzodiazepine natural products

Similarly structured elements among PBDs suggest that they might derive from similar building blocks through common biogenetic origins and biosynthetic pathways. Based on the results of feeding experiments, the biosynthesis of PBDs

requires the involvement of several amino acids and glucose. The anthranilate A-rings of PBDs are derived from L-tryptophan through the kynurenine pathway and the pyrrolidine proline C-rings are derived from tyrosine through L-dopa intermediate and losing two aromatic carbons after the aromatic ring cleavage. Methionine is also required for the biosynthesis of some PBDs to provide extra methyl groups for the *C*-methylated aromatic A-ring and the unique C-1 unit in the side chain of pyrrolidine C-ring. The only amino sugar in sibiromycin is believed to be derived from D-glucose (figure 1-05).
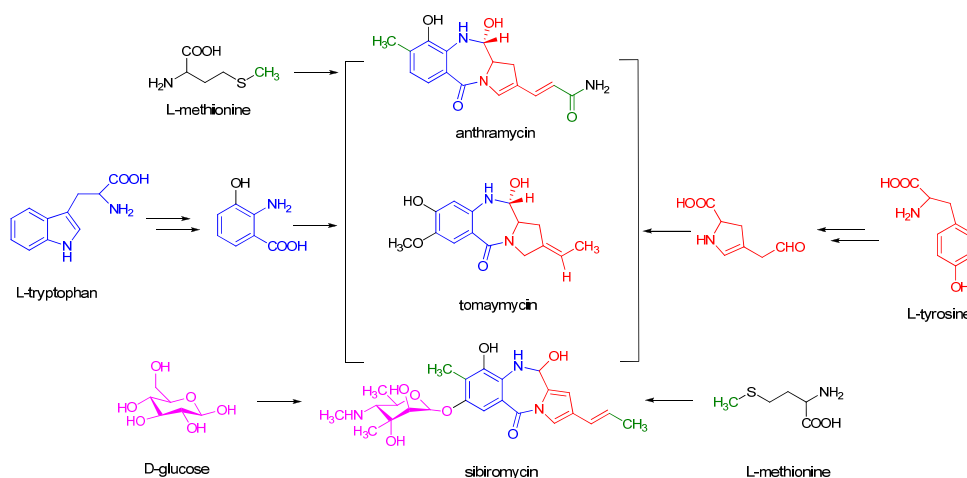
Figure 1-05. Building blocks of anthramycin, tomaymycin and sibiromycin.

To date, the biosynthesis study of PBDs has been only focused on three representative examples, anthramycin, tomaymycin and sibiromycin, primarily based on the isotopic feeding studies. The aromatic A-ring of PBDs is derived from the amino acid tryptophan with different substitutions on it for each PBD[30]. As can also be inferred from the sequence information from the identified

anthramycin biosynthetic gene cluster in its producer *S. refuineus*, the left aromatic A-ring possibly comes from tryptophan via the kynurenine primary metabolic pathway and the substitutions may occur at the kynyrenine formation stage or as the last biosynthetic step right before the final formation of PBDs.[30]

1.2.1 Biosynthetic study of anthranilate A-ring in PBDs

Anthranilic acid is the basic precursor of anthranilate A-ring in all PBDs, but [COOH-^{14}C] anthranilic acid could only be efficiently fed into tomaymycin rather than anthramycin or sibiromycin. Another possible substrate for the biosynthesis of the left anthranilate A-ring part, at least for anthramycin and sibiromycin, is 3-hydroxy-4-methylanthranilic acid. Interestingly, although anthramycin and sibiromycin share the same chemical structure for the aromatic A-ring subunit, anthramycin producer *S. refuineus* failed to take up either anthranilic acid or 3-hydroxy-4-methylanthranilic acid. Whereas sibiromycin producer *Streptosporangium sibiricum* could take up both compounds and only 3-hydroxy-4-methyl-[2-^{14}C]anthranilic acid could be incorporated into sibiromycin. Based on competition experiments among different possible substrates, kynurenine, 3-hydroxykynurenine, 3-hydroxy-4-methylkynurenine and 3-hydroxy-4-methylanthranilic acid are possible intermediates for the biosynthesis of anthranilate A-ring in sibiromycin.[30] Potential reasons for above observations are: (i) the anthranilate A-ring of tomaymycin is different from that of anthramycin and sibiromycin, so a sequentially different pathway is possibly required for the biosynthesis of the aromatic A-ring in tomaymycin, (ii) since sibiromycin shares the same anthranilate A-ring with anthramycin and can take up 3-hydroxy-4-

methylanthranilic acid as an intermediate, 3-hydroxy-4-methylanthranilic acid is possibly an important intermediate for the biosynthesis of anthramycin but the membrane permeability of anthramycin producer *S. refuineus* for this intermediate may be a problem, (iii) another possibility is that anthramycin uses a complete different biosynthetic pathway for the anthranilate A-ring. If this is the case, then three different biosynthetic pathways for the anthranilate A-ring part may exist for above three PBDs natural products.

On the anthranilate A-ring of sibiromycin, there is one additional hydroxyl group at C-7 position, which can also be found in other PBDs like tomaymycin and neothramycins. In the labeling experiments using DL-[5-^{3}H] tryptophan as a precursor, ^{3}H was detected in the C-6 position in the final chemical structure of sibiromycin probably because of the involvement of an NIH shift during the hydroxylation at C-7 position (figure 1-06).[31]
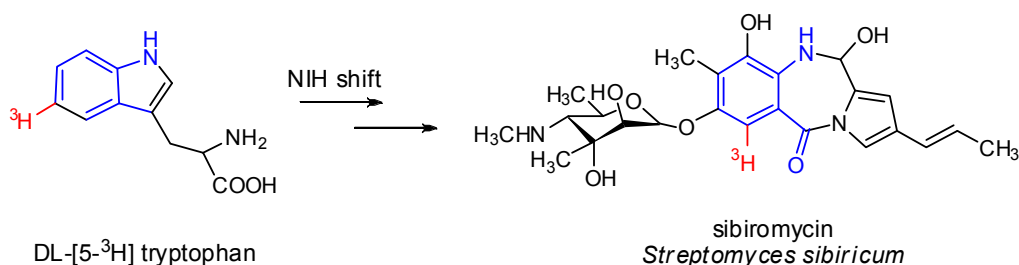
Figure 1-06. An NIH shift is proposed to be involved in sibiromycin biosynthesis.

In summary, the left aromatic A-ring is derived from tryptophan through the primary metabolic kynurenine pathway in which L-tryptophan is sequentially converted to *N*-formylkynurenine, L-kynurenine, 3-hydroxylkynurenine, 3-hydroxy-anthranilic acid and possibly 3-hydroxy-4-methylanthranilic acid. The

questions that remain to be answered are: the sequence for the bioconversion from tryptophan to 3-hydroxy-4-methylanthranilic acid and especially at what stage the C-methyl group is introduced into PBDs (figure 1-07).
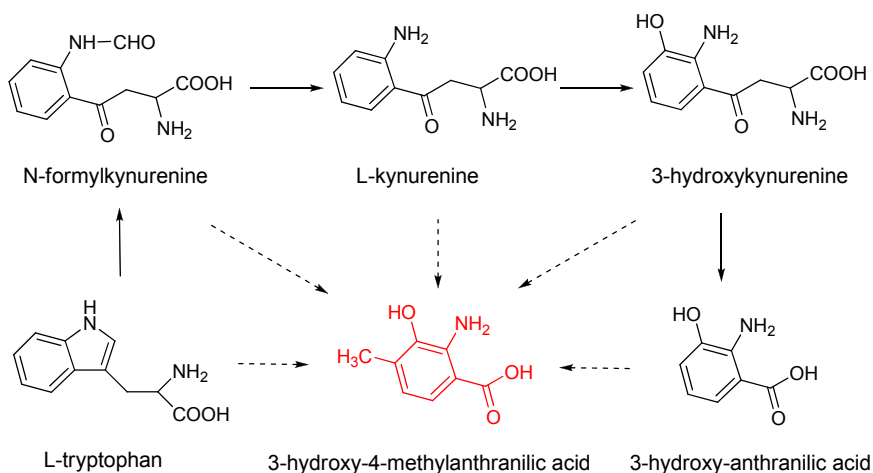
Figure 1-07. Proposed biosynthetic pathway for 3-hydroxy-4-methylanthranilic acid.

1.2.2 Biosynthetic study of dehydroproline in PBDs

The most intriguing aspect related to the biosynthesis of PBDs is the biosynthetic pathway of their C-7 branch point compounds derived from L-tyrosine. So far progresses in this area are primarily accomplished by Dr. Laurence H. Hurley lab and a series of feeding experiments using dual labeled (^{3}H/^{14}C) and ^{2}H-labeled substrates had been employed to elucidate these partial biosynthetic pathways for the five-member rings and C2/C3 side chains in PBDs.

For the formation of five-member rings in PBDs, L-tyrosine is first converted to intermediate L-dopa with subsequent cleavage of two carbon atoms in the aromatic ring of tyrosine. In terms of the cleavage of two carbon atoms, theoretically there are several possible routes for the conversion from L-tyrosine

to the five-member ring. But feeding experiments supported the route in which the π bond between C5 and C6 in tyrosine was broken prior to the formation of five-member rings and ruled out some other impossible biosynthetic pathways of the five-member rings in PBDs. Next the amine group attacks the C5 aldehyde to form a five-member ring from C2-C5, followed by the cleavage of another carbon atom at C7 position. After the formation of a C-7 compound, an extra C-1 unit will be transferred to the end of the C-2 side chain from L-methionine in the case of anthramycin and sibiromycin (figure 1-08).[32]
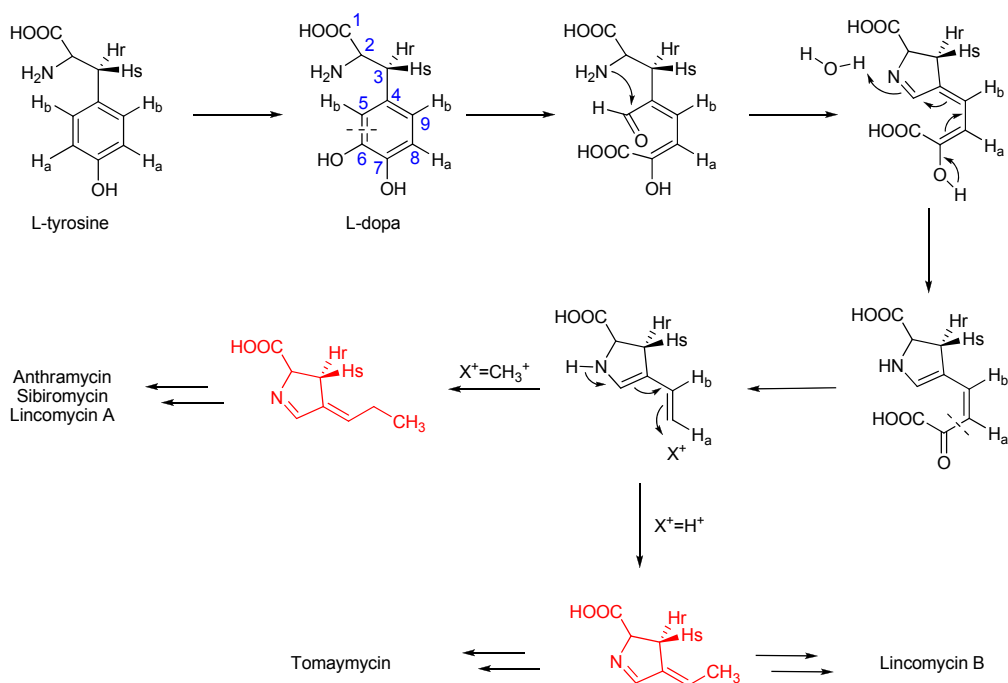
Figure 1-08. Proposed biosynthetic pathways from L-tyrosine to dehydroproline in PBDs and lincomycin.

Anthramycin and sibiromycin also contain a common structure, C3-proline moiety, at C-2 position of proline C-ring. These two PBDs are the second

example of such C3-proline structure containing natural products after the isolation and characterization of lincomycin A from *Streptomyces lincolnensis*. Another unique structure about anthramycin is the existence of an amide carbonyl group which is believed to be derived from the *S*-methyl group of methionine, whereas both sibiromycin and lincomycin A contain a methyl group instead of an amide carbonyl group at the end of the C3-proline moiety. Tomamycin and lincomycin B contain a C2-proline side chain at the same C-2 position of proline C-ring and this complete C2-proline moiety has been proven to be derived from L-tyrosine after the aromatic ring cleavage. Interestingly, only one form of PBD with either a C2 or a C3 proline moiety could be formed by its producer. But in the case of lincomycin, two forms of lincomycin with C2 or C3 proline moiety have been detected from the fermentation broth of their common producing strain (figure 1-09).

lincomycin A
Strptomyces lincolnensis

lincomycin B
Strptomyces lincolnensis
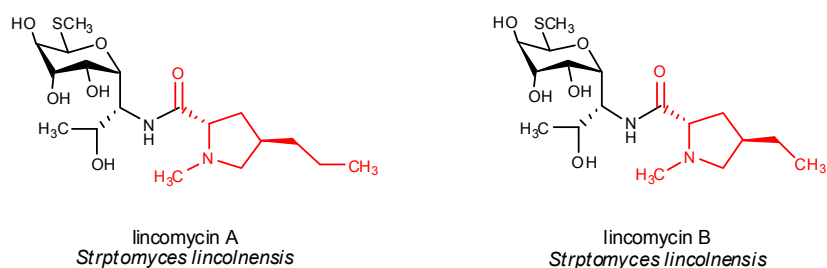
Figure 1-09. Two forms of lincomycin from *Strptomyces lincolnensis*.

1.2.3 Assembly of anthranilate A-ring and dehydroprolineacrylamide by NRPS

Among all the PBDs, only the biosynthetic gene cluster for anthramycin has been identified from its producer *S. refuineus*. The function of the

anthramycin biosynthetic gene cluster was confirmed by gene disruption methods. In anthamycin biosynthetic gene cluster, an NRPS (nonribosomal peptide synthase) is involved in the biosynthesis of anthramycin and can assemble its two important intermediates: 3-hydroxy-4-methylanthranilic acid and dehydroproline acrylamide to yield anthramycin. Notably, anthramycin is probably released from the NRPS by a reductive mechanism because of the existence of an unusual reduction domain after the thioesterase domain (figure 1-10).
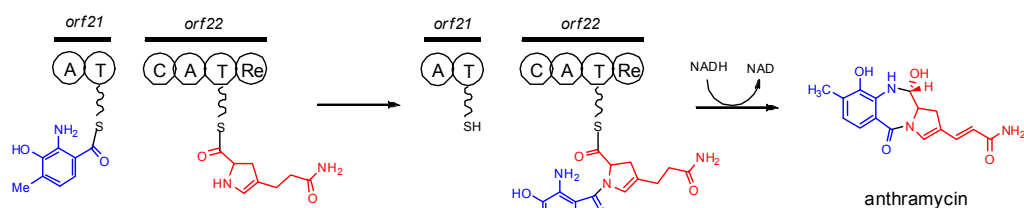
Figure 1-10. Nonribosomal assembly of anthramycin in *Streptomyces refuineus*.

The biosynthetic gene clusters for other PBDs, especially tomaymycin, have not been reported so far. Tomaymycin producer *S. achromogenes* can also produce its biologically inactive metabolite oxotomaymycin. The only structural difference between tomaymycin and oxotomaymycin is that the hydroxyl group at C-11 position in tomaymycin is replaced by a carbonyl group in oxytomaymycin (figure 1-11). Both tomaymycin and oxotomaymycin have been confirmed to be derived from the same precursors: L-tyrosine, L-tryptophan and L-methionine. Tomaymycin shows dramatic antibacterial and antitumor activities toward different cell lines, whereas oxotomaymycin is a biologically inactive products derived directly from tomaymycin. The oxidative conversion from tomaymycin to

oxotomaymycin is due to a constitutive intracellular enzyme, rather than an intercellular enzyme, produced by tomaymycin producer *S. achromogenes.*[33] Very similar to anthramycin, tomaymycin also contains a N10-C11 carbinolamine functional group. So the unidentified tomaymycin biosynthetic gene cluster from *S. achromogenes* very possibly harbors an NRPS with a special reduction domain leading to the formation of carbinolamine in tomaymycin. If there is indeed a reduction domain in the NRPS for the biosynthesis of tomaymycin or at least a reduction reaction is required right before the final formation of tomaymycin, then why tomaymycin is further oxidized to oxotomaymycin is really very interesting.

tomaymycin
Streptomyces achromogenes

oxotomaymycin
Streptomyces achromogenes
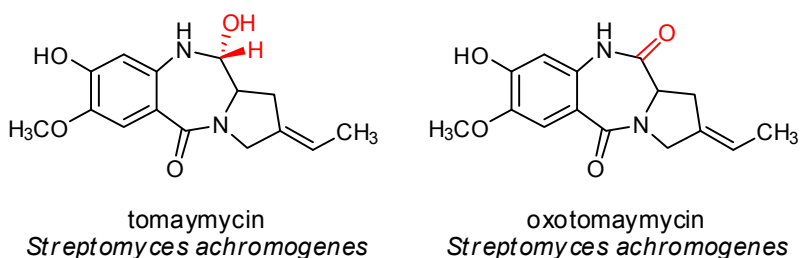
Figure 1-11. tomaymycin and oxotomaymycin from *S. achromogenes*.

1.2.4 Problems remain to be answered in the biosynthesis of PBDs

Other problems that have not been investigated in the biosynthesis of PBDs include the timing of the modifications steps for the two key intermediates, which might occur prior to or after the condensation and assembly of 3-hydroxy-4-methylanthranilic acid and dehydroproline acrylamide by NRPS. These biosynthetic steps include the substitutions on the anthranilate aromatic ring and

the proline five-member ring as well as the incorporation of C2/C3 side chains.

1.3 Biological activities of benzodiazepine natural products

PBDs are very well known for their potent biological activities, especially antitumor and antibacterial activities, because of their ability to form covalent adducts with double-stranded DNA in an irreversible manner and inactivate DNA as a template for the biosynthesis of DNA and subsequently RNA in both eukaryotic and prokaryotic cells. But PBDs have been shown to have little effect on protein synthesis.[9, 34] Because of their potent antitumor activity, some PBDs have been developed for drugs in the treatment of cancer and used clinically. However, these PBD natural products exhibit dose-limiting cardiotoxicity, which dramatically restricts their clinical applications and further development in drug discovery. But considering their unique mechanisms of action and lack of other toxicities, recently researchers have designed and synthesized diverse chemical analogs derived from PBDs to reduce their toxicity and increase their corresponding potency and activity.

PBDs like anthramycin, sibiromycin and tomaymycin share very similar mechanisms for their biological activities and compete for the same guanine residue binding sites in double-stranded DNA. These natural products have been proven to be cable to forming highly specific and stable covalent bonds between C-11 position and N-2 position of guanine in double-stranded polydeoxynucleotides without dramatic distortion of the DNA helix. The PBDs-DNA covalent adduct is believed to be formed after the nucleophilic attack from

the N2 amine group in double-stranded DNA towards N10-C11 imine intermediate via an SN_1 mechanism. Other possible positions on DNA that could be modified by anthramycin are N-7, N-3 and C-8, but these possible modifications have been ruled out by assay experiments using labeled guanine.[35] The hydrogen bonding between C9 hydroxyl group and O2 of cytosine can also further stabilize the PBD-DNA adduct. PBDs don not cross-link DNA, so probably only one covalent bond is found in PBD-DNA adducts. Unlike other natural products, for example bleomycin which requires metal ions for its therapeutic effect[36], PBDs do not require the presence of other cofactors for their potent biological activities.

In terms of DNA binding properties, the order for the degree of DNA modification of natural-occurring PBDs is sibiromycin > anthramycin > tomaymycin > DC-81 > neothramycin.[37, 38] Despite their small molecular weights, PBDs exhibit great sequence specificity and prefer covalent binding with DNA sequences flanked by purines rather than pyrimidines with 5'PuGPu as the most preferred binding sequence and 5'PyGPy the least preferred sequence for PBDs.[39] The rate for the formation of adducts between PBDs and DNA is relatively slower than that of other DNA binding compounds which can interact with DNA within a few seconds, for example it takes 1 hour for the reaction between anthramycin and DNA to complete *in vitro.*[37]

1.3.1 Biological activity of anthramycin

Anthramycin is the most extensively studied PBD natural product in the aspect of both biosynthesis and biological activity. Anthramycin is detected from

the fermentation broth of *S. refuineus* via cup-plate assay method using *Sarcina lutea* and *Bacillus sp. TA* as test strains. During the course of anthramycin extraction, anthramycin could be converted into either anthramycin methyl ether after crystallization from boiling methanol-water or anhydroanthramycin after crystallization from boiling acetone. Interconversion between anthramycin methyl ether and anhydroanthramycin could also be observed. There is no dramatic difference between the biological activities of anthramycin and anthramycin methyl ether. When anthramycin methyl ether was dissolved in water, three forms of anthramycin could be detected: anthramycin, C11-*epi*-anthramycin and anhydroanthramycin.[1]

Anthramycin can inhibit the synthesis of doubled-stranded DNA by forming covalent bond with the 2-amino group of guanine and remains hidden in the minor groove of DNA, but will not react with poly(dI)poly(dC) because they lack a N2 amine group. Based on the X-ray crystal structure analysis of a covalent anthramycin-DNA adduct, anthramycin can lead to a low twist of double-stranded DNA after its binding with DNA, but hydrogen bonds between C9 hydroxyl group and N10 as well as the acylamide bond tail and base pairs in the minor groove can also contribute to the stabilization of anthramycin-DNA adduct.[40] Anthramycin binding sites have been well studied and it has been shown that the bonding formation of anthramycin-DNA adducts is strongly influenced by the neighboring bases of guanine and exhibits dramatic specificity and selectivity. However, for anthramycin, the preferred sequence of triplets is AGA, AGG > GGA > GGG.[41] But base sequence rather than the overall base composition of DNA is believed

17

to be the determinant for the reaction rate between anthramycin and DNA. For example *Cl. perfrignens* DNA with 37% GC and more fast-reacting sites reacts faster with anthramycin than *M. lysodeikticus* with 72% GC and less fast-reacting sites. In addition, the binding of anthramycin to double-stranded DNA is very sensitive to the formation of DNA.[10]

Actually anthramycin alone can not lead to single or double strand DNA breakage, but anthramycin possesses the ability to cause *in vivo* excision-dependent single and double strand breaks in DNA. One explanation for this might be that the cleavage of DNA by anthramycin is caused by the self-repair system which is cable of recognizing anthramycin-DNA adducts. The cleavage of DNA caused from anthramycin are dose-dependent and can occur at a very low concentration of anthramycin.[42] The mechanism of anthramycin-caused DNA cleavage is very unique and dramatically different from that of other DNA-binding natural products like bleomycin and daunomycin.[43, 44] Another possible source of the biological activity of anthramycin is via the inhibition of the interaction between DNA and its transcription factor Sp1 and further inhibition of RNA biosynthesis.[45]

Anthramycin has a broad spectrum activity *in vitro* against a wide variety of tumor cell lines such as monkey kidney, rabbit kidney cells, HeLa cells, Sarcoma 180, Walker 256 carcinosarcoma, human epithelioma No.3, leukemias P388, and human adenoma, *etc*. Other pharmacological results showed that anthramycin was able to cause nuclear segregation in Kupffer cells, endocrine and exocrine pancreatic cells, proximal tubular cells of the kidney and in the

reticuloendothelial cells from spleen in rats.[46] Anthramycin has also been demonstrated to be able to inhibit gastrointestinal, breast neoplasms, lymphoma and sarcomas in its evaluation in humans.[47] But anthramycin did not show biological activity against leukemia L1210 and plasma cell tumor YPC-1.[29] As to its antimicrobial activity, anthramycin is *in vitro* most active against gram-positive bacteria, streptomycetes, mycobacteria, *Escherichia coli* B and weakly active against fungi and yeasts. But anthramycin has been found to be inactive for its antimicrobial activity *in vivo*.[29, 48] Anthramycn also exhibits some other biological activities, for example amebicidal and chemosterilant activities.

Anthramycin is a potent antitumor natural product, which can inhibit the synthesis of DNA but can not affect the functions of the DNA of its producer *S. refuineus*. Anthramycin producer *S. refuineus* has been proven to be able to take in tomamycin and sibiromycin and is sensitive to them because they can inhibit the DNA synthesis of *S. refuineus*. As mentioned above, anthramycin, tomamycin and sibiromycin share similar biological mechanisms to inhibit DNA synthesis and can competitively bind to the same sites on double-stranded DNA. Besides, it has also been shown that intact cells are necessary for the total biosynthesis of anthramycin from *S. refuineus*. Based upon above experiments, anthramycin is very possibly biosynthesized in the cell wall and periplasmic space and can not have contact with functional double-stranded DNA in *S. refuineus*.[49]

1.3.2 Biological activity of sibiromycin

Sibiromycin stands out as the only example in PBDs that contains an amino sugar (sibirosaminide) at C-7 position of aromatic A-ring and is the most

potent natural product of the PBDs.[50] The binding ability between sibiromycin and double-stranded DNA is reinforced by the electrostatic forces from this extra sugar and the phosphate groups of the backbone of DNA.[51]

Under mild acidic conditions it has been proven that sibiromycin is unstable and can easily turn into anhydrosibiromycin by losing a molecule of water. But under acidic conditions, sibiromycin tends to lose its aminosugar moiety to yield anhydrosibiromycinone. Both anhydrosibiromycin and anhydrosibiromycinone are very stable compounds but unfortunately biologically inactive.[3, 15]

Sibiromycin can inhibit the growth of some bacteria like *Bacillus mycoides*, *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli*. Sibiromycin is very well known for its activity in the treatment of cancer cells such as praegastric cells and lymphosarcoma cells.[15]

1.3.3 Biological activity of tomaymyicn

Tomaymycin is able to bind with the exocyclic 2-amino group of guanine in DNA. The neighboring bases also influence the covalent bonding between tomaymycin and double-stranded DNA. The preferred order sequence of triplets for tomaymycin is AGA > GGC, TGC > AGC with AGA being the most preferred triplet sequence for both anthramycin and tomaymycin. But there are still dramatic differences as to DNA triplet preference for tomaymycin and anthramycin, so DNA saturated with tomaymycin can still react with anthramycin.[41] Tomaymycin can lead to greater conformation changes of double-stranded DNA than anthramycin and the DNA binding sequence specificity has

great effect on the degree of bending and the reaction rate between PBDs and DNA.[52]

Based on the Corey-Pauling-Koltun (CPK) model building studies, the stereochemistry of the covalent bonding site between DNA and PBDs including anthramycin, tomaymycin, sibiromycin and neothramycins had been assigned as *S*. However, a later report derived from a recombination of fluorescence, high-field NMR and molecular modeling methods demonstrated that tomaymycin could yield 11*S*, 11a*S* and 11*R*, 11a*S* diastereomeric adducts with opposite orientations in the minor groove of double-stranded DNA because of the epimerization of tomaymycin at the C-11 position via 10,11-anhydrotomaymycin(figure 1-12).[11] Using similar approaches, it has been proposed that DNA and anthramycin with a similar N10-C11 imine structure can form covalent adduct with an 11*S* stereochemistry.[53, 54] But further characterization studies of tomaymycin-DNA adduct using proton NMR showed that tomaymycin bound to 5'-CGA with an *S* stereochemistry at C-11 position and the orientation of PBDs connected with double-stranded DNA as well as the stereochemistry at C-11 position were largely due to the DNA binding sequence and the chemical structure of PBDs.[55]

10,11-anhydrotomaymycin
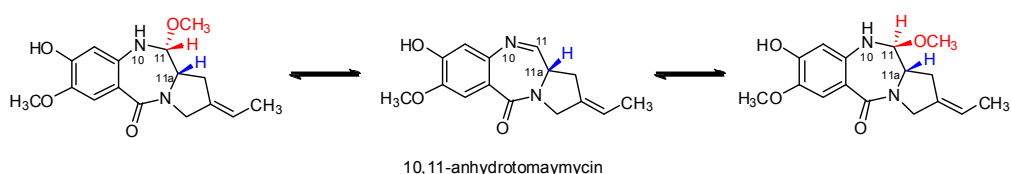
Figure 1-12. Proposed mechanism for epimerization of C-11 of tomaymycin.

There is an unsaturated double bond at C-2 position of tomaymycin. NMR spectra data as well as X-ray crystallography studies confirmed the configuration of C-2 side chain to be *E*-tomaymycin instead of *Z*-tomaymycin.[51, 56] Later, total synthesis of Z-tomaymycin has also been reported, showing similar biological activity to the naturally occurring *E*-tomaymycin (figure 1-13)[57].

E-tomaymycin
Streptomyces achromogenes
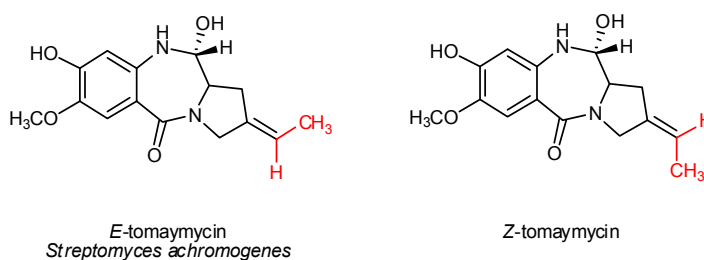
Z-tomaymycin

Figure 1-13. *E*-tomaymycin from *S. achromogenes* and its synthesized isomer *Z*-tomaymycin.

Tomaymycin exhibits strong activity against different kinds of phages like *Escherichia coli* phages, λ phage, *Bacillus subtilis* phage SP-10 as well as gram-positive bacteria.[2]

1.3.4 Biological activity of neothramycin

Neothramycin is one of the simplest compounds in PBDs. Generally the reaction rate of PBDs is much slower than other DNA-binding natural products and neomycin reacts more slowly with double-stranded DNA than other PBDs.[18] This slow reaction rate of the DNA binding ability of neothramycin may be attributed to its lack of C2 or C3 side chain at C-2 position and the unsaturated bond between C-2 and C-3 on pyrolidien C-ring, which are very important for the potent activity. Neothramycin-DNA adduct can not be further stabilized because

of the absence of a hydroxyl group at C-9 position in neothramycin.[58] Neothramycin has been shown to inhibit the growth of bacteria such as *E. coli* and exhibit activity against mouse lymphoblastoma L5178Y and HeLa cell lines.[17]

1.3.5 Biological activity of tomaymycin and DC-81

The chemical structures of tomaymycin and DC-81 are very similar. The major structural difference between them is the existence of one addition *exo* unsaturated group at C2 position in tomaymycin, which is probably the reason why tomaymycin is more potent than DC-81. Some tomaymycin analogues with similar C2 unsaturated structures have been successfully synthesized and the pharmacological experiments have proven the importance of C2-unsaturation, which can enhance both DNA-binding ability as well as cytotoxicity.

1.3.6 Relationship between the chemical structures and biological activity of PBDs

A series of PBD analogues have been synthesized to study the relationship between PBDs structure and their biological activity. Modifications on pyrrolidine C-ring and the substitutions of aromatic A-ring have been achieved based on antitumor agent DC-81. Toxicity studies showed that functional group substitutions in the pyrrolidine C-ring, particularly the C2-C3 unsaturation or C2 side chain was very important for the binding affinity and cytotoxicity.[59] The presence of either C2-C3 *endo* unsaturation in the pyrrolidine C-ring or C2 *exo* unsaturaturation can enhance the DNA binding ability and activity of PBDs in vitro.[60, 61] Neither anthranilic acid nor 3-hydroxyanthranilic acid (figure 1-14) can form adducts with double-stranded DNA and inhibit the biosynthesis of DNA and RNA. These two structural building blocks of PBDs have no biological activities

toward cell lines as their parent PBDs do and the changes of the substitutions on the aromatic A-ring did not dramatically improved the corresponding biologically activities of PBDs. This illustrates that the anthranilic acid A-ring moiety in PBDs is not solely responsible, or at least not enough, for the overall biological activities of PBDs.

anthranilic acid   3-hydroxyanthranilic acid
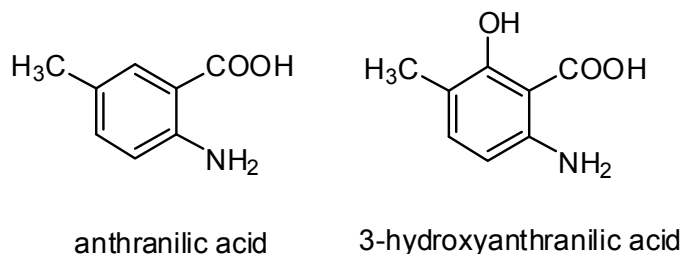
Figure 1-14. anthranilic acid and 3-hydroxyanthranilic acid.

1.3.7 Chemical synthesis of PBD analogs for better biological activity

After the identification and characterization of PBDs, tremendous efforts have been devoted to creating novel benzodiazepine compounds and a great number of analogues have been chemically synthesized for better biological activities and less cytotoxicity. Among these PBD analogs, compounds with structural modifications in the anthranilate A-ring, C2/C3 side chain as well as the unsaturation between C-2 and C-3 have exhibited different biological activities compared with their mother PBDs and also well elucidated the relationship between their chemical structures and activities, which may pay the way for further modifications of PBDs through chemical synthesis. The synthesis of some other analogs brilliantly utilized the concept of combining the active structures from PBDs and other antibiotics to retain expected activities and reduce their

cardiotoxicity. Many studies supported the conclusion that PBDs and DNA were connected by a covalent bond and further stabilized by hydrogen bonding interaction between them. Recently a structurally similar synthetic compound with a 2-naphthyl group at C-2 position of tomaymycin shows high binding affinity with double-stranded DNA and has been demonstrated to be able to stabilize the corresponding adduct predominantly by hydrophobic (van der Waals) interactions because of the planar hydrophobic groups at C-2 position.[62]

2. Biosynthesis of nitro sugar and arylnitro group in natural products

2.1 Biosynthesis of nitro sugar containing natural products

2.1.1　Biosynthesis of deoxyaminosugars

In the past twenty years, secondary metabolites from bacteria, especially a wide variety of sugar containing natural products, have been extensively studied. Glycosylated natural products exhibit potent biological activities and it has been demonstrated that decorating sugars play very important roles in the activities of their parent compounds.[63-65] For bacteria and other microorganisms, secondary metabolites are of great ecological importance and generally used as defenses against predators, parasites and for interspecies competition. Secondary metabolites produced by microorganisms can be used in drug discovery and other biotechnology fields.[66]

Deoxyaminosugars are deoxysugars containing amino groups or amino-derived groups and are biosynthesized by a variety of organisms, including

fungal, bacterial and botanical sources. Deoxyaminosugars play significant roles in the structures of lipopolysaccharides (LPS)[67, 68] and extracellular polysaccharides (EPS)[69] as well as the biological functions of natural products[70] from their producers. Of the above functions, we are mostly interested in the biosynthesis and functional studies of highly modified deoxyaminosugars produced by microorganisms, specifically bacteria.

Deoxyaminosugars are generally derived from precursors such as glucose 6-phosphate or fructose 6-phosphate through both primary metabolic pathways and secondary metabolic pathways. Genes for the biosynthesis of deoxyaminosugars can generally be identified in the complete gene clusters for the biosynthesis of their corresponding deoxyaminosugars containing natural products but are not always clustered together. The genes for the biosynthesis of deoxyaminosugars are relatively conserved and generally code for dehydratases, ketoreductases, isomerases, methyltransferases and aminotransferases etc. After a series of enzymatic transformations on their common precursors, the modified deoxysugars are attached to the final natural products by glycosyltransferases. Amino groups generally occur at the C2, C3, C4 and C6 positions on the deoxyaminosugars. C3 and C4 amino sugars containing deoxyaminosugars are mostly identified on aromatic and macrolide polyketides, nonribosomal peptides and oligosaccharides.[71, 72] C2 and C6 amino sugars are generally found in aminoglycosides.[73]

The most common precursor for the biosynthesis of deoxyaminosugars as well as other deoxysugars is glucose 6-phosphate which is derived from D-

glucose by an anomeric hexose kinase. Glucose 6-phosphate can be subsequently converted to NDP-α-D-glucose and then a key intermediate in deoxysugar biosynthesis, NDP-4-keto-6-deoxy-α-D-glucose, from which many diversely modified deoxysugars including deoxyaminosugars are derived. Deoxyaminosugars originated from NDP-4-keto-6-deoxy-α-D-glucose include TDP-D-desosamine, TDP-D-forosamine, TDP-D-vancosamine, TDP-L-daunosamine and TDP-D-mycaminose. Another common precursor for the biosynthesis of deoxyaminosugars is fructose 6-phosphate. Fructose 6-phosphate is converted to GDP-D-mannose which can be further modified to produce different NDP sugars (figure 1-15).[74]
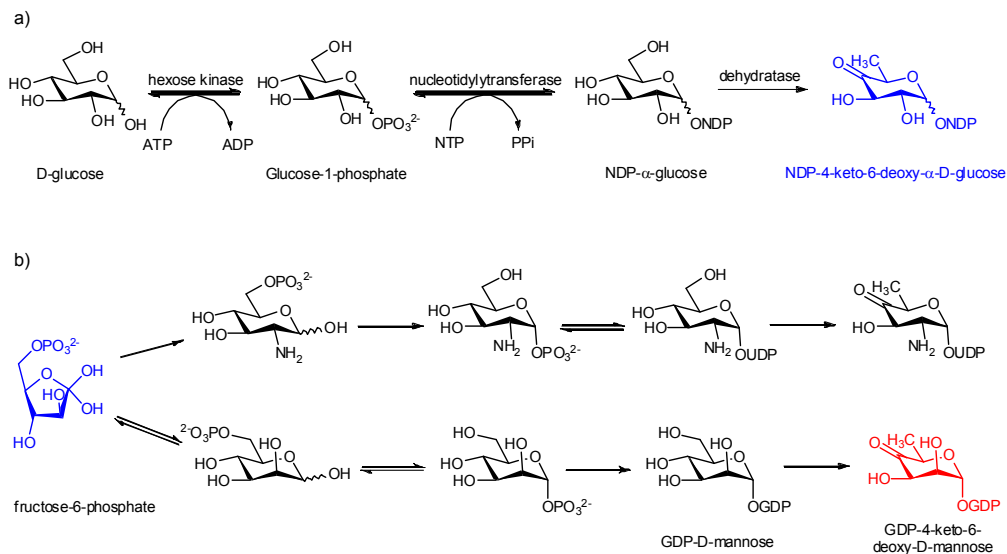
Figure 1-15. Two common precursors for deoxyaminosgurs: NDP-4-keto-6-deoxy-α-D-glucose (a) and fructose 6-phosphate (b).

The amino groups in deoxyaminosugars can be ionized *in vitro* and have the ability to interact with target molecules through the formation of hydrogen bonds or electrostatic bonds. For example, the hydrogen bond is vital for the

interaction between the 14-membered ring macrolides and the ribosome target.[75-][78] Besides, the amino group is also involved in the signal interaction among natural products or their producers.[79]

2.1.2 Examples of aminosugars from natural products

In the past several decades, a large number of amino sugars from natural products that contain diverse substitutions and structures have been reported. They are derived from the two common precursors: glucose 6-phosphate and fructose 6-phosphate. The biosynthetic pathways for some of the intriguing amino sugars from microbial natural products have been extensively investigated (figure 1-16).
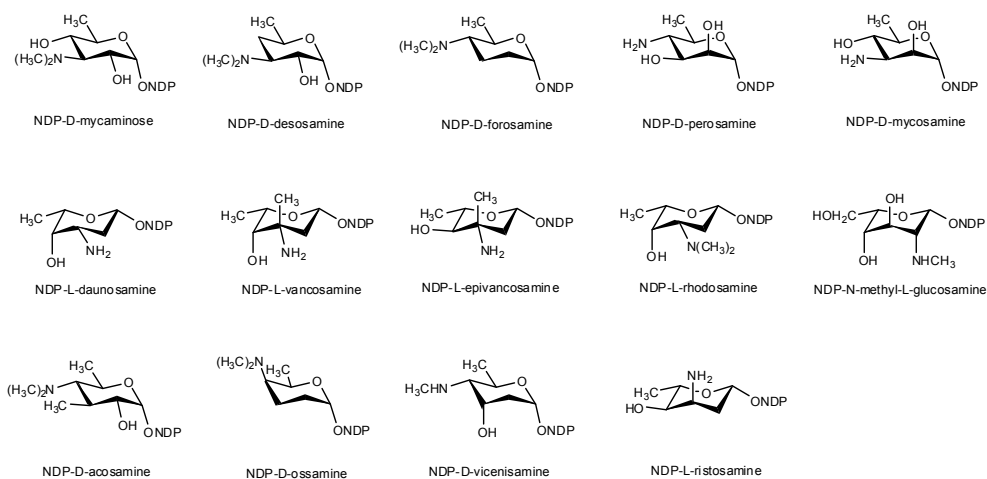
Figure 1-16. Examples of NDP-deoxyaminosugars from natural products.

2.1.2.1 D-Desosamine

D-Desosamine can be found to exist in several natural products produced

28

by different species. One of the most well known natural product is erythromycin from *Saccharopolyspora erythraea*.[80] Erythromycin possesses two sugars, D-desosamine and L-cladinose, which are attached to a 14-membered ring biosynthesized by polyketide synthase (PKS). The erythromycin biosynthetic gene cluster has been reported[81] and the biosynthesis of D-desosamine requires the involvement of five genes coding for isomerase, aminotransferase, dehydratase and 3,4-reductase and methyltransferase (figure 1-17). Other D-desosamine containing natural products include oleandomycin from *Streptomyces antibioticus*[82] and methymycin/pikromycin from *Strptomyces venezuelae*.[83]
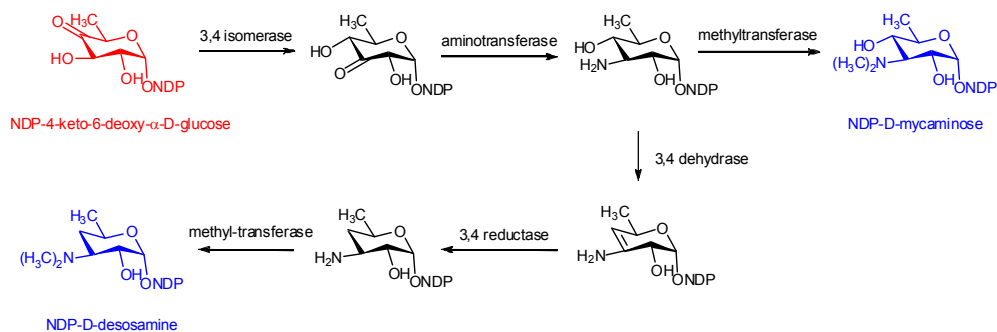
Figure 1-17. Biosynthetic pathways of NDP-D-desosamine and NDP-D-mycaminose.

2.1.2.2   D-Mycaminose

Tylosin, isolated from *Strptomyces fradiae* T59235, contains a 16-membered ring macrodide decorated with three dexosugars, one of which is mycaminose.[84] Notably, two genes for the biosynthesis of the general deoxy sugar precursor NDP-4-keto-6-deoxy-α-D-glucose, *tylAI* and *tylAII*, exist in the tylosin biosynthetic gene cluster. Subsequently, the biosynthesis of D-

29

mycaminose from NDP-4-keto-6-deoxy-α-D-glucose requires three tailoring enzymes: isomerase, aminotransferase and methyltransferase (figure 1-17).[85]

2.1.2.3   Amino sugars in vancomycin family natural products

Vancomycin is produced by *Streptomyces orientalis* and contains vancosamine, 3-amino-2,3,6-trideoxy-3-C-methyl-L-arabino-hexose. Chloroeremomycin with two epivancosamines, the 4-*epi* isomer of vancosamine, is another representative natural product in vancomycin family. Bahlimycin is structurally similar to vancomycin but possesses the 4-oxo form of 3-methyl-3-amino trideoxyhexose (figure 1-18). The biosynthetic gene clusters for both chloroeremomycin and bahlimycin have been identified and the biosynthetic pathway for epivancosamine has been well studied *in vitro*. The vancomycin biosynthetic gene cluster has not been reported to date and the exact biosynthetic pathway for vancosamine has not been studied *in vitro*. Since the only difference between vancosamine and epivancosamine is the stereochemistry of 4-OH group, stereoisomers vancosamine and epivancosamine potentially share a similar biosynthetic pathway in which the 4-keto group can be reduced to a hydroxyl group with either *axial* or equatorial stereochemistry by ketoreductase.[74]
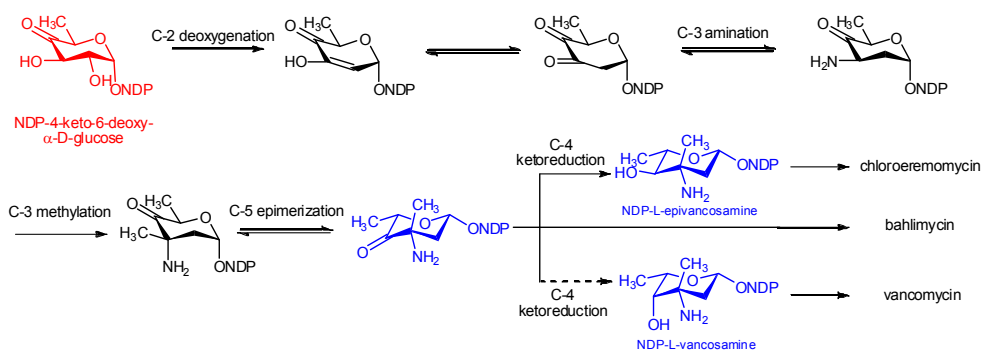
Figure 1-18. Biosynthetic pathways for NDP-L-vancosamine and NDP-L-epivancosamine.

2.1.2.4   Forosamine from Spinosyn

Spinosyns are produced by *Saccharopolyspora spinosa* and consist of more than 25 derivatives with minor structural differences from the culture of the same strain.[86, 87] Spinosyns contain two dexosugars, an amino sugar forosamine and tri-O-methylrhammnose, which are attached to a tetracyclic macrolide core biosynthesized by a polyketide synthase (PKS). Spinosyns show dramatic mosquito larvicidal and insecticidal activities probably by binding with the nervous system receptors and damaging the insect nervous system.[88] The biosynthetic gene cluster for spinosyns has been identified and feeding experiments have been utilized to elucidate the biosynthetic pathway for spinosyns.[89] In the biosynthetic gene cluster of spinosyns, five genes, *spnONQRS*, are proposed to be involved in the biosynthesis of forosamine and have been proven *in vitro* to encode 2,3-dehydratase, 3-ketoredutase, 3-dehydratase, aminotransferase and 4-dimethyltransferase respectively (figure 1-19).[89-94] Several other natural products including spiramycin, forosaminylgriseucin A and dunaimycin also

contain forosamine.[95, 96]
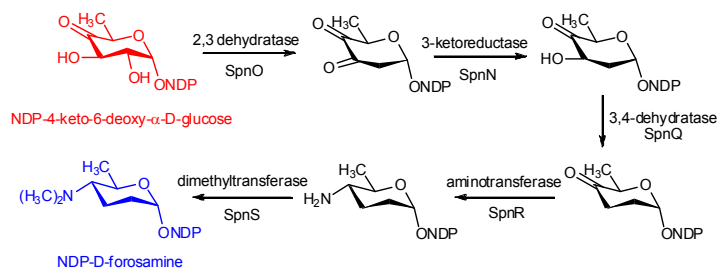
Figure 1-19. Biosynthetic pathways for NDP-D-forosamine.

2.1.2.5 Daunosamine from daunorubicin

Daunorubicin is a representative example of the anthracycline natural products with a four fused-ring aromatic system biosynthesized by PKS.[97] Daunorubicin exhibits dramatic biological activity, in particular antitumor activity, and has been used clinically.[98] Daunorubicin contains an amino sugar daunosamine which is of great importance for its biological activity.[99] At least four genes are proposed to be responsible for the formation of daunosamine from the identified daunorubicin biosynthetic gene cluster and code for dehydratase, epimerase, aminotransferase and ketoreductase respectively (figure 1-20). But after the identification of the daunorubicin biosynthetic gene cluster, the detailed functions of some of the genes and the exact biosynthetic pathway for daunosamine have not been well elucidated.[99, 100]
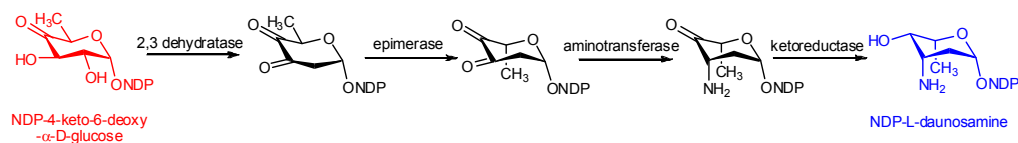
Figure 1-20. Biosynthetic pathways for NDP-L-daunosamine.

2.1.2.6 D-Mycosamine

Nystatin is a natural product produced by *Streptomyces noursei* and contains a 38-membered macrolactone ring with a deoxyaminosugar, D-mycosamine, attached at C-19 position. Other D-mycosamine containing natural products include amphotericin, candicidin, pimaricin and rimocidin.[101-105] All the above natural products exhibit antifungal activity and their biological activity stems from their ability to promote leakage of the ion channel on the membrane by binding with the sterol targets of fungal cell membranes.[106] The biosynthetic gene cluster for nystatin has been identified and three genes are proposed to be involved in the biosynthesis of D-mycosamine derived from GDP-D-mannose and fructose-6-phosphate(figure 1-21).[107]
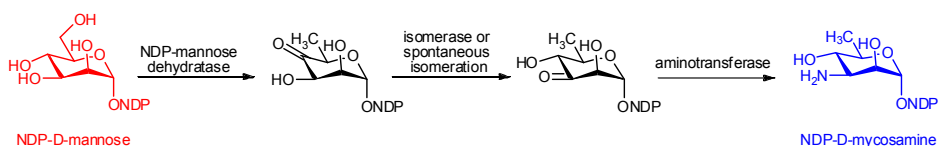
Figure 1-21. Biosynthetic pathways for NDP-D-mycosamine.

2.1.2.7   D-Perosamine

Lipopolysaccharide (LPS) is a very important component for the membrane of some gram-negative bacteria like *E. coli* O157:H7.[108] LPS plays a key role for virus infection by protecting the pathogen from the defense system of the host. LPS is composed of O-antigen, core oligosaccharide and lipidA.[109-111] O-antigen consists of four repeating units: *N*-acetyl-D-erosamine, L-fucose, D-glucose and *N*-acetyl-D-galactose.[112-114] *N*-acetyl-D-erosamine is another representative example of amino sugars derived from fructose-6-phosphate through the GDP-D-mannose intermediate. GDP-D-mannose is first oxidized by a dehydratase to GDP4-keto-D-mannose, which is subsequently converted to GDP-D-perosamine by an aminotransferase (figure 1-22).[115]
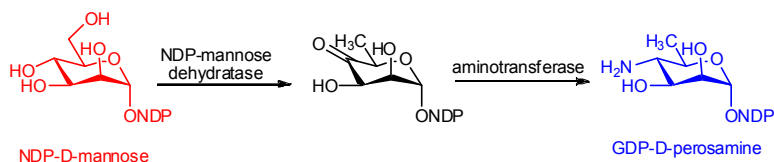
Figure 1-22. Biosynthetic pathway for GDP-D-perosamine.

2.1.2.8   Other amino sugars from natural products

Besides the amino sugars mentioned above, there are still other amino sugars that exist in identified natural products. Biosynthetic pathways for these amino sugars have not been well investigated because the corresponding biosynthetic gene clusters are not available or incomplete. These amino sugars with undetermined biosynthetic pathways include *N*-methyl-L-glucosamine in

streptomycin produced by *Streptomyces griseus* and *Streptomyces glaucescens*[116], ristosamine in ristomycin produced by *Proactinomyces fructiferi* var. *ristomycin*[117], ossamine in ossamycin produced by *Streptomyces hygroscopicus* var. *assamyceticus*[118, 119], rhodosamine in megalomicin A produced by *Micromonospora megalomicea*[120-123], and vincenisamine in vicenistation produced by *Strptomyces* sp. HC-34 (figure 1-16)[124].

2.1.3 Biosynthesis of nitro sugars from natural products

Only a small portion of amino sugars as mentioned above can be oxidized to the corresponding nitro sugars which have been proven to be very important for the potent biological activities of their mother compounds. The biosynthetic pathways of nitro sugar and the mechanism for the formation of the nitro group remained uninvestigated for a long time, after the identification and characterization of some nitro sugar containing natural products.

In the known nitro sugar containing natural products, deoxysugars share the same carbon skeleton, but stereochemistries of attached nitro groups may vary. Nitro sugar containing natural products are represented by everninomycin produced by *Micromonospora carbonacea* var. *africana* ATCC39149[125], rubradirin produced by *Streptomyces achromogenes* var. *rubradiris*[126] and kijanimicin produced by *Actinomadura kijaniata*[127] as shown in Figure 1-23. The biosynthetic gene clusters for the above three natural products have been reported.[128-130] These identified nitro sugar containing natural product biosynthetic gene clusters from their producers have provided a basis for us to

elucidate the biosynthetic pathways for the formation of their unique nitro sugars and characterize the functions of enzymes involved in the biosynthesis of nitro sugars.
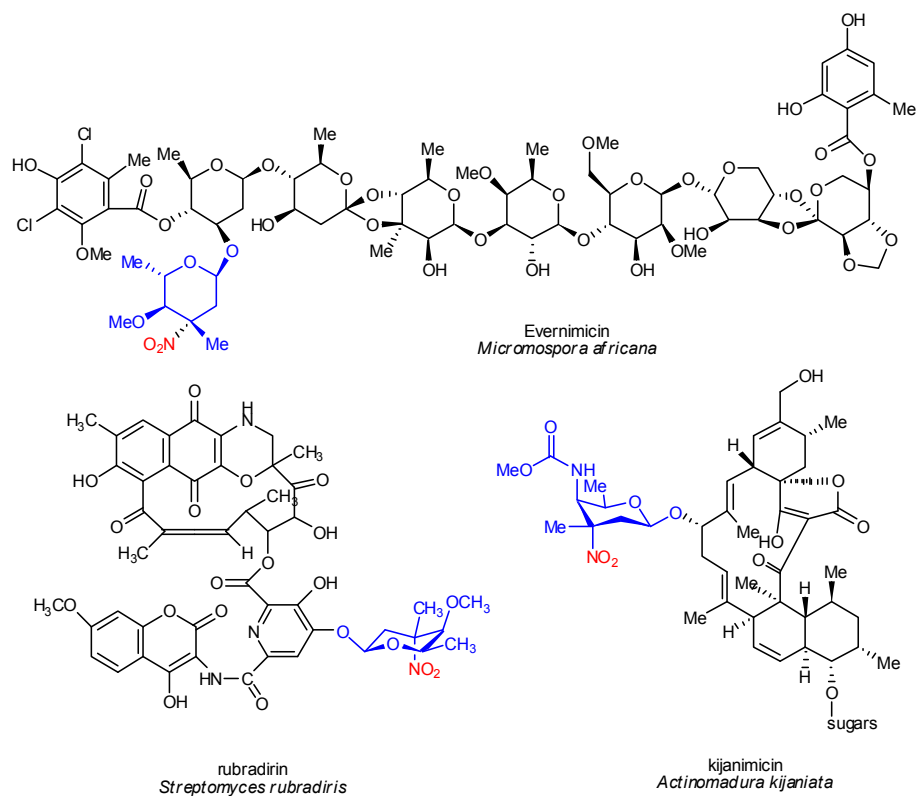
Figure 1-23. Structures of everninomycin, rubradirin and kijanimicin.

2.1.3.1   Everninomycin

Everninomycin belongs to the orthosomycin class of antibiotics and contains a nitro sugar and seven deoxysugar residues attached to modified orsellinic acid with amazing thirty-five asymmetric centers.[128] Some other natural products belonging to the orthosomyicn family of antibiotic include curamycin,

olivamycin and avilamycin. Everninomycin antibiotics exhibit broad-spectrum biological activity against both Gram-positive and Gram-negative bacteria, notably some multidrug-resistant bacteria including penicillin-resistant streptococci, methicillin-resistant staphylococci and glycopeptide-resistant enterococci as well as bacteria resistant to vancomycin, which has been known as the "antibiotic of last resort".[131, 132] The fact is that so far there are not many alternative antibiotics to conquer infectious diseases, so the occurrence of everninomycins may provide another option to combat infectious diseases caused by the above multidrug resistance bacteria which have been very common in clinical settings.

Like chloramphenicol, which is an important member of vancomycin family, everninomycin is effective against bacteria by the inhibition of protein synthesis, especially by binding to ribosomal protein L16 and subsequently affecting the functions of 50S ribosomal subunit.[133-135] Everninomycin producer *Micromonospora carbonacea* var. *africana* can produce several other structurally related secondary metabolites differing in possession of an amine, hydroxylamine, nitroso or a novel alkoxyl group in the place of nitro group in everninomycin (figure 1-24). However, these structurally similar natural products from the same producer strain *M. africana* exhibit significantly different biological activities. For example, everninomycin with a nitro group at C65 is active again gram-positive bacteria with a mean Minimum Inhibitory Concentration (MIC) of 0.03 mcg/mL for all the 22 tested Staphylococci strains, whereas the everninomycin derivative with an amino sugar at C65 is active again gram-

positive bacteria with a mean MIC 2.2 mcg/mL for all the 11 tested Staphylococci strains and gram-negative bacteria with a mean MIC of 100.4 mcg/mL for all the 57 tested strains. The range of activities in the everninomycin natural products indicates that the nitro group is critical for the biological activity of nitro group containing natural products.

	R_1	R_2	R_3	R_4	( )
everninomycin	OMe	Cl	Me	NO_2	CH_2
ever-amino	OMe	Cl	Me	NH_2	CH_2
ever-hydroxyl	OMe	Cl	Me	NHOH	CH_2
ever-nitroso	OMe	Cl	Me	NO	CH_2
Sch 58769	OH	H	Me	NO_2	CH_2
Sch 58771	OMe	H	Me	NO_2	CH_2
Sch 58773	OMe	Cl	H	NO_2	CH_2
Sch 58775	OMe	Cl	Me	NO_2	OCOH, H
Sch 49088	OMe	Cl	Me	R_5	CH_2
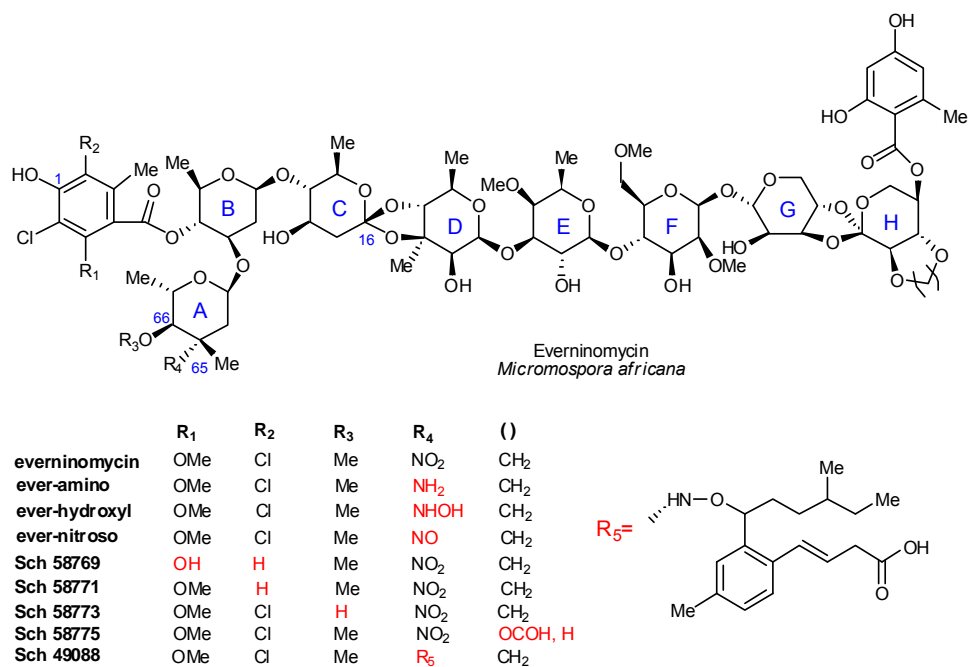
Figure 1-24. Everninomycin derivatives from *Micromonospora africana*.

Early experiments have shown that amino group, hydroxyl group and nitroso group are able to affect the biological activity of everninomycins. Lately, some other structure features have also been proven to be necessary for the activity of everniomycin. For example, the stereochemistry at C16 is vital, because the isomer of everninomycin with reverse stereochemistry at C16 does not possess activity. The orthoester linkages are also required for the activity of

everninomycin because the hydrolysis of orthoester linkages will inactive everninomycin. The phenolic hydroxyl at C1 is also imperative, because everninomycin derivatives with a methyl or allyl ether at C1 position do not exhibit activity. Interestingly, although the nitro group at C65 has been proven to have effect on everninomycin activity, the complete loss of the nitro sugar does not dramatically change biological activity of the corresponding new natural product. One everninomycin derivative with chemically modified acetamide structure at the original C65 nitro group position exhibits the highest biological activity.[136]

Previously, only everninomycin derivatives with the amino sugar, hydroxylamino sugar, nitroso sugar or nitro sugar have been isolated from the broth of everninomycin producer *M. africana*. In recent years, some other oligosaccharide secondary metabolites with structural differences at other positions have also been reported. They are Sch 49088, Sch 58769, Sch 58773, Sch58775 and Sch 58777 (figure 1-24), the structures of which have been confirmed by NMR.[137, 138] These novel oligosaccharide natural products are different from everninomycin mainly in the substitutions on the PKS derived aromatic ring, C-66 position on the nitro sugar group as well as the side chain on the H deoxysugar. Of note, natural product Sch 58773 contains a hydroxyl group instead of the everninomycin-harboring $-OCH_3$ group at C-66 position on nitro sugar, meaning that the substitution on C66 position, at least changing from $-OCH_3$ to $-OH$, does not affect the *N*-oxidation of the amino sugar precursor to the nitro sugar.

The biosynthetic gene cluster for everninomycins was reported in 2002.

The identification of everninomycins biosynthetic gene cluster greatly facilitates the elucidation of the everninomycin biosynthetic pathway, especially the formation of novel nitro sugar. In the everninomycin gene cluster that harbors more than 40 biosynthetic genes, seven genes are proposed to be involved in the biosynthesis of L-evernitrose from the common sugar precursor NDP-4-keto-6-deoxy-a-D-glucose. These seven genes function in the sequential 2,3-dehydration, amino transformation, C-methylation, epimerization, C-4 keto reduction, O-methylation and N-oxidation steps followed by the transformation of the mature deoxysugars onto everninomycin by a glycosyltransferase (figure 1-25).
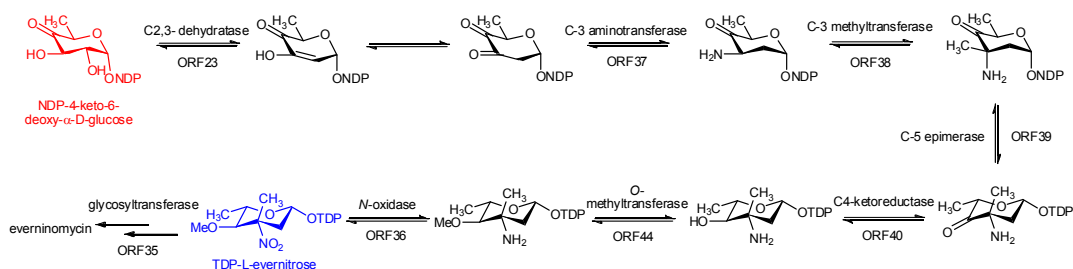
Figure 1-25. Proposed biosynthetic pathway of TDP-L-evernitrose for everninomycin.

2.1.3.2 Rubradirin

The polyketide rubradirin, first isolated in 1964, contains one nitro sugar rubranitrose and is active against a variety of Gram-positive bacteria both *in vitro* and *in vivo*.[126] Its antibacterial activity is due to its impairment of protein synthesis by selective inhibition of ribosomal functions.[139-142] To date, the stereochemical studies performed on rubranitrose reveal confusing and ambiguous results. But

based on the biosynthetic pathway (figure 1-26) of rubranitrose in which there is no epimerase and the chemical synthesis of enantiomers of rubranitrose, rubranitrose should be assigned as D configuration and share similar biosynthetic pathway with D-kijanose in kijanimicin. Another intriguing result is the isolation of protorubradirin which is structurally different from rubradirin only in the nitroso group at the original nitro group. When the isolation of rubradirin was conducted in complete darkness, protorubradirin could be isolated from the broth of rubradirin producer *S. rubradiris* and then oxidized to rubradirin after its exposure to light. So it has been proposed that protorubradirin with a nitroso sugar is the true product produced by *S. rubradiris* and rubradirin is the oxidative product of protorubradirin.[143]
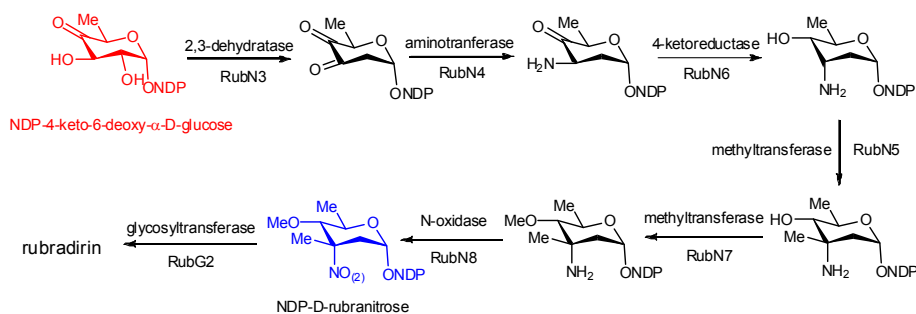
Figure 1-26. Proposed biosynthetic pathway of NDP-D-rubranitrose in rubradirin.

2.1.3.3   Kijanimicin

Kijanimicin is composed of a PKS biosynthesized pentacyclic core which is decorated by four L-digitoxoses and an unusual nitro sugar, D-kijanose. Kijanimicin is effective against some Gram-positive bacteria, anaerobic microorganisms and malaria.[127] Recently it has also been found that kijanimicin

41

possesses significant antitumor activity.[144] Sequence analysis of the identified 107.6 kb kijanimicin biosynthetic gene cluster indicates that six genes are proposed to be involved in the biosynthesis of the nitro sugar (figure 1-27), D-kijanose. One flavin dependent oxidase KijD3 shows high similarity with ORF36 in the everninomycin gene cluster as well as RubN8 in the rubradirin gene cluster and is predicted to mediate the oxidation from an amino sugar intermediate to the final nitro sugar.[130]
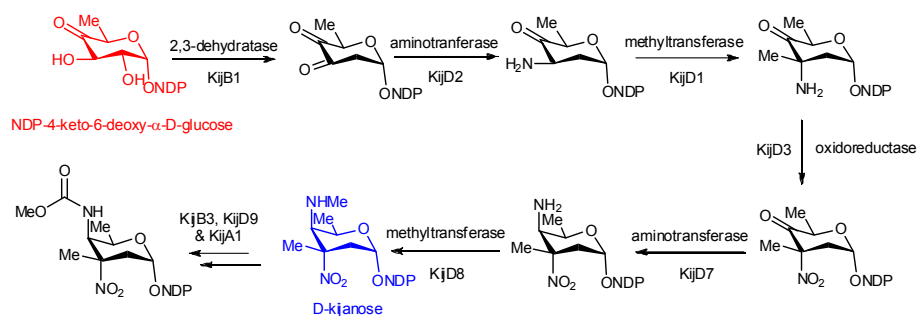
Figure 1-27. Proposed biosynthetic pathway for D-kijanose in kijanimicin.

2.2 Biosynthesis of aryl nitro group containing natural products

Nitro groups can also be found to be connected with aromatic groups in a large number of natural products. These aryl nitro natural products, mostly produced by bacteria and fungi, possess potent biological activities and have great potential in the development of drug discovery and other industries like explosives and dyes. Here we are mostly interested in the study of those microbial aryl nitro compounds with dramatic biological activities. Chloramphenicol is the first example of aryl nitro class of natural product and

isolated from *Streptomyces venezuelae*. Chloramphenicol has been used clinically for the treatment of a great variety of infectious diseases. Other representative examples of the aryl nitro natural products include dioxapyrrolomycin produced by *Streptomyces fumamus*, 1-nitroaknadinine produced by plant *Menispermaceae*, thaxtomin produced by *Stretomyces*, pyrrolnitrin produced by *Pseudomonas fluorescens* and aureothin produce by *Streptomyces thioluteus (*figure 1-28).
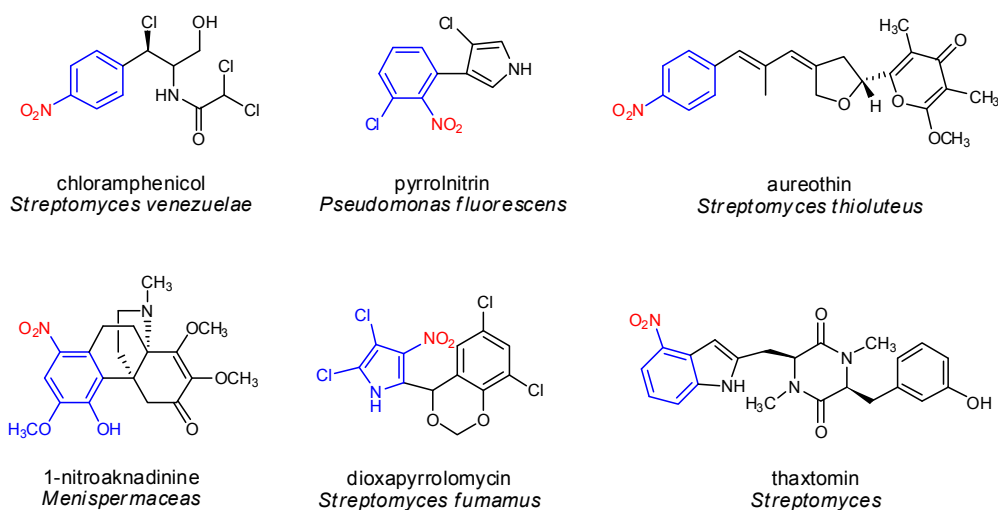
Figure 1-28. Representative examples of aryl nitro containing natural products.

Aryl nitro functional groups are different from nitro sugars not only in their chemical structures. More importantly, they possess dramatic diverse mechanisms and biosynthetic pathways compared to that of nitro sugars that have been proven to be oxidized from amino sugars by *N*-oxidases. To date, there are mainly two mechanisms for the formation of arylnitro groups: direct nitration and enzymatic *N*-oxygenation.

2.2.1 Direct nitration mechanism for the biosynthesis of aryl nitro natural product

   The first class of mechanism for the biosynthesis of aryl nitro functionality is direct elctrophilic nitration of aromatic groups by NO_2^+ through which the nitro groups are introduced into the aromatic rings of natural products. This kind of nitration mechanism is dramatically different from the mechanism for the biosynthesis of nitro sugars in which an amino sugar is oxidized to a nitro sugar enzymatically. The first example of direct nitration was reported in 1989, with a biosynthetic investigation of the nitro group in dioxapyrrolomycin.[145] Dioxapyrrolomycin as well as its proposed precursor pyrrolomycin C missing the corresponding -NO_2 at C4 can both be found from the culture of *Streptomyces fumanus*. Feeding studies have shown that the benzenoid ring is derived from three acetate units and the pyrrole ring is derived from L-proline.[146, 147] Originally, the formation of nitro group on the pyrrole was proposed to be due to the oxidation of an amino group because a secondary metabolite 3-amino-L-proline was identified. Later incorporation experiments demonstrated that the introduction of C4 nitro group was dependent on the components in the medium. If $^{15}N^{18}O_3^-$ was used as the sole source of nitrogen in the medium for *S. fumanus*, all nitrogen and oxygen atoms in C4 nitro group on the pyrrole ring would come from $^{15}N^{18}O_3^-$.[148] This direct nitration step for the biosynthesis of dioxapyrrolomycin was also confirmed by *in vitro* treatment with HNO_3 (figure 1-29).[149]
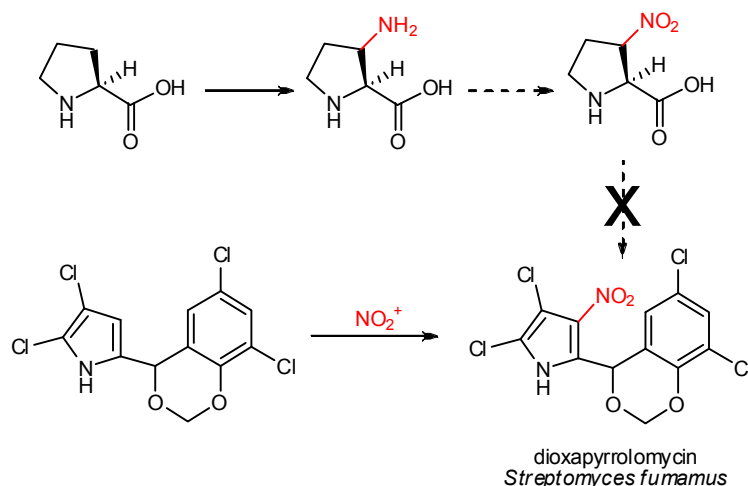
Figure 1-29. Direct nitration mechanism for the biosynthesis of dioxapyrrolomycin.

Another prominent example of direct nitration is related to thaxtomin which is a nitro-containing produced by plant pathogen *Streptomyces turgidiscabies.*[150] In the genome of *Streptomyces turgidiscabies* one *nos* gene exists upstream of thaxtomin biosynthetic gene cluster and shows high similarity with the *nos* genes in mammals.[151] Nitric oxide (NO) produced by nitric oxide synthases (NOSs) identified in mammals have been recognized as a potent signal molecular and plays important roles in many body responses in mammals.[152, 153] Gene disruption and complementation experiments have already shown the necessity of *nos* gene for the biosynthesis of thaxtomin.[154] Further biochemical studies also proved that NOS could mediate the nitration reaction to produce L-4-nitrotryptophan from the oxidation of NO during the biosynthesis of thaxtomin (figure 1-30).[155, 156]
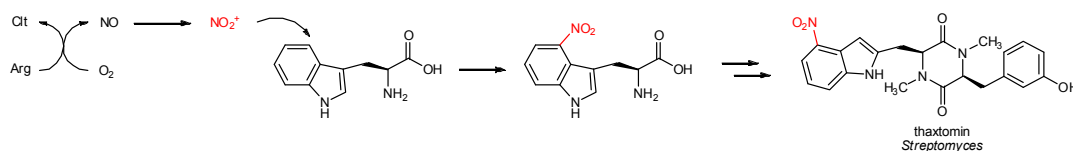
Figure 1-30. Direct nitration involved in the biosynthesis of thaxtomin.

2.2.2 Enzymatic oxidation in the biosynthesis of aryl nitro natural products

The nitro groups from some other identified aryl nitro containing natural products are oxidized from amino groups by *N*-oxidase in their biosynthetic gene clusters. Although the oxidation reaction for the biosynthesis of aryl nitro structures seems similar to that for nitro sugars at first sight, the truth is that the formations of aryl nitro groups are mediated by *N*-oxidases with dramatic different oxidation mechanisms.

The first characterized oxygenase related to the biosynthesis of aryl nitro is PrnD for the biosynthesis of pyrrolnitrin produced by *Pseudomonas fluorescens.*[157] Pyrrolnitrin is derived from tryptophan and exhibits potent and broad-spectrum activity against fungi. The biosynthetic gene cluster for pyrrolnitrin has been identified from the genome of *P. fluorescens* and four clustered genes are involved in the production of pyrrolnitrin.[158] Previous gene disruption experiments have already confirmed the function of each gene in the pyrrolnitrin biosynthetic gene cluster. PrnA can mediate the chlorination of L-tryptophan to yield 7-chloro-L-tryptophan, followed by the ring rearrangement and decarboxylation catalyzed by PrnB. PrnC is another halogenase which can introduce the second chlorine onto the aromatic ring. PrnD is the last biosynthetic

enzyme responsible for the oxidation of an amino group to a nitro group. Further biochemical experiments *in vitro* proved the function of PrnD in the formation of arylnitro group. PrnD is a novel Rieske iron-sulfur cluster *N*-oxidase which is able to catalyze the arylamine oxidation reaction from aminopyrrolnitrin to pyrrolnitrin and requires the involvement of a flavin reductase to provide reduced flavin for PrnD from NADPH (figure 1-31).[159] Because the substrate for PrnD is not easily obtained, a substrate analog pABA was used to investigate the mechanism for this arylamine oxidation reaction. In this Rieske *N*-oxygenase catalyzed oxidation reaction, the amine group is first oxidized to a hydroxyl group and then to a nitroso group before the formation of the final product with a nitro group (figure 1-32).[160]
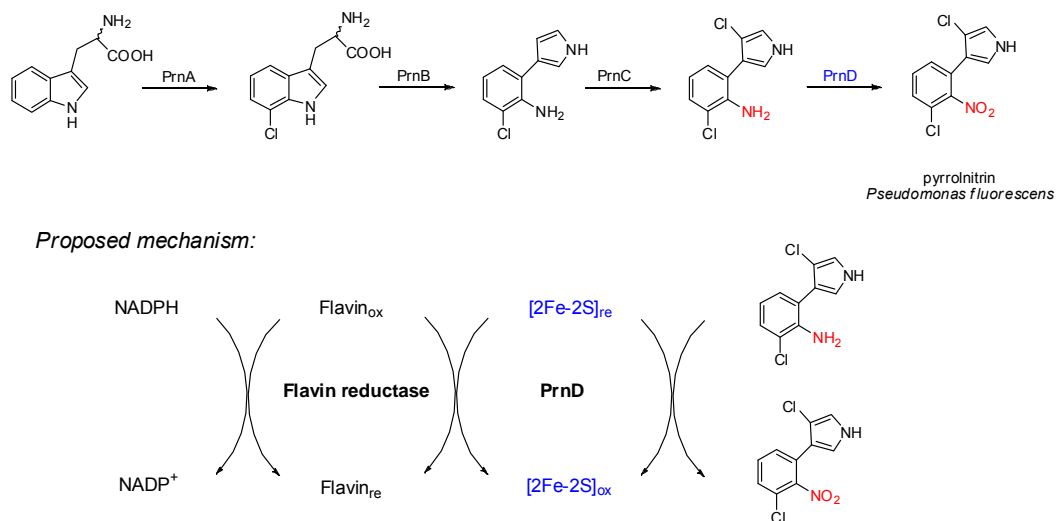
Figure 1-31. Biosynthetic pathway of pyrrolnitrin and mechanism of PrnD catalyzed reaction.
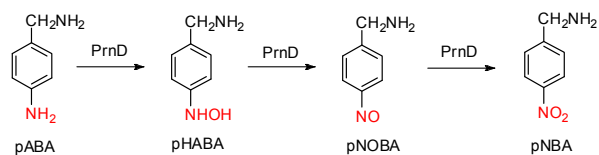
Figure 1-32. Biosynthetic mechanism of *N*-oxidazse PrnD.

N-oxidase AurF represents another example involved in the biosynthesis of aryl nitro polyketide secondary metabolite aureothin, which is produced by *Streptomyces thioluteus* and exhibits potent antifungal and antitumor activities.[161] The gene cluster for the biosynthesis of aureothin was identified from the genome of *S. thioluteus* and subsequent analysis of this gene cluster showed that the PKS was involved in the biosynthesis of aureothin.[162] The function of the aureothin gene cluster has been determined using gene disruption and heterologous expression methodologies. In the aureothin biosynthetic gene cluster, the function of AurF was suggested to be an *N*-oxidase based upon in-frame deletion results.[65] Biochemical experiments further proved the function of AurF which can catalyze the oxidation of para-aminobenzoic acid (pABA) to para-nitrobenzoic acid (pNBA) through the intermediate para-(hydroxyamino) benzoic acid (figure 1-33).[163, 164] The two oxidation reactions mediated by PrnD and AurF share very similar biosynthetic pathways, but show basically no protein sequence similarity with each other. In addition, the mechanisms for these two oxidation reactions catalyzed by PrnD and AurF are dramatically different. As

mentioned above, PrnD is a Rieske *N*-oxygenase require an iron-sulfur cluster for its activity, whereas AurF is actually a di-nuclear N-oxygenase.[163]
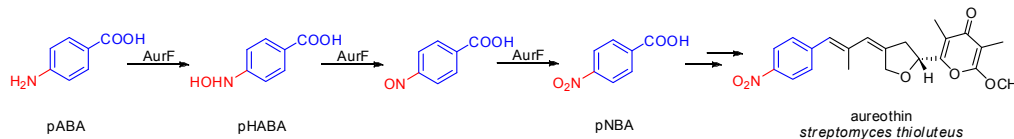
Figure 1-33. Biosynthetic pathway of aureothin.

2.2.3 Possible oxidative functions of halogenases in the biosynthesis of aryl nitro natural products

As can be seen from the structures of aryl nitro containing natural products, chlorine is often found to coexist with those aryl nitro functional groups and introduced into natural products by halogenases. So those identified halogenases from the biosynthetic gene clusters can possibly function in the oxidation of amino groups to nitro groups. Indeed, some experiments have provided some evidence showing the ability of halogenase to oxidize aryl amine groups to aryl nitro groups, however these oxidation reactions actually occurred only in extreme or non natural conditions.[165, 166] So it is very possible that the *N*-oxidation reaction catalyzed by halogenases should not happen *in vivo* and halogenases are not the true *N*-oxidases.

3. Dissertation aims

3.1 Biosynthesis of pyrrolo[2,1-c] benzodiazepine natural products

pyrrolo[2,1-c] benzodiazepine natural products represent a very important

class of natural products produced by microorganisms. PBD natural products exhibit dramatic biologically activities, especially antitumor activity and antibacterial activity. To date, a great variety of synthetic benzodiazepine containing drugs have been used clinically mainly as anxiolytic, hyptonic, anticonvulsant and muscle relaxant, etc. Since microorganisms can produce benzodiazepine natural products with dramatic biological activities, it will be very interesting and of great significance to study and investigate the biosynthetic pathways and mechanisms for benzodiazepine natural products. In the 1970's, the Hurley research group studied the biosynthetic building blocks of benzodiazepine natural products, notably anthramycin, tomaymycin and sibiramycin, by using isotopic labeling experiments. Benzodiazepine natural products have been demonstrated to be derived from three amino acids: tryptophan, tyrosine and methionine. But afterwards, very little is known in terms of the biosynthesis of microbial benzodiazepine natural products at either the genetic level or the biochemical level. So in this project, we intended to investigate the biosynthetic pathways and mechanisms of benzodiazepine natural products mainly from the genetic level. In our case, we studied the biosynthesis of anthramycin, which is produced by *S. refuineus* and is the first isolated benzodiazepine natural products. Generally the biosynthetic genes for natural products produced by bacteria are clustered on the genome, our purpose is to identify the biosynthetic gene cluster from the genome of *S. refuineus* and investigate the biosynthetic pathway of anthramycin using genetic methods. The identification of anthramycn biosynthetic gene cluster will greatly facilitate the

identification of gene clusters for other benzodiazepine natural products and pave the way for the biochemical studies of benzodiazepine natural products. In the process of studying anthramycin biosynthesis, we developed a new method which was able to recombine two partial anthramycin gene clusters into a complete anthramycin biosynthetic gene cluster on the same vector. Heterologous expression of the anthramycin gene cluster further confirmed the function of the identified anthramycin gene cluster and ruled out the possibility that genes outside this identified gene cluster were also involved in anthramycin biosynthesis.

3.2 Biosynthesis of nitrosugar containing natural products

Natural products have been demonstrated to exhibit potent biological activities and have been utilized as a great resource for drug discovery. A great many natural products, especially those produced by microorganisms, contain amino sugars or nitrosugars. The biosynthetic pathways and mechanisms for some amino sugars and nitrosugars containing natural products have been studied and elucidated in the past twenty years. Among those identified amino sugars in natural products, not all amino sugars can be converted to nitrosugars. So in the identified nitrosugar biosynthetic gene clusters, there should be some oxidases which are responsible for the oxidation of amino sugars to nitrosugars in natural products. Before our studies on nitrosugar containing natural products, several other research groups studied and investigated the biosynthetic mechanisms of aryl nitro groups in natural products. In our this project, we

intended to identified the genes coding for the oxidases for the biosynthesis of nitro groups in nitrosugar containing natural products from the identified biosynthetic gene clusters for nitrosugar containing natural products using comparative genomic method. Afterwards, the oxidases proposed for the oxidation from amino sugars to nitrosugars were expressed and purified in E. coli. The function of the identified oxidases was chacterized using the chemically prepared amino sugar substrate.

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CHAPTER II

BIOSYNTHESIS OF ANTHRAMYCIN FROM STREPTOMYCES REFUINEUS

Introduction

Anthramycin is a model compound in Pyrrolo[2,1-c] benzodiazepine (PBD) class and exhibits potent antitumor and antibacterial activity as well several other important biological activities.[1, 2] The activities of anthramycin have been demonstrated to be due to the irreversible formation of a covalent bond between anthramycin and double-stranded DNA. The anthramycin-DNA adduct is able to cause the degradation of DNA and further inhibit the synthesis of DNA and RNA from their DNA template.[3-5] Because of its dramatic biological activities and unique mechanism, the anthramycin scaffold has been developed for drug discovery and utilized clinically for cancer treatment. The existing problem for anthramycin as well as other antitumor compounds being studied in the preclinical stage, is the inherent dose-limiting cardiotoxicity for cancer patients, preventing the broad applications of anthramyicn and other structurally related PBDs in the treatment of cancer.[6] "Red/ET cloning system"

The discovery of anthramycin was reported as early as 1940's but at that time anthramycin was believed to be a protein produced by its producer actinomycete *Streptomyces refuineus* sbsp. *Thermotolerans.*[7] Later, with the development of chemical techniques, anthramycin was confirmed to be a small molecule rather than a protein. Anthramycin was isolated and purified from the

broth of *S. refuineus* and the chemical structure of anthramycin was solved using NMR[1]. The total synthesis of anthramycin in 1960s confirmed the chemical structure of anthramycin.[8] Anthramycin producer *S. refuineus* is kind of unusual in that *S. refuineus* is a thermotolerant strain and anthramycin is produced at 47°C. *S. refuineus* belongs to genus Streptomyces, but interestingly, its morphology is very similar to that of *E. coli* when it is cultured in liquid medium or solid medium.

The biosynthesis of anthramycin has been extensively studied after its isolation. The excellent research was mainly accomplished by the Hurley group[9, 10] in the 1970s. For a long time, the elucidation of the anthramycin biosynthetic pathway was based mainly on the results of feeding labeled precursors into the anthramycin producer *S. refuineus*. Those feeding studies have provided insightful information related to the biosynthesis of anthramycin and made the anthramycin biosynthetic pathway much clearer than before. We can also notice that there are still many biosynthetic questions remained to be solved, especially within the partial biosynthetic pathway of the dehydroproline five-member ring of anthramycin and the overall biochemical mechanism for the formation of anthramycin.

After the early research work for anthramycin that had been carried on in the Hurley laboratory, there seems to have been little progress in the biosynthetic study of anthramycin as well as other PBD natural products. In recent years, the anthramycin biosynthetic gene cluster, the first gene cluster for PBD natural products, was identified from the genome of anthramycin producer *Streptomyces*

refuineus by our collaborators in Ecopia Bioscience Inc. The identification of anthramycin biosynthetic gene cluster greatly facilitates the biosynthetic study of PBD compounds at both genetic and biochemical levels. In our laboratory, we mainly study the biosynthesis of anthramycin from the genetic perspective and the research work focusing on anthramycin biosynthesis in our laboratory includes:

1)   We developed a high-efficient genetic manipulation system suitable for the anthramycin producer *Strptomyces refuineus* via E. coli-Streptomyces intergenetic conjugation. Using this mature transformation method, we were able to transform plasmids or cosmids containing *oriT* fragment into *S. refuineus* and manipulate the biosynthetic gene cluster of anthramycin as designed.

2)   We disrupted several key genes in the anthramycin gene cluster: *orf12*, *orf19*, NRPS (*orf21* & *orf22*) to confirm that the identified DNA locus on the genome of anthramycin producer *S. refuineus* was indeed the anthramycin biosynthetic gene cluster. For the disruption of our target genes, we utilized a new gene disruption method, a "Red/ET cloning system"[11], to mutate certain genes in the anthramycin gene cluster.

3)   Additionally, we determined the boundaries of anthramycin biosynthetic gene cluster using the "Red/ET cloning system". We mutated five genes: *orfAS10*, *orf1*, *orf23*, *orf24* and *orfS11* in the boundary regions of the anthramycin gene cluster and demonstrated that *orf1* and *orf25* were the configurations boundaries of anthramycin biosynthetic gene cluster.

4)   We elucidated a partial anthramycin biosynthetic pathway: the pathway for

intermediate 3-hydroxy-4-methylanthranilic acid. Since we had the mutant strains for several disrupted genes in anthramycin gene cluster and some possible intermediates, we did chemical complementation experiments to elucidate this partial anthramycin biosynthetic pathway.

After the identification and functional determination of the anthramycin biosynthetic gene cluster, several other biosynthetic gene clusters of benzodiazepine natural products have also been reported. In 2008, the biosynthetic gene cluster for diazepinomicin/ECO-4601[12](figure 2-01), a novel farnesylated dibenzodiazepinone currently in clinical trials for the treatment of brain, breast, prostate, and pancreatic tumors, was identified from *Micromospora* sp. 046-ECO11.[13, 14] Labeled feeding experiments have been carried out to elucidate the diazepinomicin biosynthetic pathway which is proposed to be derived from 3-hydroxyanthranilic acid and 3-amino-5-hydroxybenzoic acid.[12] The biosynthetic gene cluster of lincomycin, a natural product biosynthetically related to anthramycin, was also found from its producer *Streptomyces lincolnensis* in 2008.[15] The function of this lincomycin gene cluster was confirmed by using heterologous expression in *Streptomyces coelicolor*. Elucidation and comparison of the identified biosynthetic pathways of PBDs and structurally related natural products may provide further insights into our understanding of their biosynthetic mechanisms. Manipulations of benzodiazepines biosynthetic gene clusters on the genome of their producers can possibly increase the production of desired natural products or produce novel benzodiazepines with superior or novel biological activities.
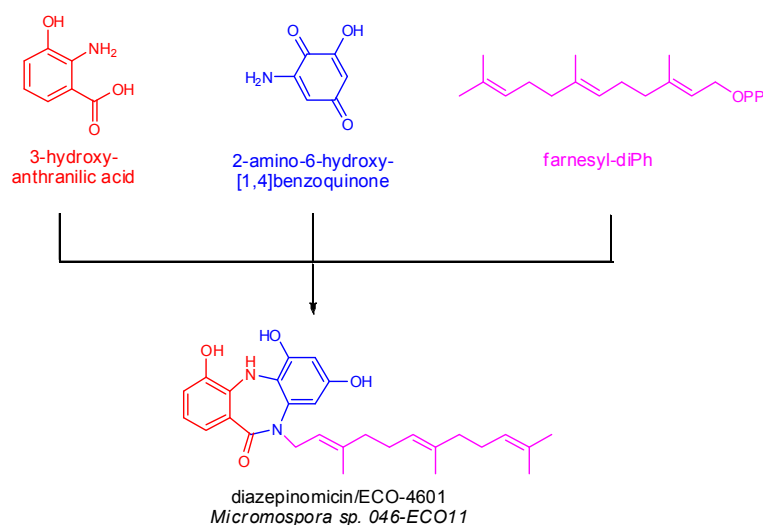
Figure 2-01. Biosynthetic building blocks of diazepinomicin/ECO-4601.

Results and discussion

1. Identification of anthramycin biosynthetic gene cluster from *Streptomyces refuineus*

Using isotopic labeling experiments, Hurley et al. proposed that anthramycin was derived from two halves, each derived by oxidative ring opening of an aromatic amino acid followed by subsequent biosynthetic tailoring steps. The 4-methyl-3-hydroxyanthranilic acid half of anthramycin was proposed to be derived from tryptophan via the primary metabolic kynurenine pathway followed by *S*-adenosylmethionine dependent aromatic *C*-methylation. The "dehydroproline acrylamide" moiety was derived from L-tyrosine via the oxidative ring opening of intermediate DOPA.

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The identification of anthramycin biosynthetic gene cluster was accomplished by Ecopia Biosciences Inc. In this study, the DNA locus for biosynthesis of anthramycin was identified using a rapid genome-scanning approach.

Briefly, a two-tiered genomic DNA library (of 1.5–3 kb and 30–50 kb fragments) was constructed from sonically and enzymatically sheared high-molecular-weight DNA, respectively, isolated from *Streptomyces refuineus* sbsp. *thermotolerans*. The short insert library was cloned into a pBluescript SK+ derivative. A total of 486 genomic sequence tags (GSTs) were generated by sequencing randomly selected clones. Clones were analyzed for sequence similarity to secondary metabolic genes in the NCBI nonredundant protein database. In this manner, a single GST was identified containing sequence similarity to the predicted requisite NRPS. An 864-member cosmid insert library, corresponding to approximately 3- to 4-fold coverage, was created from the large fragment DNA. The NRPS GST was labeled and used as colony-hybridization probe for the identification of anthramycin cluster containing cosmid candidates. The fragments on those cosmid candidates were sequenced by a short-gun sequencing approach.

Two cosmids 024CA and 024CO, were identified, each containing portions of the anthramycin biosynthetic gene cluster with an approximate 10 kb overlap DNA region. The identified DNA locus appeared to harbor the complete gene cluster for the biosynthesis of anthramycin (figure 2-02). Sequence analysis reveals that the open reading frame anthramycin cluster contains genes

consistent with the biosynthesis of the two halves of anthramycin: 4 methyl-3-hydroxyanthranilic acid and a "dehydroproline acrylamide" moiety. These non-proteinogenic amino acid precursors are condensed by a two-module nonribosomal peptide synthetase (NRPS) terminated by a reductase domain, consistent with the final hemiaminal oxidation state of anthramycin. The boundaries of the gene cluster were deduced by comparative genomics.
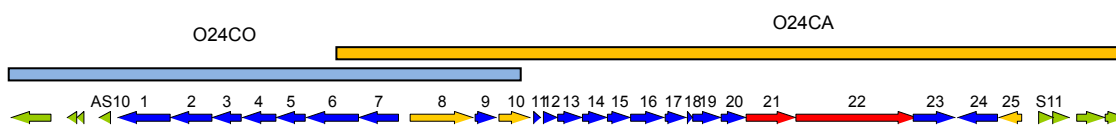
Figure 2-02. DNA locus that contains proposed anthramycin biosynthetic gene cluster.

The functions of the identified genes in the biosynthetic gene cluster of anthramycin were proposed by comparing their protein sequences with the sequences deposited in the National Center for Biotechnology Information (NCBI) protein database. In the identified anthramycin biosynthetic gene cluster, 25 genes are proposed to be involved in the biosynthesis of anthramycin and consist of genes for 4-methyl-3-hydroxy-anthranilic acid biosynthesis, genes for the biosynthesis of the pyrrolylacrylamide moiety, a bimodular NRPS system as well as gene candidates for regulation, resistance, and transport (table 2-01).

Table 2-01. Open Reading Frame Analysis for Anthramycin Gene Cluster from *Streptomyces refuineus*

ORF	#AA	GenBank Homology	% Identity/Similarity	Proposed Function
AS10	138	AAU19321.1	46/68	unknown, hypothetical protein
1	624	BAB12569.1	57/68	amidotransferase
2	500	CAD30313.1		aldehyde dehydrogease
3	354	EAO60654.1	50/65	alcohol dehydrogenase
4	410	CAJ23858.1	49/63	Cytochrome P-450 hydroxylase
5	352	487713	79/87	lmbA, methyltranferase
6	621	CAA55746.1	73/80	lmbW, unknown function
7	487	ABF90321.1	38/54	FAD oxidoreductase
8	764	AAL06654.1	59/75	drug-resistence pump
9	256	CAB55527.1	27/40	putative hydroxylase/glyoxylase
10	377	EAL16816.1	41/66	transporter
11	89	–	–	none
12	169	CAA55747.1	48/63	lmbB1, L-DOPA 2,3-dioxygenase
13	302	CAA55748.1	42/54	lmbB2, L-tyrosine 3-hydroxylase
14	297	CAA55772.1	50/63	lmbY, unk. lincomycin biosynth.
15	276	CAA55771.1	34/41	lmbX, unk. lincomycin biosynth.
16	413	42543461	37/57	kynureninase
17	261	4753870	37/52	tryptophan 2,3-dioxygenase
18	58	–	–	none
19	348	37542638	43/63	aromatic C-methyltransferase
20	296	EAU10758.1	36/49	aryl formamidase
21	600	CAD92850.1	35/51	NRPS
22	1146	ABF90459.1	32/46	NRPS
23	500	ABF39686.1	32/49	kynurenine 3-monooxygenase
24	475	ABF87356.1	36/50	flavin-containing oxidoreductase
25	273	BAC79018.1	46/64	repressor-response regulator
S11	174	BAC69182.1	63/68	Zn-dependent hydrolase
S12	199	EAU12469.1	31/53	unknown, hypothetical protein

4-methyl-3-hydroxyanthranilic acid biosynthesis in anthramycin

Several genes putatively assigned for the biosynthesis of 4-methyl-3-hydroxyanthranilic acid are comprised of homologs of primary metabolic genes

involved in 3-hydroxyanthranilic acid biosynthesis (*orf16*, *orf17*, *orf20* and *orf23*) as shown in table 2-01. These four genes are proposed to be involved in the biosynthesis of 3-hydroxyanthranilic acid and show high similarity with the genes in the biosynthetic gene clusters of other secondary metabolites containing the 3-hydroxyanthranilic acid structural unit, for example from the gene cluster of actinomycin produced by various Streptomyces.[18] Notably, the chemical structure of actinomycin contains a building block of 4-methyl-3-hydroxyanthranilic acid. So anthramycin and actinomycin very possibly share the same or similar biosynthetic pathway for the formation of 4-methyl-3-hydroxyanthranilic acid or 3-hydroxyanthranilic acid intermediate. The proposed biosynthetic pathway for 3-hydroxyanthranilic acid that is derived from L-tryptophan and requires the modifications by *orf16*, *orf17*, *orf20* and *orf23* in anthramycin biosynthetic gene cluster is shown in figure 2-03. The existence of a *C*-methyl group on the anthranilate A-ring suggests that a *C*-methyltransferase should be encoded by a gene inside the anthramycin biosynthetic gene cluster. Actually, there are two genes putatively encoding methyltransferases in the anthramycin biosynthetic gene cluster, *orf5* and *orf19*. Methyltransferase ORF5 is probably involved in the biosynthesis of dehydroproline acrylamide building block and addition of the unique C1 unit because it shows high sequence similarity with another *C*-methyltransferase in the biosynthetic gene cluster of lincomycin which shares a similar dehydroproline structure.[19] So we speculate that ORF19 should be the *C*-methyltransferase to append the aryl methyl group. *Orf24* is proposed to encode a flavin-dependent oxidase or an amine oxidase and seems to not be involved in

the biosynthesis of dehydroproline. In the identified DNA locus, *orf24* is clustered with *orf23*, a gene for the biosynthesis of 3-hydroxyanthranilic acid. Thus, *orf24* is possibly related to the biosynthesis of 3-hydroxyanthranilic acid but the precise function of this gene has not been well investigated.
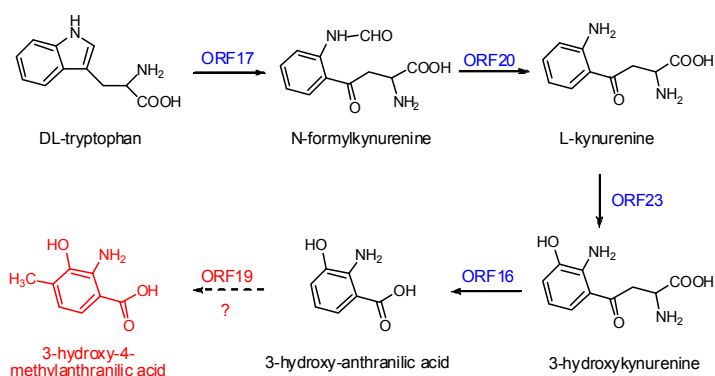
Figure 2-03. Proposed partial biosynthetic pathway for 3-hydroxy-4-methylanthranilic acid.

Dehydroproline acrylamide biosynthesis in anthramycin

The dehydroproline acrylamide building block present in anthramycin is very similar to the 4-propyl-4,5-dehydroproline structure from lincomycin A, a secondary metabolite produced by *Strptomyces lincolnensis*.[19] Seven genes (*orf1*, *orf2*, *orf3*, *orf4*, *orf5*, *orf12* and *orf13*) in anthramycin biosynthetic gene cluster show high sequence similarities with the genes identified from lincomycin gene cluster. Feeding experiments have demonstrated that the dehydroproline building blocks in anthramycin and lincomycin A are derived from L-tyrosine and the terminal C-1 carbon atom in the side chain is provided by methionine.[20] The

above seven genes can be assigned to be involved in the biosynthetic pathway for the unique dehydroproline acrylamide intermediate after the analysis of anthramycin and lincomycin biosynthetic gene clusters. A proposed biosynthetic pathway for dehydroproline acrylamide in anthramycin is shown in figure 2-04.
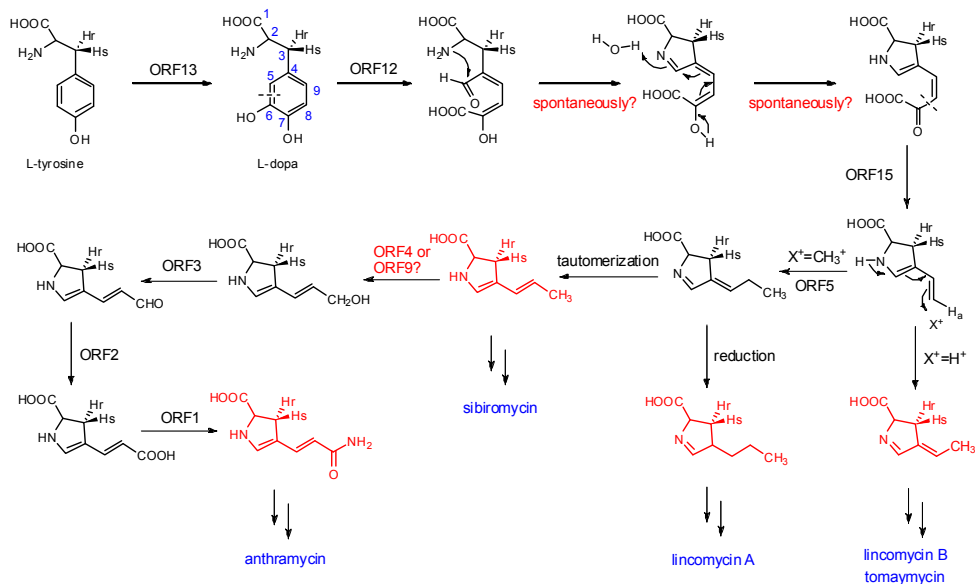
Figure 2-04. Proposed biosynthetic pathways of dehydroproline subunits for anthramycin, sibiromycin,tomaymycin and lincomycin.

The biosynthetic pathway of dehydroproline acryamide is one of the most interesting portions in anthramycin biosynthesis. Previous incorporation experiments have provided evidences the biosynthetic pathway for the formation of the dehydroproline acryamide in anthramycin. Unfortunately, very little is known about this partial anthramycin biosynthetic pathway at either the genetic or the biochemical level. To date, only Imb B1 (showing 48% identity with ORF12

from anthramycin gene cluster) in lincomycin biosynthetic gene cluster has been reconstituted *in vitro* and demonstrated to be a Fe^{2+} dependent L-DOPA 2,3-dioxygenase which is responsible for the C5-C6 cleavage in L-DOPA.[21] The functions of other genes proposed to be involved in the dehydroproline biosynthetic pathway have not been characterized and need further investigation.

Nonribosomal peptide synthase in anthramycin biosynthesis

4-methyl-3-hydroxyanthranilic acid and dehydroproline acrylamide are two potential precursors of anthramycin. The biosynthetic genes of these two compounds can be identified within anthramycin biosynthetic gene cluster. NRPS has been shown to be a common paradigm system for the biosynthesis of amide bonds in structural diverse small peptides isolated from bacteria and fungi. The anthramycin biosynthetic gene cluster contains two genes encoding NRPS, which is probably responsible for the amide bond formation of above two intermediates. *Orf21* encodes an adenylation domain (A domain) and a thioesterification domain (T domain). *Orf22* encodes a condensation domain (C domain), an A domain and a T domain as well as a putative reduction domain (Re domain). Notably, the existence of an unusual *C*-terminal reductase domain in the second module suggests that the release of anthramycin from magasynthetase NRPS is accomplished by the reduction of the dipeptide attached to NRPS instead of a direct hydrolysis of this dipeptide by the T domain in ORF22 as evidenced by BLAST homology of C-terminal region to NADH binding motifs (figure 2-05). This reductase domain can also be identified in other

NRPS magasynthetases from the biosynthetic gene clusters of saframycin, myxochelin, nostocyclo peptide and equistin.[22-25] A similar reduction reaction before the release from NRPS is also required for the biosynthesis of those natural products. Another unusual aspect of the NRPS for anthramycin biosynthesis is the peptide sequences of A domains in the two modules of the NRPS, which show relative low similarity with other known A domains, especially the A domain that has been previous demonstrated to activate 4 methyl-3-hydroxyanthranilic acid in the biosynthesis of actinomycin.[26]
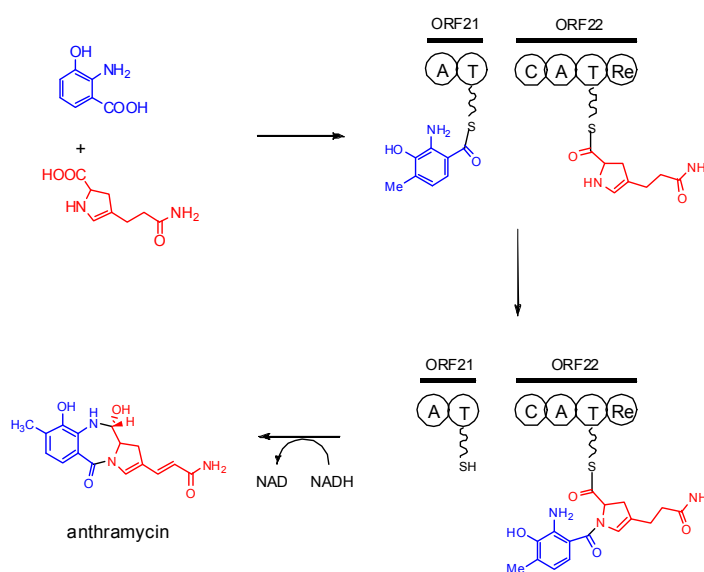
Figure 2-05. Nonribosomal Assembly and Reductive Release of Anthramycin.

Resistance and regulation

In the anthramycin biosynthetic gene cluster, *orf8* shows 59% identity to resistance gene *DrrC* in the daunorubicin gene cluster[27] and *orf10* is a homologous gene of antibiotic transporters in other natural product biosynthetic

gene clusters. So *orf8* and *orf10* are probably the components of the resistance system for anthramycin producer *S. refuineus*. *Orf25* is the only gene in the anthramycin gene cluster that encodes an amino acid sequence with similarity to response regulators for other natural products, suggesting *orf25* may play a significant important role in the regulation of anthramycin biosynthesis.[28]

2. Functional determination of anthramycin biosynthetic gene cluster

After the identification of the DNA locus potentially harboring the anthramycin biosynthetic gene cluster from the genome of *Streptomyces refuineus*, the function of the identified gene cluster needed to be characterized before further genetic and biochemical studies for anthramycin could be performed.

Generally a very straightforward method for determining the function of a natural product biosynthetic gene cluster is to disrupt several key genes inside this gene cluster. If the production of a natural product is completely abolished after the disruption of a gene or a DNA fragment in the identified gene cluster, then the function of this gene cluster can be determined and assigned to the natural product biosynthetic gene cluster.[29] Subsequently, genetic and chemical complementation can also be utilized to further confirm the function of a natural product biosynthetic gene cluster by reintroducing the complete genes into mutant strains.

To mutate target genes in a natural product biosynthetic gene cluster within a genome, a suitable genetic manipulation system needs to be established

for a producer strain. Since more than 70% of antibiotics that have been discovered so far are produced by Streptomcyes[30], tremendous efforts have been devoted to the development of genetic system for various Streptomyces strains in the past forty years. Three main methods have been employed for the transformation of DNA vectors or DNA fragments into Streptomyces strains: *polyethylene* glycol induced protoplast preparation, electroporation and E. coli-Streptomyces intergenetic conjugation.[31] The first two methods have been proven to be laborious and the transformation efficiency can not be guaranteed for all Streptomyces strains. E. coli-Streptomyces conjugation is a relative new transformation method that has been widely used for the genetic study of Streptomyces because it can be applied to a great variety of Streptomyces strains and exhibits higher efficiency for DNA transformation.[32, 33]

In our laboratory, we have successfully developed a genetic manipulation system for the anthramycin producer *Streptomyces refuineus* based on E. coli-Streptomyces conjugation. We were able to transform DNA vectors of different sizes from *E. coli* to *S. refuineus* with very high efficiency. *E. coli* ET12567/pUZ8002 was chosen as the donor strain for conjugation because it contains a large plasmid pUZ8002 which is able to mobilize DNA vectors containing *oriT* fragment to Streptomyces.[31] The establishment of a genetic manipulation system by using the conjugation method greatly facilitated the subsequent gene mutations in anthramycin biosynthetic gene cluster from *S. refuineus.*[34]

Traditionally, to mutate a single gene or a DNA fragment inside a natural

product biosynthetic gene cluster on the genome of its producer, a selective marker is put inside two homologous sequences from both sides of the target gene and this DNA fragment is cloned into a suitable plasmid followed by the transformation of this recombinant plasmid into the natural product producer. Because of the existence of homologous sequences between the genome and the DNA fragment on the plasmid, recombination can occur between homologous sequences and the target gene will be replaced by the selectable marker.[35]

In 2000, a new gene mutation method called "Red/ET cloning system" for the efficient disruption of biosynthetic genes in Streptomyces was reported by researchers from John Innes Center in England.[11] This new approach is based on the discovery that in the presence of Red α (exo), Red β (bet) and Red γ (gam) proteins of phage λ, recombination can occur between a target gene and a selectable marker with homologous nucleotide sequence as few as 40 bp on both sides, resulting in the replacement of the target gene by a selectable marker.[36-40] In this manner, a target gene in a biosynthetic gene cluster which has been cloned into a vector such as a cosmid or fosmid can be replaced. Subsequently, the vector containing a partial biosynthetic gene cluster with a target gene disruption is transformed into the natural product producer strain. There are generally more than 10 kb homologous sequences on both sides of a target gene, therefore recombination efficiency will be greatly increased using this approach, compared with the traditional gene disruption method as mentioned above. Dr. Keith F. Chater kindly provided us with this "Red/ET cloning system" by which we were able to develop the gene disruption method

for anthramycin biosynthetic genes in *S. refuineus* (figure 2-06).
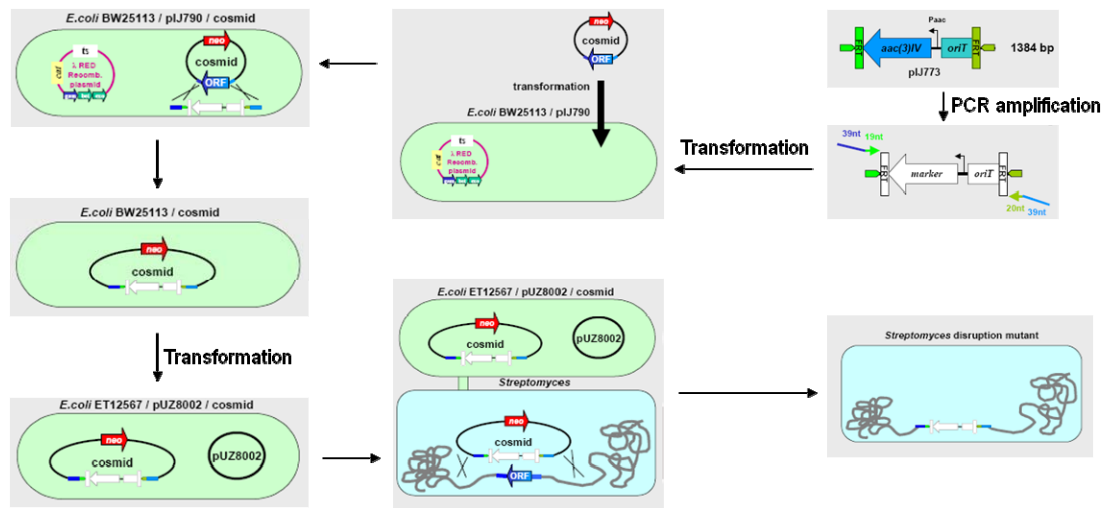
Figure 2-06. Gene disruption strategy in "Red/ET cloning system" (copyright of John Innes Center, UK).

In order to determine the function of this cloned DNA locus to be anthramycin biosynthetic gene cluster, we decided to mutate several key genes in this DNA locus. If anthramycin production could not be detected after the disruption of these genes, then this gene cluster could be assigned as anthramycin biosynthetic gene cluster.

We first disrupted a proposed essential component of the anthramycin gene cluster, *orf21* & *orf22* which encode the NRPS proposed to be responsible for the assembly of two intermediates to yield anthramycin. We cloned the apramycin resistance gene *acc(3)IV* from plasmid pIJ773 and about 40 bp homologous DNA sequences were added on both sides of this selectable marker via designed PCR primers. Then the cloned apramycin resistance gene and cosmid 024OCA containing the partial anthramycin biosynthetic gene cluster

were transformed into *E. coli* BW25113 harboring the plasmid pIJ790 expresses the proteins to facilitate DNA homologous recombination. After the induction of L-arabinose, recombination occurred between the homologous sequences of PCR products and cosmid 024CA, resulting in the replacement of *orf21* & *orf22* by the apramycin resistance gene on the partial anthramycin gene cluster in cosmid 024CA. Subsequently, this recombinant cosmid was transformed into anthramycin producer *S. refuineus* and the target genes *orf21* & *orf22* were replaced by apramycin resistance gene on the genome of *S. refuineus*. Correct double crossover mutant stains were selected for an apramycin resistance (Apr^R) and kanamycin sensitive (Kan^S) phenotype (figure 2-07).
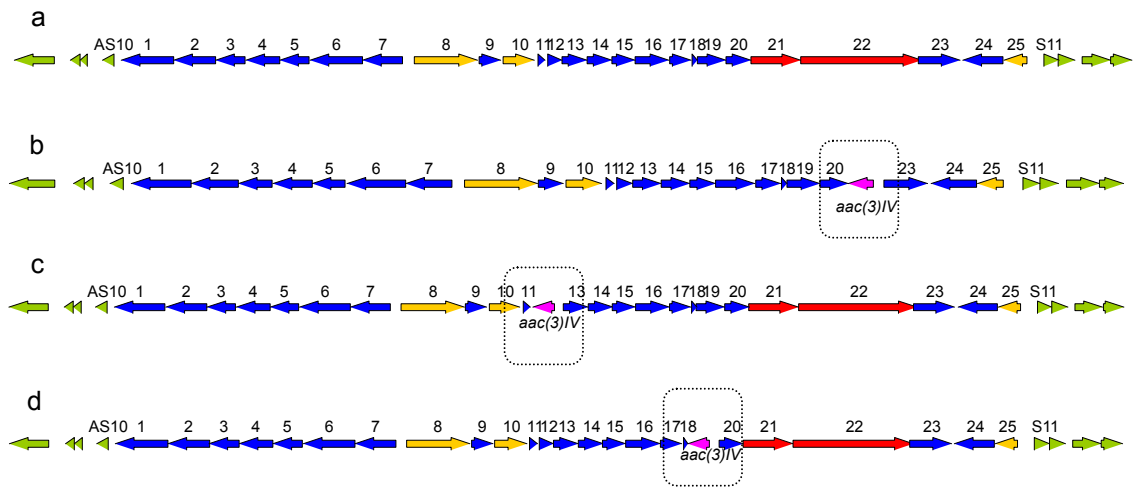
Figure 2-07. Anthramycin biosynthetic gene cluster (a), NRPS (*orf21*&*orf22*) mutant strain (b), *orf12* mutant strain (c) and *orf19* mutant strain (d).

Following the gene disruption protocol for *orf21* & *orf22*, we utilized apramycin resistance gene to replace *orf12* which was proposed to encode an L-DOPA-dioxygenase involved in the biosynthesis of dehydroproline acrylamide and *orf19* which was proposed to code for the *C*-methyltransferase for the *C*-methylation on anthranilate aromatic A-ring, respectively. Corresponding mutant strains for these two genes were selected based on Apr^R and Kan^S phenotype (figure 2-07).

The thusly obtained mutant strains for *orf12*, *orf19* and NRPS (*orf21*&*orf22*) were confirmed by PCR using primers from outside the target genes and inside apramycin resistance gene. Subsequently, the mutant strains were subject to liquid culture for the isolation of anthramycin. The detection of anthramycin from these three mutant strains was carried out by using HPLC-MS analysis. After deletion of *orf12*, *orf19* and NRPS (*orf21*&*orf22*), no anthramycin could be detected in the fermentation broth for any of these three mutant strains, with the isolated anthramycin from its producer *S. refuineus* under the same culture conditions as a positive control (figure 2-08).
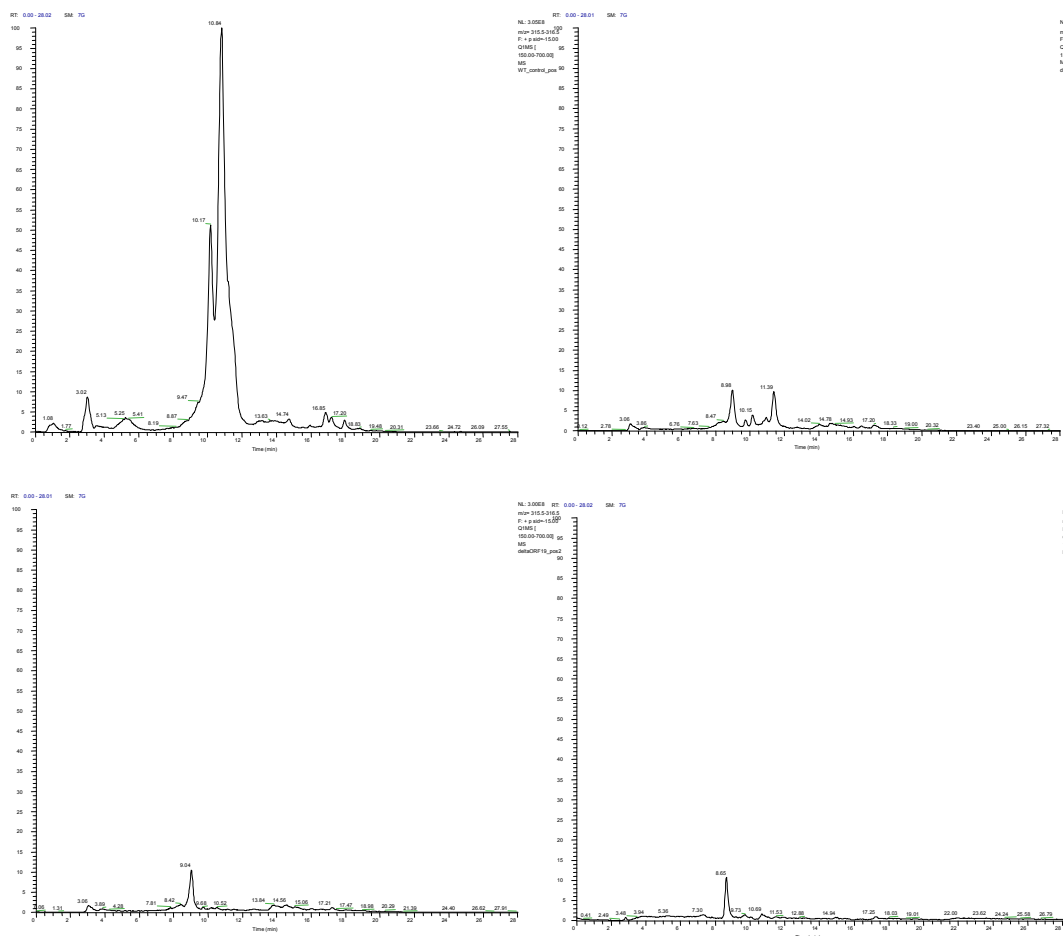
Figure 2-08. Anthramycin production from *S. refuineus*(up left), *orf12* mutant (up right), *orf19* mutant (down left) and *orf21*&*orf22* mutant (down right).

The above anthramycin abolishment results from the anthramycin mutant strains of *orf12*, *orf19* and NRPS (*orf21*&*orf22*) confirmed that the DNA locus we identified from the genome of *S. refuineus* was indeed the anthramycin gene cluster and responsible for the biosynthesis of anthramycin.

In summary, a genomic DNA library of anthramycin producer *Streptomyces refuineus* was constructed and the DNA locus proposed to contain the anthramycin biosynthetic gene cluster was identified from the genome of *S.*

refuineus. From the genomic DNA library, two cosmids containing the potential anthramycin biosynthetic gene cluster were sequenced and sequence analysis suggested that this DNA locus probably harbored the complete anthramycin biosynthetic gene cluster with the biosynthetic genes for intermediates 4-methyl-3-hydroxyanthranilic acid and dehydroproline acrylamide as well as NRPS to assemble these two intermediates into anthramycin. A high-efficient genetic manipulation system for anthramycin producer *S. refuineus* was developed in our laboratory. We disrupted several key genes (*orf12*, *orf19* and NRPS (*orf21&orf22*)) in this proposed anthramycin gene cluster using a new gene disruption method, "Red/ET cloning system". The gene disruption results confirmed the function of this DNA locus to be the anthramycin biosynthetic gene cluster.

3. Boundaries determination of anthramycin biosynthetic gene cluster

A series of gene disruption experiments that have been carried out in our laboratory providing supportive evidence for the DNA locus identified from the genome of *S. refuineus* as the anthramycin biosynthetic gene cluster. Previous sequence analysis of this anthramycin biosynthetic gene cluster, after comparing the protein sequences against the protein database in NCBI, suggested that the anthramycin biosynthetic gene cluster containing 25 genes was proposed to start at *orf1* and conclude at *orf25*.

Biosynthetic genes for natural products produced by bacteria are generally clustered together with very few exceptions. Anthramycin biosynthetic genes

were also proposed to be clustered on the genome based upon the sequence analysis of the DNA locus containing anthramycin gene cluster, with *orf1* and *orf25* proposed to be the boundaries of this gene cluster base on the BLAST results from NCBI. Since we have already successfully established a genetic manipulation system for anthramycin producer *S. refuineus* and are able to utilize the "Red/ET cloning system" to mutate target genes as designed, the boundaries of anthramycin gene cluster can also be identified by gene inactivation studies. The biosynthetic genes in the proposed boundary regions of the anthramycin gene cluster can be replaced separately using "Red/ET cloning system". After mutation of individual genes required for the biosynthesis of anthramycin, the production of anthramycin will be completely abolished in the mutant strains. Correspondingly, the mutation of genes not essential to anthramycin biosynthesis will not have effect on the biosynthesis of anthramycin and anthramycin should still be produced by these mutant strains. Using this strategy, we should be able to determine the boundaries on both sides of anthramycin biosynthetic gene cluster by analyzing the results of anthramycin production from the mutant strains we generate.

Orf1 was proposed to be the left boundary of the anthramycin biosynthetic gene cluster. In order to determine the left boundary of this gene cluster, we disrupted *orf1* and *orfAS10* which resides next to *orf1* and right outside the proposed anthramycin gene cluster. Similar to the gene disruptions we achieved before, we utilized the apramycin resistance gene to replace *orfAS10* and *orf1* separately through double crossover homologous recombination on the genome.

Corresponding knockout strains for these separate genes were selected based on Apr^R and Kan^S phenotype and further confirmed by PCR using primers from outside the target genes and inside the apramycin resistance gene (figure 2-09). HPLC-MS and bioactivity were utilized to detect the production of anthramycin from the fermentation broth of *orfAS10* and *orf1* mutation strains with anthramycin from its producer *S. refuineus* as a positive control. *Orf1* was proposed to code for an amidotransferase responsible for the formation of the unique carboylamide structure in dehydroproline acrylamide intermediate of anthramycin. After the deletion of *orf1* from the genome of *S. refuineus*, the production of anthramycin was completely abolished and no anthramycin production could be detected in the extracts from fermentation broth of *orf1* mutant strain, suggesting that *orf1* is a key gene related to anthramycin biosynthesis. Whereas the disruption of *orfAS10*, a gene encoding a protein with unknown function, did not affect the production of anthramycin and anthramycin could still be detected from the fermentation medium of the *orfAS10* mutant strain, indicating *orfAS10* is not related to anthramycin biosynthesis. These results demonstrated that *orf1* was the left boundary of anthramycin biosynthetic gene cluster and played a very important role in the biosynthesis of anthramycin, which was consistent with our prediction based on the sequence analysis of the DNA locus containing anthramycin gene cluster (figure 2-10).
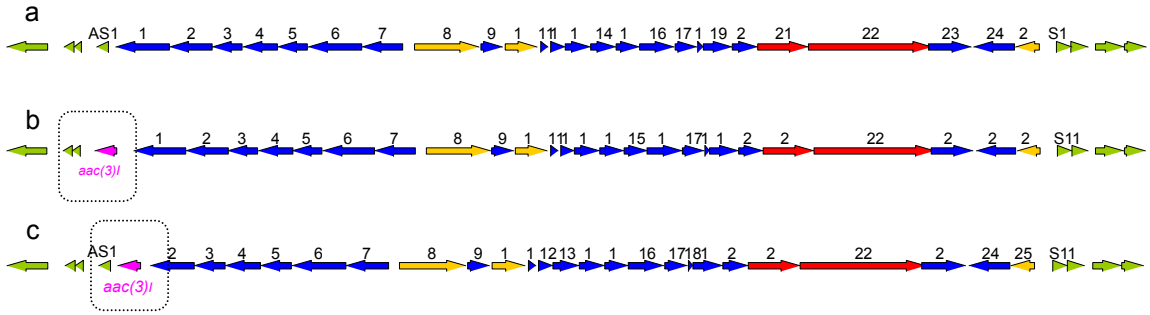
Figure 2-09. Anthramycin biosynthetic gene cluster (a), *orfAS10* mutant strain (b) and *orf1* mutant strain (c).
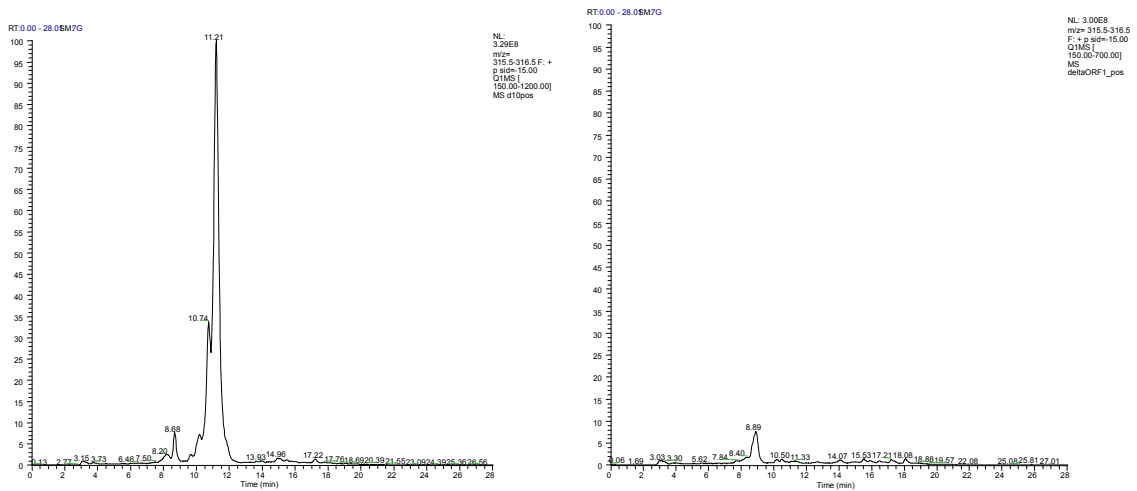
Figure 2-10. Anthramycin production from *orfAS10* mutant and *orf1* mutant.

Similarly, to determine the right boundary of anthramycin biosynthetic gene cluster, we also disrupted several genes (*orf23*, *orf24* and *orfS11*) in this boundary region. Our prediction, after the analysis of anthramycin gene cluster, was that *orf25* encoding a regulatory gene should be the right boundary and the genes upstream of *orf25* should be related to anthramycin biosynthesis and within anthramycin biosynthetic gene cluster. However, the disruption of regulatory gene *orf25* should either up-regulate or down-regulate the production

of anthramycin. So the disruption result of *orf25* can not provide direct evidence in terms of whether this gene is involved in anthramycin biosynthesis. Since *orf25* is the only regulatory gene in this DNA locus, it is very possible that *orf25* plays a very important regulatory role in the production of anthramycin. To characterize the functions of biosynthetic genes on both sides of *orf25*, we mutated these three genes separately using the previous developed gene disruption method we have developed in our laboratory (figure 02-11). The fermentation results demonstrated our prediction that *orf23* and *orf24* were involved in the biosynthesis of anthramycin and *orfS10* was not related to anthramycin production because the mutant strains of *orf23* and *orf24* failed to produce anthramycin whereas anthramycin could still be detected from *orfS10* mutant strains. Based on gene disruption results together with sequence information of anthramycin biosynthetic gene cluster, *orf25* was determined to be the right boundary of anthramycin gene cluster from *S. refuineus* (figure 2-12).
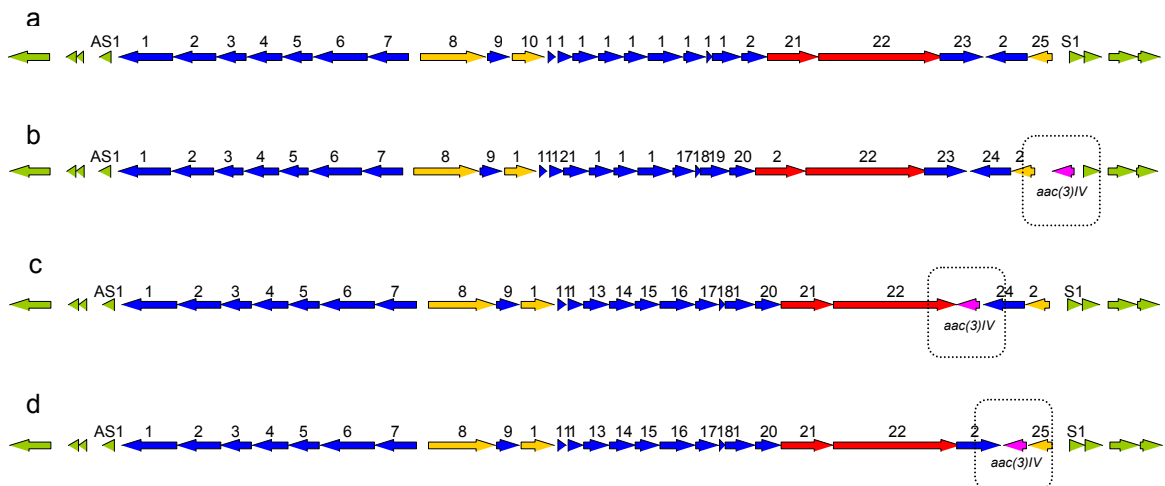
Figure 2-11. Anthramycin biosynthetic gene cluster (a), *orfS11* mutant strain (b) and *orf23* mutant strain (c) and *orf24* mutant strain (d).
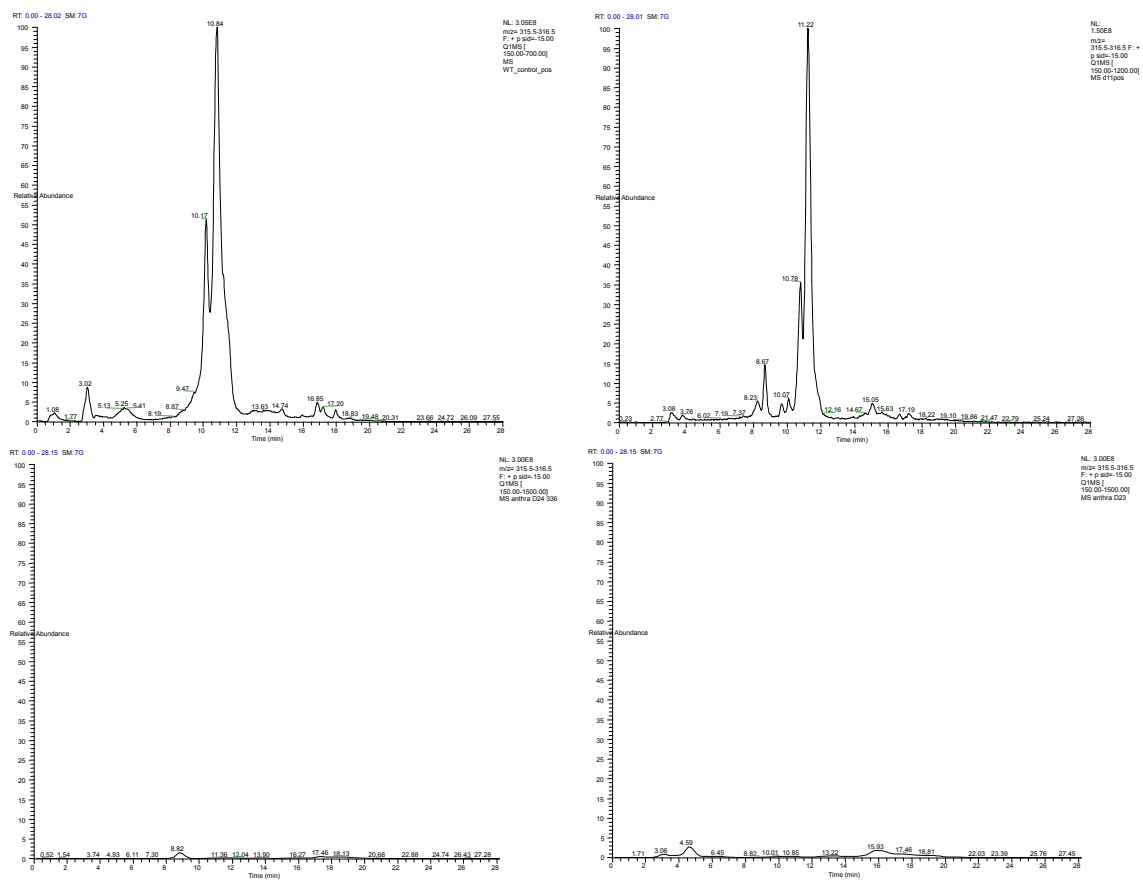
Figure 2-12. Anthramycin production from *S. refuineus*, *orfS11* mutant, *orf23* mutant and *orf24* mutant.

In summary, gene disruptions were carried out in our laboratory to determine the boundaries of anthramycin biosynthetic gene cluster using a new gene disruption method, "Red/ET cloning system", for anthramycin producer *S. refuineus*. The gene mutation results of *orfAS11*, *orf1*, *orf23*, *orf24* and *orfS10* in the boundary regions of anthramycin gene cluster together with DNA sequence information demonstrated that *orf1* and *orf25* were the boundaries of the anthramycin gene cluster and strongly supported our previous prediction derived from the sequence analysis of the anthramycin gene cluster.

4. Partial elucidation of anthramycin biosynthetic pathways

The anthramycin biosynthetic gene cluster that has been identified from the genome of *S. refuineus* putatively contains the genes necessary for the biosynthesis of 3-hydroxyanthranilic acid. ORF17 shows high similarity to tryptophan 2,3-dioxygenases and probably can catalyze the oxidation of the double bond in the five member-ring of tryptophan to yield *N*-formylkynurenine. ORF20 probably catalyzes the next biosynthetic step in which *N*-formylkynurenine is reduced to L-kynurenine and the aldehyde group is removed. ORF23 which is probably a kynurenine 3-monooxygenase can introduce a hydroxyl group to the aromatic ring and yield 3-hydroxykynurenine. ORF16 is a kynureninase and probably responsible for the transformation from 3-hydroxykynurenine to 3-hydroxy-anthranilic acid with L-alanine as a byproduct. ORF19 is proposed to be *C*-methyltransferase for *C*-methylation of the anthranilate aromatic A-ring to yield 3-hydroxy-4-methylanthranilic acid (table 2-01).

In the anthramycin biosynthetic gene cluster, *orf24* is clustered with *orf23* and previously we have demonstrated *orf24* is involved in the biosynthesis of anthramycin. Protein sequence analysis has shown that ORF24 is proposed to encode a flavin-dependent oxidoreductase or an amine oxidase but seems not to be involved in the biosynthesis of dehydroproline. So we speculated that *orf24* is possibly related to the biosynthesis of 3-hydroxy-anthranilic acid.

In our previous studies to determine the function and boundaries of anthramycin biosynthetic gene cluster, we generated several anthramycin mutant

strains, especially *orf23* and *orf19*, which were proposed to be involved in the biosynthesis of 3-hydroxy-anthranilic acid. The intermediates in 3-hydroxy-anthranilic acid biosynthetic pathway are either commercially available or can be prepared by chemical synthesis in our laboratory. We are then able to feed these intermediates to *orf23* and *orf19* mutant strains to chemically complement the function of these genes. Since *orf24* is also potentially involved in 3-hydroxy-anthranilic acid biosynthesis and we have the *orf24* mutant strain on hand, we can feed these possible intermediates into the fermentation broth of the *orf24* mutant strain and determine whether they could chemically complement anthramycin production from mutant strains.

Chemical complementation results strongly suggested that 3-hydroxy-anthranilic acid was a late stage intermediate for anthramycin, because it could be fed into gene knockout strains and lead to the formation of a demethylanthramycin. A related case study is diazepinomicin/ECO-4601 which shares a similar aromatic A-ring with anthramycin. Feeding experiments have already demonstrated that 3-hydroxy-anthranilic acid is an important precursor of diazepinomicin because $4,6-D_2$-3-hydroxyanthranilic acid could be successfully incorporated into diazepinomicin. The feeding studies of 3-hydroxyanthranilic acid into diazepinomicin are consistence with our chemical complementation results for the restoration of anthramycin in *orf19* and *orf23* mutant strains by 3-hydroxyanthranilic acid (table 2-02).
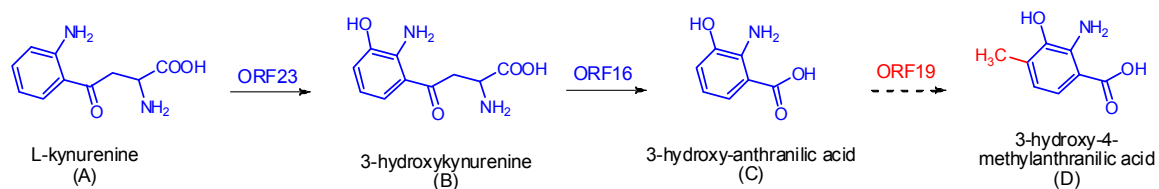
Figure 2-13. Proposed precursors and biosynthetic pathways for 3-hydroxyl-4-methylanthranilic acid.

Table 2-02. Chemical complementary results to elucidate 3-hydroxy-4-methylanthranilic acid biosynthetic pathway (A: L-kynurenine, B: 3-hydroxykynurenine, C: 3-hydroxy-anthranilic acid, D: 3-hydroxy-4-methylanthranilic acid).

ORF	A Th	A Obs	B Th	B Obs	C Th	C Obs	D Th	D Obs
19	-	-	302	-	302	302	316	-
23	-	-	302	302	302	302	316	-
			316	316				
24	302	-	302	-	302	302	316	-
	316		316	316				

Orf23 was proposed to the oxidase take part in the biosynthesis of 3-hydroxyanthranilic acid with 3-hydroxykynurenine as its direct product. After we fed 3-hydroxykynurenine into *orf23* mutant strain, anthramycin production could be restored. These chemical complementation results confirmed that *orf23* was involved in the biosynthesis of anthramycin with 3-hydroxykynurenine as the direct product of *orf23* and precursor of anthramycin.

Orf19 was proposed to be the methyltransferase for the *C*-methylation of anthranilate aromatic A-ring, but we could not detect anthramycin production from the fermentation broth of *orf23* and *orf19* mutant strains after feeding 3-

hydroxy-4-methylanthranilic acid into these two mutant strains. Based on the isotopic feeding studies in the Dr. Hurley lab, 3-hydroxy-4-methylanthranilic acid was a very possible intermediate for both sibiromycin and anthramycin. The results of these experiments were contradictory. However, it has also been demonstrated that actually 3-hydroxy-4-methylanthranilic acid was not incorporated into the anthramycin producer *S. refuineus* probably because of the lack of permeability for this compound with *S. refuineus*. Our feeding results were consistent with previous labeling studies for anthramycin biosynthesis. Despite the fact that 3-hydroxy-4-methylanthranilic acid could not restore anthramycin production for *orf23* and *orf19* mutant strains, 3-hydroxy-4-methylanthranilic acid could not be definitely denied as an important intermediate for anthramycin biosynthesis and *in vitro* biochemical experiments might possibly determine the function of ORF19 as a *C*-methyltransferase.

Protein sequence analysis has shown that *orf24* codes for a flavin-dependent oxidoreductase. *Orf17*, *orf20*, *orf23* and *orf16* seem to be sufficient for the biosynthesis of intermediate 3-hydroxyanthranilic acid. However, our feeding studies have shown that both 3-hydroxyanthranilic acid and 3-hydroxykynurenine could chemically complement demethylanthramycin or anthramycin production from *orf24* mutant strain, which was consistent with the chemical complementation results for *orf23* mutant strain. Based on sequence analysis of the genes in the anthramycin biosynthetic gene cluster, we still prefer that the hydrolase *orf20* which encodes an aryl formamidase, rather than *orf24,* is involved in 3-hydroxyanthranilic acid biosynthesis. Since *orf23* and *orf24* are

clustered in anthramycin gene cluster, the disruption of *orf24* may affect the function of *orf23*, very possibly at the genetic level. The function of oxidereductase ORF24 can not be definitely assigned to be related to 3-hydroxyanthranilic acid biosynthesis and needs further investigation.

We also performed chemical complementation by feeding a possible intermediate *N*-formylkynurenine into above three mutant strains. *N*-formylkynurenine failed to lead to anthramycin restoration, which was as expected. The reason for this is probably that *N*-formylkynurenine actually occurs prior to the biosynthetic steps mediated by ORF23 and ORF19 (figure 2-13 & table 2-02).

In summary, the biosynthesis of 3-hydroxy-4-methylanthranilic acid of anthramycin has been demonstrated to be derived from kynurenine pathway. 3-hydroxy-anthranilic acid is a key intermediate for anthramycin biosynthesis. ORF23 can catalyze the conversion from L-kynurenine to 3-hydroxykynurenine. Whether ORF24 is related to the biosynthesis of 3-hydroxy-4-methylanthranilic acid and when the timing of C-methylation on this aromatic group remains unresolved of this time. On the basis of chemical complementary results, the biosynthetic pathway for 3-hydroxy-4-methylanthranilic acid is provided in figure 2-14.
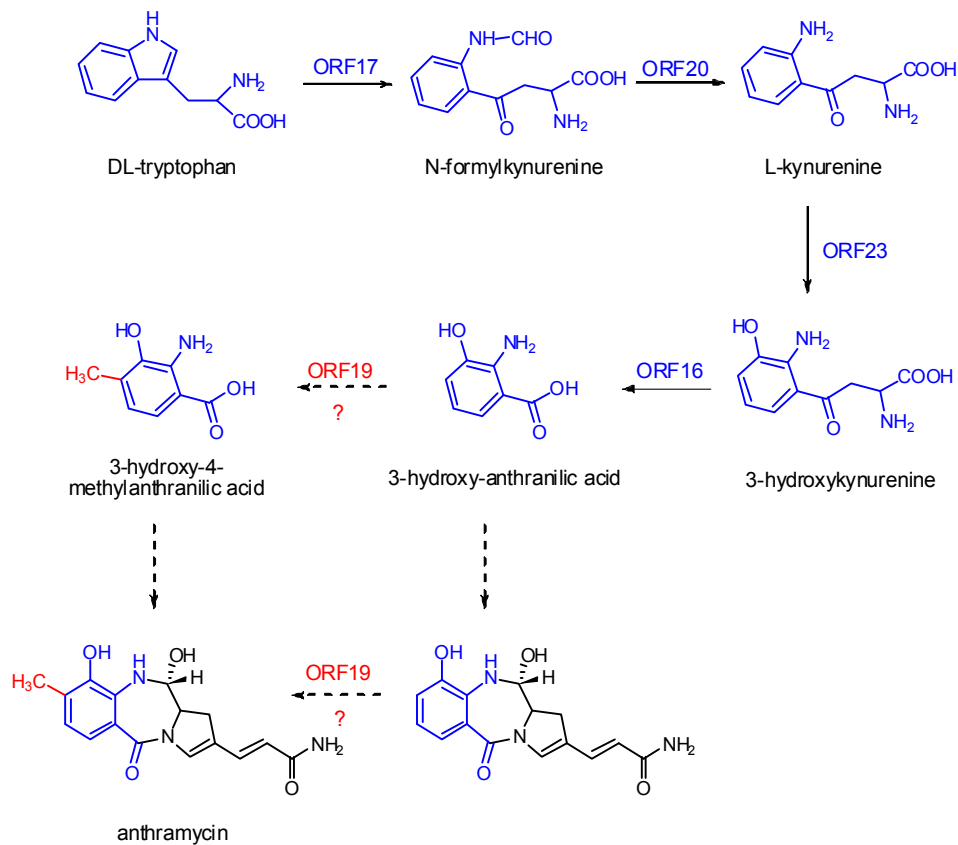
Figure 2-14. Proposed partial biosynthetic pathway for 3-hydroxy-4-methylanthranilic acid on the base of chemical complementary results.

Materials and methods

Bacterial Strains and Culture Conditions

  Streptomyces refuineus var. *thermotolerans* (NRRL 3143) and its derivatives were maintained and grown on either ISP4 medium or TSB medium with appropriate antibiotics at 37°C. For anthramycin production, *S. refuineus* and its derivatives were cultured in production medium (1% corn starch, 2%

peptonized milk, and 0.3% yeast extract at pH 7.0) at 47°C. *Bacillus* sp. TA (NRRL B-3167) was cultured in nutrient agar and used as a test organism for the antibacterial activity of *S. refuineus* and its derivatives. DH10B (Invitrogen) served as host for *S. refuineus* genomic library construction. *E. coli* BW25113 containing plasmid pIJ790 was used for targeted gene disruption in *S. refuineus*. *E. coli* ET12567 containing the RP4 derivative pUZ8002 was used for intergeneric conjugation between *E. coli* and *S. refuineus*. *E. coli* strains were grown in LB medium supplemented with appropriate antibiotics for selection of plasmids.

Plasmids and General DNA Procedures

SuperCos-1 derivatives were used to construct genomic libraries of *S. refuineus*. DNA isolation and manipulation, cosmid preparation, and gel electrophoresis were conducted according to standard methods. Cosmid DNA was isolated from *E. coli* strains by using Qiagen miniprep kits. Isolation of DNA fragments from agarose was carried out by using Qiagen gel extraction kit. Genomic DNA from *S. refuineus* and its derivatives were isolated by using Wizard genomic DNA purification kit (Promega).

Genomic Sampling Library Construction and Screening

SuperCos-1 derivatives were used to construct genomic libraries of *S. refuineus*. High molecular weight DNA was isolated by established protocols, and its integrity was verified by field inversion gel electrophoresis (FIGE) with preset program number 6 (switch time ramp 0.4–1.5 s, linear shape, forward voltage 180, reverse voltage 120) of the FIGE MAPPER power supply (BIORAD, Inc.).

To generate the small insert DNA library, genomic DNA was randomly sheared by sonication. DNA fragments having a size range between 1.5 and 3 kb were fractionated on agarose. The ends of the DNA fragments were repaired by using T4 DNA polymerase (Roche) as described by the supplier. The repaired DNA fragments were subcloned into a derivative of pBluescript SK+vector (STRATAGENE). Plasmid DNA carrying the *Streptomyces refuineus* genomic DNA fragments was extracted by the alkaline lysis method, and the insert size of 1.5 to 3 kb was confirmed by electrophoresis on agarose gels. Using this procedure, a library of small-size random genomic DNA fragments representative of the entire genome of *Streptomyces refuineus* was generated. The small insert DNA library was analyzed by sequence determination of the cloned genomic DNA inserts to generate genomic sequence tags, GSTs. GST sequencing was performed by using a 3700 ABI capillary electrophoresis DNA sequencer (Applied Biosystems). The average length of the DNA sequence reads was ~700 bp. Further analysis of the obtained GSTs was performed by sequence homology comparison to various protein-sequence databases. The DNA sequences of the obtained GSTs were translated into amino acid sequences and compared to the National Center for Biotechnology Information (NCBI) nonredundant protein database by using known algorithms. A total of 486 *Streptomyces refuineus* GSTs were generated and analyzed by sequence comparison with BLAST. Sequence alignments displaying an E value of at least e-5 were considered as significantly homologous and retained for further evaluation. One GST clone identified by BLAST analysis as encoding a fragment of a nonribosomal peptide

synthetase (NRPS) enzyme was selected for the generation of an oligonucleotide probe, which was then used to identify the gene cluster harboring this specific NRPS gene(s) in the large insert cosmid library.

Cosmid Library Construction and Screening

A cosmid library was constructed from the *Streptomyces refuineus* high molecular weight genomic DNA by using a SuperCos-1-derived cosmid vector (STRATAGENE). The cosmid arms were prepared as specified by the manufacturer. The cosmid library consisted of 864 isolated cosmid clones in *E. coli* DH10B (Invitrogen). These clones were picked and inoculated into nine 96-well microtiter plates containing LB broth, which were grown overnight and then adjusted to contain a final concentration of 25% glycerol. These microtiter plates were stored at -80°C and served as glycerol stocks of the cosmid library. Duplicate microtiter plates were arrayed onto nylon membranes and crosslinked onto the membranes by UV irradiation by using a GS GENE LINKER UV Chamber (BIORAD, Inc). Considering an average size of 8 Mb for an actinomycete genome and an average size of 35 kb of genomic insert in the cosmid library, this library represents roughly a 4-fold coverage of the microorganism's genome. A GST clone identified by BLAST analysis as encoding a fragment of a nonribosomal peptide synthetase (NRPS) enzyme was selected for the generation of an oligonucleotide probe, which was then used to identify the gene cluster harboring this specific NRPS gene(s) in the cosmid library. Hybridization oligonucleotide probes were radiolabeled with P32 by using T4 polynucleotide kinase (New England Biolabs, Inc.) in 15 ml reactions

containing 5 pM of oligonucleotide and 6.6 pM of g-P32 ATP in the kinase reaction buffer supplied by the manufacturer. Positive clones were identified, cosmid DNA was extracted from 30 ml cultures by the alkaline lysis method, and the inserts were entirely sequenced by a shotgun sequencing approach. Two overlapping cosmid clones that were detected by the oligonucleotide probe derived from the original NRPS GST clone were completely sequenced to provide approximately 60 Kb of information.

Retrofitting Cosmids with Origin of Transfer

To enable gene deletion, cosmids 024CO and O24CA were retrofitted with *oriT* by replacing kanamycin resistance gene (*neo*) with *oriT* and streptomycin resistance-gene (*aad(A)*) cassette from plasmid pIJ778 with primers Neo-OS (pIJ778 homologous sequence underlined) and then replacing *aad(A)* with neo with primers OS-Neo (*neo* homologous sequence underlined) through "Red/ET cloning system" (primers in table 2-03). Cosmids 024CO and O24CA containing the *oriT* insertion are designated 024OCO and 024OCA, respectively, and used for the disruption of *orfS10*, *orf12*, *orf19*, and NRPS (*orf21*/*orf22*).

Table 2-03. Primers for retrofitting cosmids with origin of transfer (pIJ778 homologous sequence underlined).

Target ORF	Primer
Neo-OS	5' TCAAGATCTGATCAAGAGACAGGATGAGGATCGTTTCGC<u>GCGGCATCTTATTTGCCGAC</u> 3'
	5' GCGTCG CTTGGTCGGTCATTTCGAACCCCAGAGTCCCGC<u>GTTCCCGCCAGCCTCGCAG</u> 3'
OS-neo	5' TATGCAGAGGCCGAGGC 3'
	5' TATATCGTGCGAAAAAGGATGGATATACCGAAAAAATCGC<u>CAGAGTCCCGCTCAGAAG</u> 3'

Targeted Replacement of orf12, orf19, and NRPS (orf21/orf22)

Apramycin resistance gene (*aac(3)IV*) was amplified from plasmid pIJ773 with forward and reverse primers with flanking regions appropriate for gene

replacement. The resulting PCR products were transformed into *E. coli* BW25113/pIJ790 containing cosmid 024OCO or 024OCA. *Orf12*, *orf19* and NRPS (*orf21/orf22*) were separately replaced by *aac(3)IV*. The resulting cosmids pBOB3001, pBOB3002, pBOB3003, and pBOB3004 were transformed into *E. coli* ET12567/pUZ8002 and then introduced into wild-type *S. refuineus* strain by intergeneric conjugation. The double-crossover homologous recombination mutant strains were selected for an apramycin-resistant and kanamycin-sensitive phenotype. *Orf12*, *orf19*, and NRPS (*orf21/orf22*) mutants were tested by PCR amplification with the primers internal to apramycin-resistance marker *aac(3)IV* and to regions external to the replaced genes. The corresponding mutants are designated BOB3001, BOB3002, BOB3003, and BOB3004, separately.

Targeted Replacement of orfS10

Apramycin resistance gene (*aac(3)IV*) was amplified from plasmid pIJ773 with forward and reverse primers with flanking regions appropriate for gene replacement. The resulting PCR products were transformed into *E. coli* BW25113/pIJ790 containing cosmid 024OCO. *OrfS10* was replaced by *aac(3)IV* and the resulting cosmids pBOB3001 were transformed into *E. coli* ET12567/pUZ8002 and then introduced into wild-type *S. refuineus* strain by intergeneric conjugation. The double-crossover homologous recombination mutant strains were selected for an apramycin-resistant and kanamycin-sensitive phenotype. *OrfS10* mutant were tested by PCR amplification with the primers internal to apramycin-resistance marker *aac(3)IV* and to regions external to the replaced genes. The corresponding mutants are designated BOB3001.

Targeted Replacement of orf1, orf23, orf24 and orfAS11

The cassette comprising the apramycin-resistance gene (*aac(3)IV*) and *oriT* was amplified from plasmid pIJ773 with appropriate forward primers and reverse primers. The resulting PCR products were transformed into *E. coli* BW25113/pIJ790 containing cosmid 024CO (024CA). Orf1 and *orfAS11* were replaced separately by *aac(3)IV* and *oriT* cassette and the resulting cosmids pBOB3005, pBOB3006 were transformed into *E. coli* ET12567/pUZ8002 and then introduced into wild-type *S. refuineus* strain by intergeneric conjugation. The double-crossover homologous recombination mutant strains were selected for by using apramycin-resistant and kanamycin-sensitive phenotypes. *Orf1*, *orf23*, *orf24* and *orfAS11* mutants were tested by PCR amplification with the primers internal to apramycin resistance marker *aac(3)IV* and to regions external to the replaced genes. The corresponding mutants were designated BOB3005 and BOB3006, separately. Primers for gene disruptions in anthramycin biosynthetic gene cluster were listed in table 2-04. Primers to test anthramycin mutant strains were listed in table 2-05.

Table 02-04. Primers used in gene replacement experiments for anthramycin biosynthetic gene cluster from *Streptomyces. refuineus*.

Target ORF	Primer
AS10	DAS10-F: 5' GGCGCCCGAGCCCGCGTCGGCGGCCTGGCCCCGGGCCGGGGGAATAGGAACTTATGAGC 3'
	DAS10-R: 5' CCGCCCATCGAGCCGGTCGTCCCGCGGCAACGGCGCGGCCGCTGACGCCGTTGGATAC 3'
1	D1-F: 5' TGCCGCTGCGGCCCCGAGGAGGGCGGGCGCACCCGTCTCAGGAATAGGAACTTATGAGC 3'
	D1-R: 5' AAAACCGTGTGGACGTCAATCGCCCCCTGACCTCCGGCAGAAGTTCCCGCCAGCCTC 3'
12	D12-F: 5' GCCGACCATTGAGGCCGATTGCTTCCACTCCGCGGAGACATCTAGAGGAATAGGAACTTATGAGC 3'
	D12-R: 5' CCCCCGCCCGGTGCGGCGGCATGTCCTCGGCCGTTGCTCAACTAGTGCTGACGCCGTTGGATAC 3'
19	D19-F: 5' ACGGGCCGGCGTGGGAGGCCACCGGCCTGCCGGGAGGCACTCTAGAGGAATAGGAACTTATGAGC 3'
	D19-R: 5' GGTGCTCGGCGAGTACTCGCGGTCCAGCCGGCCTGGCTCAACTAGTGCTGACGCCGTTGGATAC 3'
21/22	DNRPS-F: 5' CCGAGCCCACACGAGAAGGGACGGTGACATCCGCGCGATGTCTAGAGGAATAGGAACTTCATGAGC 3'
	DNRPS-R: 5' GTCCGGCGCCGATGACCACCACCGAGCGCCTGTGCAGCTCAACTAGTGCTGACGCCGTTGGATAC 3'
23	D23-F: 5' GATCCACTACGGACGCGCGTGCGTCGACGTGGACACCGGGGAATAGGAACTTATGAGC 3'
	D23-R: 5' GACCCCGGCCATCCGGCCGAAGACGTCGGGGGAACTCCGTAAGTTCCCGCCAGCCTC 3'
24	D24-F: 5' GCATCGCGGACCGTGCGGACCGCCGTGTCCGTGGACAGCAGGAATAGGAACTTATGAGC 3'
	D24-R: 5' GAATCCGCCTCGGCCATGGCGTGGGCCCGCTACGAGTCGGAAGTTCCCGCCAGCCTC 3'
S11	DAS11-F: 5' GCCCGCTCCCGGACGAGGTCGAGCCGGCCCCGCCGTGCCGCGGAATAGGAACTTATGAGC 3'
	DAS11-R: 5' CCACAAATCCGCAGCAGCCCAACCGTGCCGATCCTGGTTCAAGTTCCCGCCAGCCTC 3'

Table 02-05. Primers pairs for confirmation of gene deletion in anthramycin biosynthetic gene cluster from *Streptomyces. refuineus*.

Target ORF	Primer
AS10	TDAS10-F: 5' CTGCCACGACGAACACGCTG 3', Am-F: 5' CGAGAATGACCACTGCTG 3' TDAS10-R: 5' GTGACCCTCGACTGGTG 3', Am-R: 5' GTAACCCCAAGGTTGAG 3'
1	TD1-F: 5' CTCGATGGGCGGTCGGGTGG 3', Am-F: 5' CGAGAATGACCACTGCTG 3' TD1-R: 5' TGGACCCGGAGCCTGAG 3', Am-R: 5' GTAACCCCAAGGTTGAG 3'.
12	TD12-F: 5' CTGTTTTCATGCCGCTTTCC 3', Am-F: 5' CGAGAATGACCACTGCTG 3' TD12-R: 5' ACGCAGGCGGTCAGCAC 3', Am-R: 5' GTAACCCCAAGGTTGAG 3'.
19	TD19-F: 5' GGTCGCCTACCAGTTCTCC 3' , Am-F: 5' CGAGAATGACCACTGCTG 3' TD19-R: 5' GTTGCGTGCCGCGAAG 3', Am-R: 5' GTAACCCCAAGGTTGAG 3'
21/22	TDNRPS-F: 5'TACCTGCGCCAGCACGAG 3' , Am-F: 5' CGAGAATGACCACTGCTG 3' TDNRPS-R: 5' ACAGCAGGTGATGGGTGTC 3', Am-R: 5' GT AACCCCAAGGTTGAG 3'
23	TD23-F: 5' CTTCGAACGGGAGCCG 3' , Am-F: 5' CGAGAATGACCACTGCTG 3' TD23-R: 5' GCGGCACAGCGAGTCC3', Am-R: 5' GTAACCCCAAGGTTGAG 3'
24	TD24-F: 5' AGGATGTACGCGCCCG 3' , Am-F: 5' CGAGAATGACCACTGCTG 3' TD24-R: 5'CTATCTGAAGGAGATGGG 3', Am-R: 5' GTAACCCCAAGGTTGAG 3'
S11	TDAS11-F: 5' AGGCACGGGATTTCCCGGAC 3', Am-F: 5' CGAGAATGACCACTGCTG 3' TDAS11-R: 5' CCCGCAAGAGACAGGC 3', Am-R: 5' GTAACCCCAAGGTTGAG 3'

Preparation of Possible Biosynthetic Intermediates of Anthramycin

L-kynurenine, 3-hydroxy-L-kynurenine, hydroxyanthranilic acid were purchased directly from Sigma Corporation. 4-methyl-3-hydroxyanthranillic acid was prepared by catalytic hydrogenation of 4-methyl-3-hydroxy-2-nitrobenzoic acid (Aldrich, Inc.) with Pd/C and H_2 at 1atm in ethanol.

Chemical complementation of anthramycin

For chemical complementation, corresponding compounds were added directly to the production medium to a final concentration of 2 mM beginning of fermentation.

Production and Detection of Anthramycin

S. refuineus and its mutant derivatives were cultured in 50 ml seed medium at 47°C for 24 hr. A 5% inoculum was then added to 50 ml production medium and cultured at 47°C for 24 hr. Anthramycin was extracted from the production medium with 50 ml butanol. Butanol extracts were concentrated in vaccuo and redissolved in MeOH. Antibacterial activity of anthramycin was detected by thin-layer chromatography bioautography. Anthramycin butaol

extracts (dissolved in MeOH) were run on 25DC-Alufolien Kieselgel plates with solvent MeOH:CHCl$_3$ (1:9); LB agar containing indicator strain Bacillus sp. TA was overlaid on TLC plates and cultured at 37°C for 20 hr to detect anti-*Bacillus* activity (evidenced by growth inhibition zones) of anthramycin. Anthramycin production was further confirmed by HPLC/MS. Mass spectrometry was performed by using ThermoFinnigan (San Jose, CA) TSQ Quantum triple quadrupole mass spectrometer equipped with a standard electrospray ionization source outfitted with a 100 mm I.D. deactivated fused Si capillary. The injection volume was 10 ml. Anthramycin was separated from cometabolites by using a Jupiter minibore 5 mm C18 column (2.0 mm x 15 cm) with a linear wateracetonitrile gradient (ranging from 95:5 to 5:95 H$_2$O:CH$_3$CN) containing 10 mM ammonium acetate. The flow rate was 0.2 ml/min. The mass spectrometer was operated in the positive (or negative) ion mode, and the electrospray needle was maintained at 4,200 V. The ion transfer tube was operated at 35 V and 342°C (~35 V and 300°C for negative). The tube lens voltage was set to 85 V (~220 V for negative). Source CID (offset voltage between skimmer and the first ion guide, Q00) was used at 15 V. The mass spectrometer was operated in full scan mode with Quad 1. The mass spectral resolution was set to a peak width of 0.70 u (full width at half maximum, FWHM). Full scan spectra were acquired from m/z 150.0 to 700.0 (m/z 150.0 to 1200.0 for negative) over 1.0 s. Data were acquired in profile mode. The electron multiplier gain was set to 3 x 10^5.

References

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CHAPTER III

REASSEMBLY AND HETEROLOGOUS EXPRESSION OF ANTHRAMYCIN BIOSYNTHETIC GENE CLUSTER

Introduction

Numerous drugs that have been used clinically are derived from natural products produced by bacterial, fungi or botanical sources.[1-4] Generally biosynthetic genes of natural products with potent biological activities are clustered together on their host genome. Over the past three decades, a large amount of gene clusters have already been identified as being responsible for the biosynthesis of natural products that have been demonstrated to play important roles for the survival and growth of their producers.[5-10] Those natural products can be developed into a variety of active compounds, some of which have been employed in clinical settings.

Cloning of natural product gene clusters has made the study of the biosynthesis of natural products relatively easier, because researchers are able to deduce biosynthetic pathways based on their chemical structures together with corresponding biosynthetic gene clusters. The biosynthetic knowledge for natural products has enabled subsequent modifications and rearrangements of these natural products biosynthetic gene clusters, called combinatorial biosynthesis, aimed at generating novel "natural products".[11-13]

In order to confirm the function of cloned biosynthetic gene clusters from the genome of their producing organisms and investigate the biosynthetic

pathways of natural products, genetic manipulation systems for the producers of target natural products have to be established first. Afterwards, gene disruptions are normally utilized to demonstrate whether a gene cluster is related to the biosynthesis of a certain natural product.[14, 15] One question for the study of natural product biosynthesis is that the genetic systems for all microorganisms can not be treated in a similar fashion and it is not easy to establish mature genetic manipulation systems for all microorganisms. Even for the well-studied Streptomyces, which can produce more that 70% of known antibiotics, to date there are only a few transformation methods that can be employed and not all Streptomyces strains are capable of being genetically manipulated as expected.[16] In terms of fungi, which have been proven to produce a great number of secondary metabolites with novel structures and potent biological activities, the genetic systems are notoriously difficult to handle[17, 18] and the research work dependent on the genetic manipulation for fungi derived natural products is more risky. Additionally, most slow growing fungi and bacteria are susceptible to contamination from other fast growing bacteria like E. coli, bringing another technical obstacle for the biosynthetic study on the direct producers of natural products.

An alternative and increasingly applied approach for natural product biosynthetic studies is to heterologously express natural product biosynthetic gene clusters. In this method, the targeted natural product gene cluster is cloned into a suitable vector and subsequently transformed into other genetically-related hosts which can be easily manipulated. The function of a given biosynthetic gene

cluster can be validated if the target natural product can be detected in the modified heterologous host. In the past decade, heterologous expression has become a very useful method for the biosynthetic study of natural products, especially those produced by microorganisms without suitable genetic manipulation systems.[19, 20] The natural products from culturable microorganisms account for only approximately 1% of all the natural products in nature. The majority of natural products are produced by those unculturable organisms that are difficult to culture using present known culture conditions.[21, 22] What we can do so far is simply to isolate genomic DNA from environmental samples and identify biosynthetic gene clusters for natural products from inside. For those natural product biosynthetic gene clusters from unculturable organisms or microorganisms that can not be genetically manipulated, heterologous expression of gene clusters is probably the best, or perhaps the only, choice for further genetic and biochemical studies.

Typically, after the isolation of a microorganism, genomic DNA is cloned into a vector such as a cosmid, fosmid or bacterial artificial chromosomes (BACs) with DNA fragments of a certain size in the vectors.[23-26] If the DNA fragment in a single vector can harbor a complete natural product biosynthetic gene cluster, the vector containing this DNA fragment can be transformed into another host for heterologous expression. In most cases, however, the biosynthetic gene clusters for natural products are very large with the size of some gene clusters up to 200 kb and can not be maintained in a single vector.[27, 28] To solve this problem, some laboratories utilized recombinant genetic methods to tether different portions of a

natural product biosynthetic gene cluster and then clone this complete gene cluster into a single vector for heterologous expression.[29] This method has been proven to be viable, but the size and complexity of a biosynthetic gene cluster makes it very difficult to find suitable restriction sites for reconstructing the complete biosynthetic gene clusters from partial gene clusters using traditional digestion/ligation methods. This approach is relatively impractical because of the difficulty in genetically manipulating long natural product biosynthetic gene clusters due to the difficulty in identifying unique restriction sites in natural product biosynthetic gene clusters.

To circumvent these problems, a new method called "Red/ET cloning system"[30] has recently been developed for the recombination of homologous sequences in *E. coli*, which is probably the easiest host for DNA manipulation. The basic principle of this recombination system is based on the presence of several proteins that can facilitate DNA homologous recombination in the same *E. coli* cells[31-33] as have been described in chapter two. Originally this recombination method was designed and utilized for the targeted replacement of biosynthetic genes in the genome of *E. coli* and later for manipulation of biosynthetic gene clusters in Streptomyces.[24, 34, 35] This method has been demonstrated to be very useful and successful, especially for the genetic work related to Streptomyces. The broad applicability of this new approach has revolutionized the traditional gene replacement methods in prokaryotic cells.

In the past several years, several new methods derived from the "Red/ET cloning system" were developed for the recombination of partial biosynthetic

gene clusters from separate vectors into complete natural product gene clusters and have been demonstrated to be more convenient compared with previous genetic methods. Recombination systems derived from "Red/ET cloning system" for the functional reconstruction of large natural product biosynthetic gene clusters from fragments on separate vectors were first applied for the reconstruction of gene clusters for myxobacterial metabolites. Myxobacteria are capable of producing a great variety of natural products with novel structures and activities and have aroused the interests of researchers from all over the world.[36-38] However, the biosynthetic study of natural products from myxobacteria has encountered difficulties. For example it is not easy to isolate and purify a single myxobactrial strain from the environment, the growth of myxobacteria is relatively slow and can be easily contaminated and the genetic systems for myxobacteria have not been well established, *etc.*[39, 40] Rolf Muller's research group has done a lot of work for the improvement of methods to heterologously express the biosynthetic gene clusters they have identified from myxobacteria, which will greatly facilitate further genetic and biochemical work.

In 2005, Dr. Muller's group developed the first recombination based approach through which they were able to recombine a 3.5 kb DNA fragment containing a minority of the myxochromide S gene cluster together with the zeocin-resistance gene into another vector with the majority of the myxochromide S gene cluster using "Red/ET cloning system".[41] The complete myxochromide S biosynthetic gene cluster, which was identified from the genome of *Stigmatella aurantiaca*, was reconstructed from two partial gene clusters and successfully

expressed in a heterologous host *Pseudomonas putida* (figure 3-01).
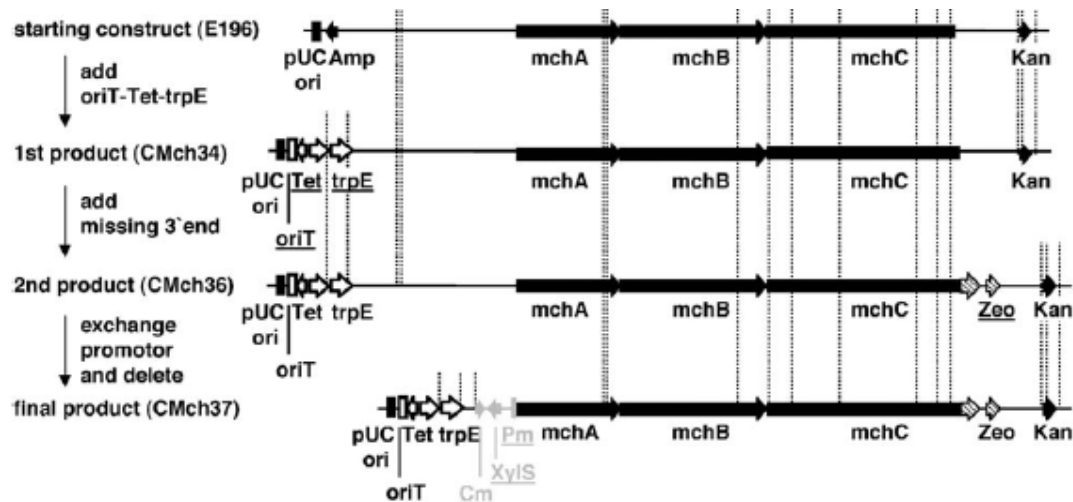
Figure 3-01. Genetic method to recombine myxochromide S biosynthetic gene cluster from *Stigmatella aurantiaca* on the base of "Red/ET cloning system" (copyright of Dr. Rolf Muller research group).

Subsequently, Muller's group developed another method through which they first inserted two different cassettes into two partial myxothiazol gene clusters via "Red/ET cloning system" and then reconstituted them into a complete gene cluster from two cosmids through traditional ligation (figure 3-02).[42] Although the myxothiazol originally produced by *Stigmatella aurantiaca* could be heterologously expressed after its biosynthetic gene cluster was transferred into *Myxococcus xanthus*, one dramatic drawback for this reconstitution method was the requirement to insert additional DNA sequence, at least one restriction enzyme site with 6 bp, into myxothiazol biosynthetic gene cluster, which might effect the function of the complete gene cluster and the production of a natural product.
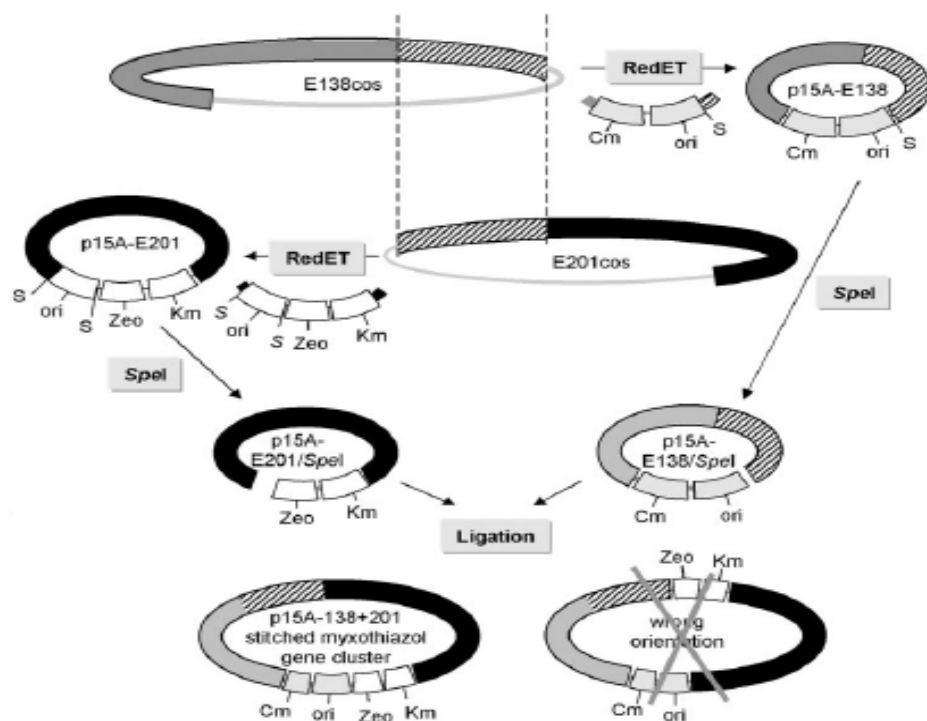
Figure 3-02. Genetic method to recombine myxothiazol biosynthetic gene cluster from *Stigmatella aurantiaca* on the base of "Red/ET cloning system" (copyright of Dr. Rolf Muller research group).

The research work for heterologous expression of natural product biosynthetic gene clusters from myxobacteria through recombination technology derived from "Red/ET cloning system" has demonstrated the feasibility of using this novel technique to reconstruct and reassemble natural product biosynthetic gene clusters from different sources. However, despite the successful expression of natural product biosynthetic gene clusters as described above, those two reconstruction approaches, which are based on the principle of "Red/ET cloning system" seem not to be the final "answer" and still need further optimization or renovation because of some limitations associated with them.

Results and Discussions

In our laboratory, we have successfully applied "Red/ET cloning system" in *Streptomyces refuineus*, the producer of antitumor natural product anthramycin.[43] The function and boundaries of anthramycin biosynthetic gene cluster have been demonstrated using this new gene mutation approach. A more direct method, heterologous expression, can also be utilized to further confirm the function of this anthramycin gene cluster. The anthramycin biosynthetic gene cluster can be cloned into a suitable vector and the recombinant vector harboring the complete anthramycin gene cluster will be transformed into other genetic related hosts for heterologous expression.

The size of the anthramycin biosynthetic gene cluster is relative large (more than 30 kb) and we had two cosmids on hand, each harboring partial anthramycin gene cluster with a roughly 10 kb DNA overlap region. In order to recombine these two partial anthramycin clusters into the same vector, we developed a new recombination method, which was based on the "Red/ET cloning system" and took advantage of the 10 kb overlap region as a homologous sequence for the recombination of larger DNA fragments.

The basic principle of the gene replacement method, "Red/ET cloning system", is that recombination will occur between homologous sequences in *E. coli* BW25113/pIJ790 which can express certain proteins to facilitate recombination between DNA homologous sequences. Since there is an approximate 10 kb overlap region between these two partial anthramycin biosynthetic gene clusters, we expected that this homologous region could be

used as a homologous sequence on one side. The remaining problem was to identify and add additional homologous DNA sequences on the other side in order to reassemble two anthramycin partial gene clusters.

Serendipitously, we noticed that in plasmids pIJ773 and pIJ778 that were commonly used in "Red/ET cloning system"[30], there was a homologous sequence region of 341 bp between the origin of transfer (*oriT*) and apramycin resistance gene (*aac(3)IV*)/streptomycin resistance gene (*aadA*). We speculated that the reason might be that the coding region of *aac(3)IV* in pIJ773 was replaced by *aadA* to yield pIJ778 using the "Red/ET cloning system" and the promoter region of *aac(3)IV* was still left on the plasmid. This homologous promoter region could be exploited to generate recombinogenic cassttes for the recombination of partial gene clusters by inserting this homologous region into certain locations into the vectors. Thus, we could amplify the apramycin resistance gene (*aac(3)IV*) from pIJ773 and the streptomycin resistance gene (*aadA*) from pIJ778, with the promoter region as the homologous sequence on the same side. The *Xba*I restriction site was added outside the homologous region with 39 bp flanking sequences on both sides of the PCR products for PCR-targeting replacement. Other restriction sites that could not be identified from the gene cluster could also be utilized and placed before the homologous region. Flanking sequences were selected from the anthramycin biosynthetic gene cluster for homologous recombination to insert *aac(3)IV* and *aadA* into those corresponding target regions. The *aac(3)IV* gene was utilized to replace a 240 bp fragment inside *orf7* of the anthramycin gene cluster in cosmid 024CA to

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yield cosmid FCA and *aadA* was used to replace a 200 bp fragment located 4425 bp upstream of *orf1* of the anthramycin gene cluster to yield cosmid FCO.

The linear PCR products of *aac(3)IV* and *aadA* with homologous sequence on the upstream side were transformed into *E. coli* BW25113/pIJ790 strains containing cosmids 024CA and 024CO, respectively. In the 10 kb overlapped region between the two anthramycin partial gene clusters, *Dra*I was recognized as a single-cut restriction enzyme site for anthramycin biosynthetic gene cluster. After inserting the *aadA* cassette into cosmid CO to produce cosmid FCO, FCO was cut with *Xba*I and *Dra*I to yield a 15.9 kb DNA fragment with DNA sequences homologous to cosmid FCA on both sides of this DNA fragment. The 15.9 kb DNA fragment was purified after enzymatic digestion and transformed into *E. coli* BW25113/pIJ790 containing cosmid FCA. Recombination occurred on both sides at the promoter homologous sequence and the overlapped DNA region in the anthramycin gene cluster. Cosmid CAO, containing the complete anthramycin biosynthetic gene cluster, was selected by isolating streptomycin resistant and apramycin sensitive colonies because *aac(3)IV* in cosmid FCA was replaced by *aadA* from the linear 15.9 kb DNA fragment. PCR was used to verify the correct orientation of the inserted sequence and sequencing analysis proved the correctness of the recombinant cosmid.

To integrate cosmid CAO into the genome of other Strepomyces hosts, an attachment site of phage c31 (*attP*) was inserted into the vector. After transformation of the recombinant vector with *attp* site into Streptomyces, vector harboring the biosynthetic gene cluster could be integrated into *attB* site on the

genome with high efficiency. An integrase gene (*int*), attachment site of phage c31 (*attP*) together with the tetracycline-resistance gene were cut from plasmid pIJ787[44] as a DNA fragment by a *DraI*/*BsaI* digestion and utilized to replace the ampicillin-resistance gene (*bla*) in cosmid CAO to yield comid CAO-2. To facilitate efficient transformation between E. coli and Streptomyces strains, an *oriT* was inserted into CAO-2 to yield cosmid CAO-3 by replacing the kanamycin resistance gene (*neo*) with a cassttee containing the apramycin resistance gene (*aac(3)IV*) and *oriT* that was cloned from plasmid pIJ773. Cosmid CAO-3 was transformed into *E. coli* ET12567/pUZ8002 and subsequently transformed into *S. lividans* TK24 and *S. coelicolor* M595 through intergeneric conjugation. Integration of cosmid CAO-3 was confirmed by the apramycin resistance phenotype and PCR amplification of DNA sequences from both the anthramycin biosynthetic gene cluster and the vector. The detailed strategy for the reassembly and heterologous expression of anthramycin biosynthetic gene cluster was elucidated in figure 3-03.
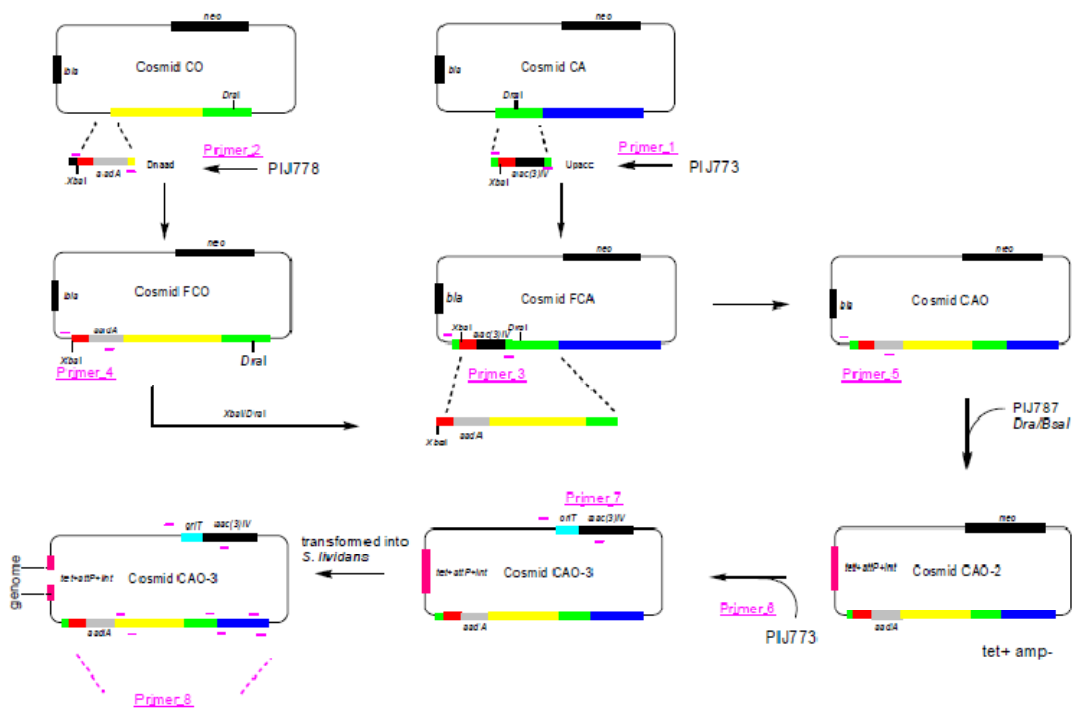
Figure 3-03. Complete cloning strategy for reassembly and heterologous expression of anthramycin biosynthetic gene cluster.

The fermentation broth of *S. lividans* TK24/CAO-3 and *S. coelicolor* M595/CAO-3 was subjected to the detection of anthramycin by using TLC bioautography (anti-*Bacillus* activity) and HPLC-MS analysis. Anthramycin could be heterologous expressed in *S. lividans* TK24 instead of *S. coelicolor*. Anthramycin could not be heterologously expressed in *S. coelicolor* probably because *S. coelicolor* was not a suitable host for anthramycin biosynthetic gene cluster or the regulation and promoter systems of anthramycin gene cluster were not compatible with *S. coelicolor*. We could detect the formation of anthramycin from the fermentation broth of *S. lividans* TK24/CAO-3. Interestingly, anthramycin could not be produced when this producing strain was cultured at 28°C, the normal temperature for the culture of *S. lividans*. When we changed the cultural

temperature to 37°C, 47°C and 52°C, anthramycin production was only detected at 47°C but not 37°C and 52°C, suggesting a temperature dependent expression mechanism of the anthramycin biosynthetic gene cluster (figure 3-04). It has also been observed that the stability of anthramycin in *S. lividans* TK24 was not very high. Only in the first two days could anthramycin be detected from the fermentation broth and after the third day we failed to observe anthramycin in production medium, perhaps because anthramycin has a high toxicity and *S. lividans* has a self-protection mechanism to inactive anthramycin after its production.
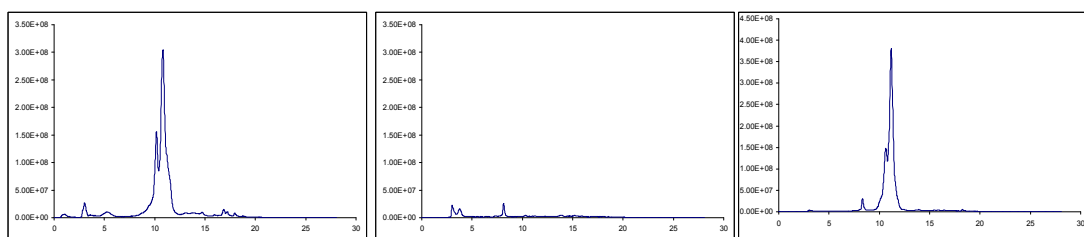
Figure 3-04. HPLC/MS results of the anthramycin biosynthetic gene cluster heterologous expression. (Left: anthramycin from *S. refuineus*. Middle: negative control from *S. lividans* TK24. Right: anthramycin from *S. lividans* TK24/CAO-3.)

In our laboratory, on the basis of "Red/ET cloning system", which was originally designed to mutate target genes on the genome, we developed a new method to recombine partial biosynthetic gene clusters and used anthramycin biosynthetic gene cluster as a case study to prove the feasibility of this new method. The successful reassembly and heterologous expression of the anthramycin biosynthetic gene cluster in *S. lividans* TK24 demonstrated our

method could be utilized to assemble fragmented gene clusters into complete biosynthetic gene clusters and could be applied for the heterologous expression of many other natural product biosynthetic gene clusters, notably those from unculturable organisms.

Materials and methods

General DNA Procedures

DNA manipulation was conducted according to standard methods. Cosmid DNA was isolated and purified from E. coli strains by using plasmid miniprep and gel extraction kits (Qiagen). All transformations in E. coli were performed by electroporation by using a GenePulser electroporator (Biorad) with a 100 V, 30 ms pulse. Genomic DNA from Streptomyces strains was isolated by using the Wizard^{TM} genomic DNA purification kit (Promega). Primers were synthesized by Operon Biotechnologies (Huntsville, AL, USA). Intergeneric conjugation was performed according to Kieser et al. with modifications as described below.

Bacterial strains and media

Targeted gene replacement experiments were performed in *E. coli* strain BW25113 that contained the plasmid pIJ790. *E. coli* ET12567 that contained the RP4 derivative pUZ8002 was used for intergeneric conjugation between E. coli and Streptomyces. The heterologous expression host was *Streptomyces lividans* strain TK24 and *Streptomyces coelicolor* M595. *Bacillus* sp. TA (NRRL B-3167) was used as an indicator strain to test the activity of anthramycin. E. coli strains were maintained in LB medium that contained the appropriate antibiotics for

selection. *S. refuineus* was grown in SRS medium (0.5% N-Z Amine B, 0.2% yeast extract, 0.2% soytone, 1.0% potato starch, 0.5% mannitol, 0.015% FeSO$_4$, 2% agar, pH 7.0) and anthramycin production was performed in production medium AP1 (1% corn starch, 2% peptonized milk, 0.3% yeast extract, pH 7.0). AS1 medium (0.1% yeast extract, 0.02% L-alanine, 0.02% L-arginine, 0.05% L-asparagine, 0.5% starch, 0.25% NaCl, 1% Na$_2$SO$_4$, 2% agar) and MS medium (2% mannitol, 2% soya, 2% flour agar) were used for conjugation.

Retrofitting donor cosmids with recombination cassettes

The apramycin-resistance gene (*aac(3)IV*) cassette was amplified by PCR from plasmid pIJ773 by using primers UPaacF/UPaacR (table 3-01) and transformed into *E. coli* BW25113/pIJ790/CA. Transformants were selected for resistance to apramycin at 30 µg/mL. The resulting cosmid FCA, in which a 240 bp DNA fragment in the anthramycin biosynthetic gene ORF7 was exchanged with PCR-targeted recombination, was purified by gel electrophoresis. A 200 bp DNA fragment that was 4425 bp upstream of the anthramycin biosynthetic cluster on cosmid CO was exchanged in a similar fashion. In this case, the streptomycin-resistance gene (*aadA*) cassette was amplified from plasmid pIJ778 with primers DNaadF/DNaadR (table 3-01) and transformed into *E. coli* BW25113/pIJ790/CO. Transformants that were resistant to streptomycin (30 µg/mL) contained cosmid FCO, which was subsequently purified by gel electrophoresis. The insertion of recombinogenic cassettes was verified by PCR using primers TFCA-1/ UPaacR and TFCO-1/ DNaadR (table 3-01).

Table 3-01. PCR-targeting primers to reassemble anthramycin biosynthetic gene cluster (Restriction sites are in bold; the homologous targeting sequence is underlined).

primer	sequence
UPaccF	5'-ACCGAGCCGATCGGGTAGAGCACCGCACCGTAGCGGTCGC**TCTAGA**<u>GCTGACGCCGTTGGATAC</u>-3'
UPaccR	5'-CCCTGGTACGACGTGTGGCTCCCCGGGTCCGCCGTGGAGG**ACTAGT**<u>GGAATAGGAACTTATGAGC</u>-3'
DNaddF	5'-CACGTGCGGCCGCACGAGGGCGAGTCGGCCGCCGCGGAGG**TCTAGA**<u>GCTGACGCCGTTGGATAC</u>-3'
DNaddR	5'-GCGCCACCGTCACGCTCTCCACCCGCAGCCCCGGCACCAT**ACTAGT**<u>GCGGCATCTTATTTGCCGAC</u>-3'
ATF	5'-TCAAGATCTGATCAAGAGACAGGATGAGGATCGTTTCGCGGAATAGGAACTTATGAGC-3'
ATR	5'-GCGTCGCTTGGTCGGTCATTTCGAACCCCAGAGTCCCGCAAGTTCCCGCCAGCCTC-3'
TFCO-F1	5'-CTGCGCCACTTCCTCGAC 3'
TFCA-F1	5' AAGTGGGGTTCCACTACC 3'
TF	5'-GTAACCCCAAGGTTGAG-3'
TR	5'-TGTCCAATTATGTCACACCAC-3'
TD-1	5' -CTCGATGGGCGGTCGGGTGG- 3'
T1-R2	5'-TGGACCCGGAGCCTGAG- 3'
TD-10	5'-CTGCCACGACGAACACGCTG-3'
T10-R2	5'-GTGACCCTCGACTGGTG-3'
TD-11	5'-AGGCACGGGATTTCCCGGAC-3'
TS11-R2	5'-CCCGCAAGAGACAGGC-3'

Reconstruction of the complete anthramycin pathway

Cosmid FCO was restricted with *Xba*I/*Dra*I and the resulting gel-purified 15.9 kb fragment was transformed into *E. coli* BW25113/pIJ790/024FCA by electroporation. Transformants that contained the resulting cosmid CAO, which contained a 15.9 kb fragment that was exchanged into cosmid FCA were selected by resistance to streptomycin (30 µg/mL). Cosmid CAO was purified by gel electrophoresis and confirmed by PCR with primers TFCA-F1/ DNaddR (table 3-01).

Retrofitting fusion cosmid with integrase and origin of transfer

Insertion of origin of transfer (*oriT*) and integrase (*int*) genes into the reconstructed cosmid was performed according to established protocols with minor modifications. Plasmid pIJ787 was digested with *Dra*I and *Bsa*I, and the resulting 5 kb DNA fragment, which contained the integrase cassette flanked by

approximately 100 bp of *bla* sequence upstream and 300 bp of *bla* sequence downstream of the integrase cassette, was gel purified and transformed into *E. coli* BW25113/pIJ790/CAO. Selection for tetracycline resistant (5 µg/mL) clones resulted in strains that contained cosmid CAO-2. The apramycin-resistance/origin of transfer gene (*aac(3)IV/oriT*) cassette was amplified from plasmid pIJ773 with primers ATF/ATR (table 3-01). The resulting gel-purified PCR product was transformed into *E. coli* BW25113/pIJ790/CAO-2, so that apramycin resistance could replace the kanamycin resistance gene in cosmid CAO-2 with *aac(3)IV/oriT*. The resulting cosmid 024CAO-3 was purified and transformed into *E. coli* ET12567/pUZ8002.

Heterologous expression of the anthramycin cluster

Cosmid CAO-3 was transformed into *Streptomyces lividans* TK24 and *Streptomyces coelicolor* M595 by intergenetic conjugation with *E. coli* ET12567/pUZ8002/024CAO-3. Transconjugants were selected on MS or AS-1 medium (50 µg/mL apramycin) and exconjugates were verified by amplifying isolated genomic DNA with four sets of primers on bracketing ends of the gene cluster and in the cosmid sequence (Table 3-01). A *Streptomyces lividans* TK24 or *Stretomyces coelicolor* integrant that harbored cosmid CAO-3 was cultured in AP1 seed medium (50 mL) at 47°C for 24 h. A 5% inoculum was then added to production medium AP1 (50 mL) and incubated at 47°C for 24 h. Anthramycin was extracted from the production medium with butanol (50 mL). Butanol fractions were dried over anhydrous $MgSO_4$, concentrated in vacuo, and redissolved in MeOH directly prior to analysis.

Assays for anthramycin production

Antibacterial activity of anthramycin was detected by thin-layer chromatography bioautography and HPLC–MS. Anthramycin (dissolved in MeOH) was chromatographed on 25DC-Alufolien kieselgel plates (Merck) that were eluted with MeOH/CHCl$_3$ (1:9) and then visualized by bioautography. LB agar (~55°C) that was inoculated with indicator strain *Bacillus* sp. TA was poured on TLC plates and cultured at 37°C for 20 h to detect anti-*Bacillus* activity of anthramycin. Anthramycin production was further confirmed by HPLC–MS analysis by using a Jupiter^{TM} minibore C-18 column (2.0 mm x 15 cm; Phenomenex, Torrance, CA, USA) with a linear water/acetonitrile gradient (H$_2$O/CH$_3$CN, 95:5 to 5:95) that contained NH$_4$OAc (10 mm) at 0.2 mL/min flow rate. Mass spectrometry was performed by using ThermoFinnigan TSQ Quantum triple quadrupole mass spectrometer equipped with a standard electrospray ionization source that was outfitted with a deactivated fused Si capillary (100 mm i.d.). The injection volume was 10 mL. Mass spectrometery was performed in the positive and negative-ion mode, and the electrospray needle was maintained at 4200 V. The ion transfer tube was operated at 35 V and 342°C (~35 V and 300°C for negative). The tube lens voltage was set to 85 V (~220 V for negative). Source CID (offset voltage between skimmer and the first ion guide, Q00) was used at 15 V.

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CHAPTER IV

CHARACTERIZATION OF A NOVEL NITROSOSYNTHASE ORF36 FROM EVERNINOMYCIN BIOSYNTHETIC GENE CLUSTER IN MICROMONOSPORA AFRICANA

Introduction

Natural products produced by bacteria, fungi and plants are a great source of valuable compounds or novel chemical structures that have been demonstrated to have potential in industry, especially in drug discovery. A variety of natural products have been extracted and purified from their corresponding producers and shown to possess potent biological activities that we have used medically for thousands of years.[1-3] The last 100 years have witnessed many scientific developments in natural product biosynthesis and total synthesis of these interesting and bioactive compounds.

Among the natural products that have been discovered so far, an intriguing class of natural products contain deoxysugars which have been experimentally found to be bonded to core chemical structures that are derived from PKS or NRPS.[4-6] The functions of these side-chain deoxysugars actually play very important roles in their biological activities and should not be undervalued.[7, 8] The biosynthesis of these deoxysugars has become much clearer after the identification of their biosynthetic genes.[9-11] Typically these dexosugars are derived from the common precursors such as NDP-4-keto-6-deoxy-α-D-glucose and fructose 6-phosphate, as has been discussed in Chapter One. The biosynthesis of deoxysugars requires the involvement of dehydratases,

ketoreductases, isomerases, methyltransferases and aminotransferases. These deoxysugars are utilized in the biosynthetic pathway in the form of NDP sugars. Glycosyltransferases are responsible for the attachment of deoxysugars to the core structures in natural products after the formation of the mature dexosugars.[12]

A great many deoxysugars harbor free amino groups at C3 or C4 positions.[13-15] The amino groups are transferred onto deoxysugars by the pyridoxal 5'-phosphate (PLP)/pyridoxamine 5'-phosphate (PMP)-dependent transamination reaction catalyzed by aminotransferases.[6] Despite the existence of large quantities of oxidases *in vivo*, most of the amine groups in amino sugars can not undergo further oxidation and only approximately less than ten nitro/nitroso sugars have been identified from deoxysugar-containing natural products, including everninomycin, rubradirin, kijanimicin, respinomycins, arisostatins and viriplanins.[16-23] Both amino sugars and nitro sugars have been demonstrated to be very important for the biological activities of their precursor natural products, for example, in the interaction between natural products and their targets or the signal interaction among their producers.[24, 25] However, natural products with different amino sugars and nitro sugars have diverse biological activities even if the rest of the natural product structure remains unchanged. Everninomycin represents a very good example for elucidation of the functions and roles of amino sugars and nitro sugars in natural products. The everninomycin producer *Micromonospora africana* can actually produce several structurally related natural products, among which are everninomycin with a nitro

sugar as the major product, and "compound 5", which is similar to everninomycin except for an amino sugar at position C65 instead of a nitro sugar as shown in Figure 4-01. Despite this minor structural difference, everninomycin and "compound 5" exhibit dramatically different biological activities toward a spectrum of bacteria. The range of activities in the everninomycin antibiotics indicate that the nitro group is critical for the biological activity of nitro group containing natural products. Thus the question of how the nitro sugars in natural products are biosynthesized has aroused great interest.

Everninomycin R=NO$_2$
"Compound 5" R=NH$_2$
Micromonospora africana
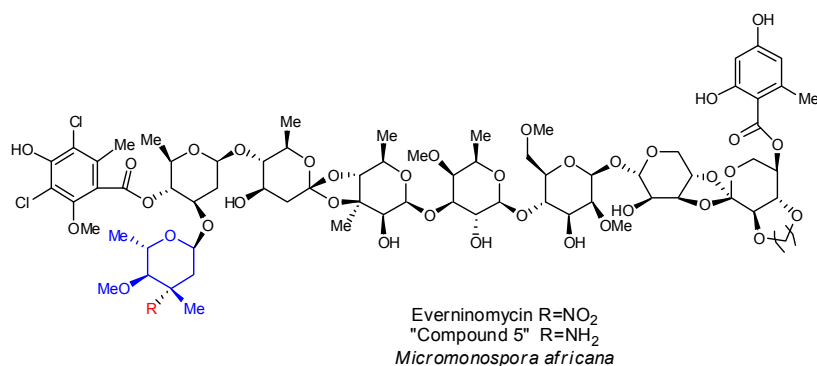
Figure 4-01. Everninomycin and "compound 5" produced by *Micromonospora africana*.

Before our biosynthetic studies of nitro sugars in natural products, the formation of several other classes of nitro groups in natural products have been well investigated. These previously studied nitro groups containing natural products include small aromatic compounds containing nitro groups. The biosynthetic mechanisms for the formation of nitro groups can be divided into two major groups: direct electrophilic nitration of aromatic groups by NO$_2^+$ and enzymatic oxidation by *N*-oxidases. In the first group, NO$_2^+$ is produced *in vivo*

and able to attack the aromatic group by an electrophilic reaction to yield an aryl nitro functional group. Two representative examples for this class of nitration mechanism are thaxtomin produced by the plant pathogen *Streptomyces turgidiscabies*[26] and dioxapyrrolomycin produced by *Streptomyces fumanus*[27] as previously introduced in Chapter One. In the second group, the nitro groups on the aromatic rings are derived from amino groups and certain *N*-oxidases from natural product biosynthetic gene clusters are responsible for the oxidation of amino groups to nitro groups. To date, only two examples for the biosynthesis of aryl nitro groups catalyzed by *N*-oxidase have been reported: pyrrolnitrin produced by *Pseudomonas fluorescens*[28] and aureothin from *Streptomyces thioluteus (*figure 4-02*).*[29-33] In the biosynthetic gene cluster of pyrrolnitrin, PrnD has been demonstrated to be an *N*-oxidase that can oxidize an aryl amino group to an aryl nitro group.[34] AurF is another *N*-oxidase identified in the biosynthetic gene cluster of aureothin and capable of catalyzing the oxidation of para-aminobenzoic acid (pABA) with an amino group to para-nitrobenzoic acid (pNBA) with a nitro group.[30, 35] Although the above two *N*-oxidases are able to mediate the oxidation of an amino group to a nitro group through the same intermediates: a hydroxylamine group and a nitroso group, the mechanisms involved in these oxidation reactions are dramatic different: PrnD is an Fe-S cluster *N*-oxidase while AurF is a di-nuclear *N*-oxidase that requires two metal ions at its active site for its *N*-oxidase activity.
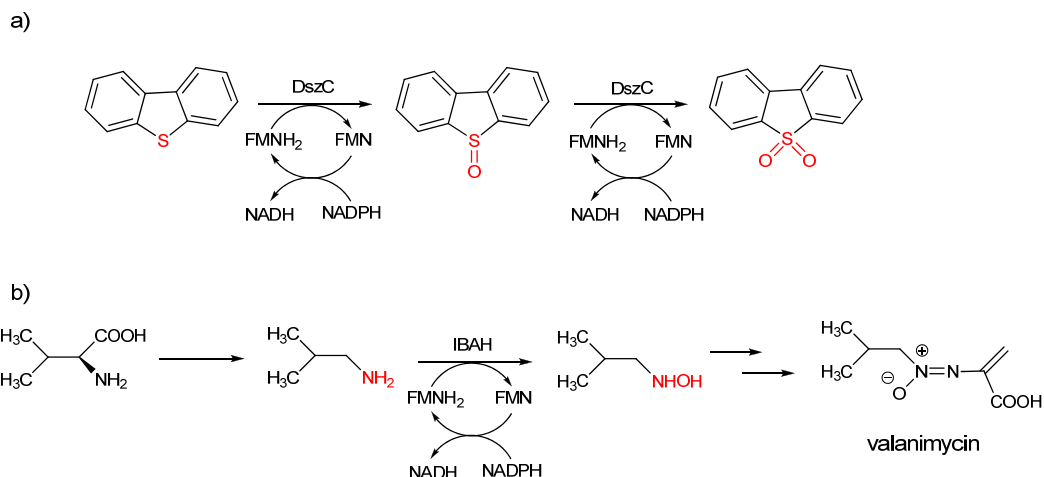
Figure 4-02. Flavin-dependent oxidation reactions catalyzed by DszC from *Rhodococcus erythropolis* (a) and IBAH from *Streptomyces viridifaciens* (b).

In the case of nitro sugar biosynthesis, we know through isolation studies that natural products harboring amino sugars can also be produced by microorganism producers for nitro sugar containing natural products, we speculate that those nitro sugars are derived from amino sugars through the oxidation reactions catalyzed by *N*-oxidases. The oxidation of amino sugars to nitro sugars very possibly occurs after the deoxy sugar activation by NDP because in general the modifications of deoxysugars are carried out at nucleotide sugar stage.[24] A hydroxylamine group and a nitroso group are proposed to be the two possible intermediates for this *N*-oxidation reaction.

Here in our laboratory, we used TDP-L-evernitrose in everninomycin as a model compound to study the biosynthetic mechanism of nitro groups on sugars. We identified a flavin-dependent oxidase ORF36 from the reported everninomycin biosynthetic gene cluster based on the comparative genomic

analysis of everninomycin biosynthetic gene cluster and the gene cluster of avilamycin which is a natural product structurally similar to everninomycin but does not harbor a nitro sugar. ORF36 also shows similarity with other flavin-dependent oxidases. For example, monooxidase DszC from *Rhodococcus erythropolis* has been demonstrated *in vitro* to oxidize a sulfide to a sulfone[36] and IBAH which has been shown to oxidize an aliphatic amine group to an aliphatic hydroxylamine group during valanimycin biosynthesis.[37-39] The characterizations of these flavin-dependent oxidases further support our prediction for the function of oxidase ORF36 from the evernionmycin gene cluster in nitro sugar biosynthesis.

We cloned gene *orf36* from the genomic DNA of everninomycin producer *M. africana* and expressed ORF36 in *E. coli* BL21(DE3) to obtain soluble protein ORF36. The function of ORF36 was demonstrated *in vitro* to be a flavin-dependent nitrososynthase responsible for the oxidation of an amino sugar to a nitroso sugar with a hydroxylamine sugar as an intermediate.

Results and Discussion

Identification of a nitrososynthase from Everninomycin biosynthetic gene cluster

Everninomycin is an oligosaccharide natural product that is composed of two dichloroisoeverninic acid moieties that are derived from an iterative PKS and eight deoxysugars, one of which is a unique nitro sugar moiety. Everninomycin can be produced by several different microorganisms such as *Micromonospora carbonacea* var. *africana* NRRL 15099 and *Micromonospora carbonacea* var.

aurantiaca NRRL 2997. The biosynthetic gene clusters of everninomycin from those producing strains have already been reported. In the everninomycin biosynthetic gene clusters, there are more than forty genes that are proposed to be responsible for the biosynthesis of everninomycin and at least six genes encoding oxidases, the functions of which have not been investigated. Therefore, it is hard to identify the target *N*-oxidase involved in the biosynthesis of nitro sugar from these reported everninomycin biosynthetic gene clusters only on the base of their sequence information.

Avilamycin produced by *Streptomyces viridochromogenes* is another representative example of oligosaccharide natural products and is structurally similar to everninomycin. Avilamycin contains a heptasaccharide chain with seven dexosugars and one PKS-derived dichloroisoeverninic acid moiety. The major difference between everninomycin and avilamycin is the absence of a nitro sugar in avilamycin. In 2001, the biosynthetic gene cluster for avialmycin was also been reported from its producer *Streptomyces viridochromogenes.*[40] The availability of both the everninomycin and avilamycin biosynthetic gene clusters facilitates the identification of biosynthetic genes for the unique nitro sugar, especially for the formation of nitro group, from everninomycin gene cluster through comparing their identified biosynthetic gene clusters (figure 4-03).
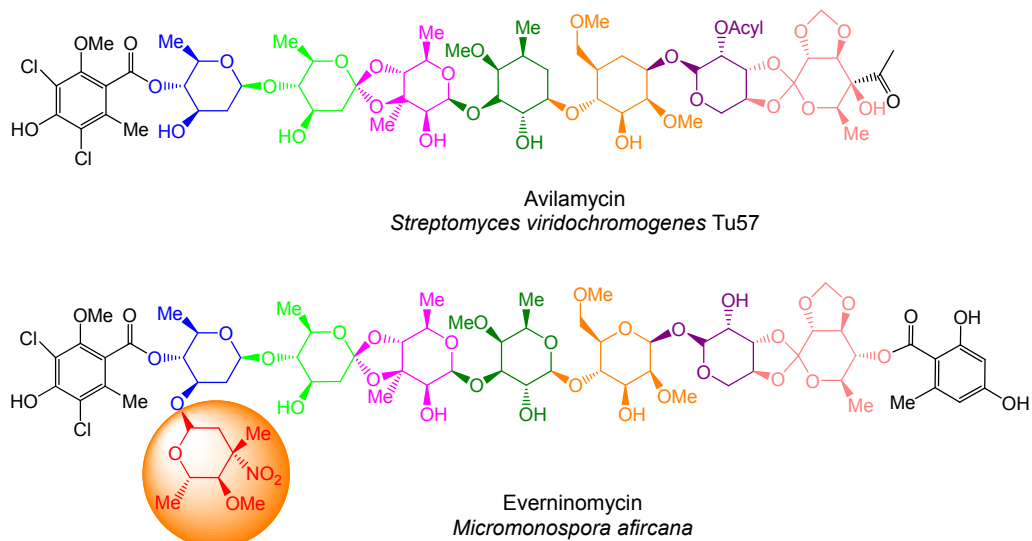
Figure 4-03. Chemical structures of avilamycin from *Streptomyces viridochromogenes* Tu57 and everninomycin from *Micromonospora afircana.*

After comparing the biosynthetic gene clusters of everninomycin and avilamycin, nine genes (*orf37/43*, *orf38/44*, *orf39/45*, *orf40/46*, *orf41/47*, *orf18/3*, *orf19/4*, *orf35/21* and *orf36/42*) were identified to be present in everninomycin gene clusters but absent in the gene cluster of avilamycin. We speculated that some of these genes were responsible for the formation of the unique nitro sugar, L-evernitrose, in everninomycin. Sequence analysis of these genes suggested that four of the identified genes (*orf37/43*, *orf38/44*, *orf39/45* and *orf40/46*) were probably involved in the biosynthesis of an amino sugar on the basis of their high similarities with the biosynthetic genes for epivancosamine in chloroeremomycin produced by *Amycolatopsis orientalis.*[41] *Among the remaining four genes, two of them were proposed to encode oxidases: orf19/4 was a copper-dependent oxidase and orf36/42* was a flavin-dependent oxidase. ORF19/4 was proposed to

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be an enzyme related to primary metabolism and involved in membrane biosynthesis. The flavin-dependent oxidase ORF36/42 was analogous to DszC which has been previously demonstrated to oxidize a sulfide to a sulfone and IBAH which was capable of oxidizing an aliphatic amine group to an aliphatic hydroxyl amine group. Based upon sequence analysis of the everninomycin and avilamycin biosynthetic gene clusters together with the sequence similarity between ORF36/42 and other flavin-dependent oxidases, we proposed that ORF36/42 encoded a flavin-dependent *N*-oxidase which was responsible for the oxidation reaction from an amino sugar to a nitro sugar at the nucleotide stage.

Analogs of ORF36/42 were also identified from the identified biosynthetic gene clusters of two other nitro sugar containing natural products: rubradirin produced by *Streptomyces achromogenes* var. *rubradiris* and kijanimicin produced by *Actinomadura kijaniata*. ORF36/42 showed high similarity with RubN8 from the rubradirin biosynthetic gene cluster and KijD8 from the kijanimicin biosynthetic gene cluster. We speculated that all three of these flavin-dependent oxidases were responsible for the formation of the corresponding nitro groups in these nitro sugar containing natural products.

An NDP activated amino sugar was proposed to be the precursor of the nitro sugar existing in everninomycin based upon the fact that "compound 5" could also be produced by the everninomycin producer *M. africana* and the existence of five genes possibly involved in the biosynthesis of an NDP-amino sugar. Among these five proteins in NDP-amino sugar biosynthesis, ORF23/ORF38 could be identified in both everninomycin and avilamycin

biosynthetic gene clusters, but we still speculated this protein was involved in the biosynthesis of the unique nitro sugar in everninomycin because this protein was proposed to catalyze the 2,3-dehydration of NDP-4-keto-6-deoxy-α-D-glucose, a key biosynthetic step for sugars in natural products. ORF37/ORF43 was proposed to be an aminotransferase based upon its similarity with EvaB in NDP-L-epivancosamine biosynthesis, RubN4 in NDP-D-rubranitrose biosynthesis and KijD2 in NDP-D-kijanose biosynthesis. ORF38/ORF44 was proposed to be a *C*-methyltransferase because of its high similarity with EvaC in NDP-L-epivancosamine biosynthesis, RubN7 in NDP-D-rubranitrose biosynthesis and KijD1 in NDP-D-kijanose biosynthesis. ORF40/46 was proposed to be a ketoreductase on the base of its similarity with EvaE in NDP-L-epivancosamine biosynthesis and RubN6 in NDP-D-rubranitrose biosynthesis. ORF39/45 was proposed to be a *C*-5 epimerase because it showed high similarity with EvaD in NDP-L-epivancosamine biosynthesis. In the biosynthetic pathways of NDP-D-rubranitrose and NDP-D-kijanose, the absence of a *C*-5 epimerase suggested that these two nitro sugars should be assigned the D-configuration. In NDP-D-kijanose, the C-4 keto group should not be reduced and an amino group was proposed to be transferred to this C-4 position because of the existence of one additional aminotransferase, which was different from the biosynthetic pathways of the three other amino/nitro sugars (table 4-01). The biosynthetic pathway for TDP-L-evernitrose from everninomycin (*M. africana*) is elucidated in figure 4-04.

Table 4-01. Biosynthetic genes for the biosynthesis of NDP-nitrosugar/aminosugar in everninomycin (*M. africana*), everninomycin (*M. aurantiaca*), rubradirin (*S. rubradiris*), kijanimicin (*Actinomadura kijaniata*), chloroeremomycin (*Amycolatopsis orientalis*), dibenzothiophene (*Rhodococcus erythropolis*) and valanimycin (*Streptomyces viridifaciens*). The protein sequences from everninomycin (*M. africana*) were used as the basis with identity/similarity underneath other proteins. C-4-aminotransferase KijD7 is unique to kijanimicin and does not have homolog in other biosynthetic gene clusters.

Natural product	C2,3-dehydrase	C-3-aminotransferase	C-3-methyltransferase	C4-ketoreductase	C-5-epimerase	C-4-aminotransferase	oxidase
everninomycin (*M. africana*)	ORF23	ORF37	ORF38	ORF40	ORF39		ORF36
everninomycin (*M. aurantiaca*)	ORF38 69/78%	ORF43 78/85%	ORF44 81/85%	ORF46 64/75%	ORF45 74/85%		ORF42 80/86%
rubradirin	RubN3 49/63%	RubN4 69/78%	RubN5 71/81%	RubN6 53/65%			RubN8 63/73%
Kijanimicin	KijB1 51/64%	KijD2 75/83%	KijD1 71/80%			KijD7	KijD3 65/76%
chloroeremomycin	ORF23 46/60%	ORF25 72/82%	ORF14 67/79%	ORF24 52/67%	ORF26 58/74%		
dibenzothiophene slufone							DszC 23/35%
valanimycin							IBAH 23/38%
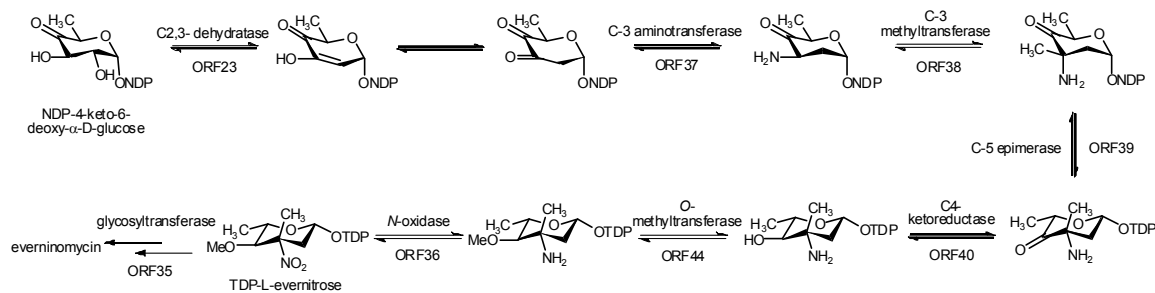
Figure 4-04. Biosynthetic pathway for TDP-L-evernitrose from everninomycin (*Micromonospora. africana*).

In 1992, a very interesting phenomenon was reported related to the biosynthesis of rubradirin: when the rubradirin producer *Streptomyces rubradiris* was cultured in the complete darkness, another structurally related natural product, protorubradirin with a nitroso group, instead of rubradirin, was detected from the fermentation broth and the purified. Protorubradirin could be converted to rubradirin after it was exposed to light.[42] Therefore, protorubradirin was most

likely the direct product of *Streptomyces rubradiris*. The oxidation of a nitroso sugar to a nitro sugar was proposed to be catalyzed by an *in vitro* chemical reaction. On the basis of the report about the identification of protorubradirin and the conversation of protorubradirin to rubradirin, we speculated that an NDP-amino sugar should be first oxidized to an NDP-nitroso sugar and then attached to its aglycon acceptor by a glycosyltransferase. The oxidation of the nitroso sugar was proposed to occur after the excretion of nitroso sugar containing natural product.

Expression of nitrososynthase from E. coli BL21(DE3) and preparation of TDP-amino sugar

Several different Micromonospora strains have the ability to produce everninomycins. We chose one everninomycin producer, *Micromonospora carbonacea* var. *africana*, as the model stain to investigate the biosynthesis of nitro sugar moiety. The biosynthetic gene cluster of everninomycin was identified from the genome in *M. africana* and one gene, *orf36*, was proposed to encode a flavin-dependent nitrososynthase for the oxidation of an NDP-amino sugar to an NDP-nitro sugar.

M. africana (NRRL 15099) was provided by Agricultural Research Service Culture Collection. Genomic DNA of *M. africana* was extracted and used as template for the PCR amplification of *orf36*. *Orf36* was cloned into *Nde*I/*Hind*III sites of plasmid pET28a to yield recombination plasmid pET-28N for the expression of an *N*-terminal hexahistidine fusion protein with ORF36. ORF36 with an *N*-terminal his tag was successfully expressed in *E. coli* BL21(DE3), after the induction by isopropyl β–D-1-thiogalactopyranoside (IPTG), as a soluble

protein. Subsequently, ORF36 was purified to homogeneity and desalted using Fast protein liquid chromatography (FPLC) (figure 4-05). ORF36 which has been cloned from the genomic DNA of *M. africana* **(**NRRL 15099) and purified from *E. coli* BL21(DE3) was used for further enzymatic characterization of its nitrososynthase activity.
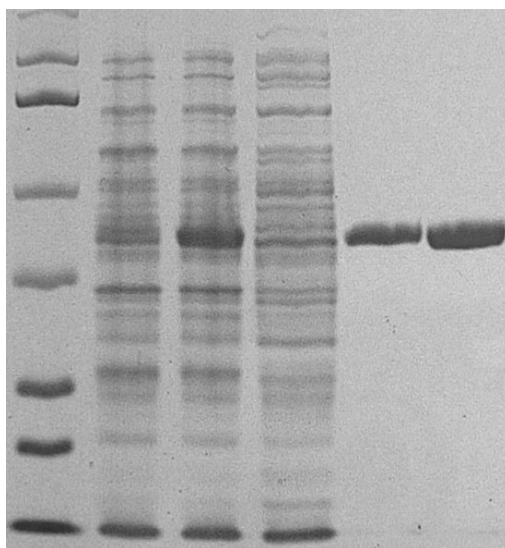

Figure 4-05. Expression of ORF36 in *E. coli* BL21(DE3) (From left to right, Lane 1: Molecular weight markers; Lane 2: Uninduced total protein extract; Lane 3 Induced total protein; Lane 4: Flow-through of Ni^{2+} column; Lane 5: Pooled fractions from Ni^{2+} column; Lane 6: Desalted fraction).

Everninomycin contains an ʟ-epivancosamine with a nitro group at the C3 position. As has been previously described, an NDP-amino sugar was proposed to be the direct precursor of the nitrososynthase ORF36. However, this required NDP-amino sugar for everninomycin biosynthesis was not commercially available, so this proposed substrate has to be synthesized to complete the enzymatic

characterization of ORF36 from everninomycin producer *M. africana*.

To date there are mainly three approaches for the preparation of NDP activated deoxysugars: (1) total chemical synthesis through multiple steps to obtain NDP-dexosygars, (2) utilizing the reversible reaction mediated by glycosyltranferase to obtain NDP-dexosygars from sugar containing natural products in the presence of excess NDP (figure 4-06), (3) preparation of NDP-amino sugars from their commercially available substrate through multiple enzymatic reactions (figure 4-07). The problem with applying these approaches to prepare the NDP-amino sugar for the characterization of ORF36 was that it is not trivial to synthesize this structurally unique NDP-amino sugar through either chemical or enzymatic syntheses, although these methods were demonstrated to be feasible for the production of NDP-deoxysugars.
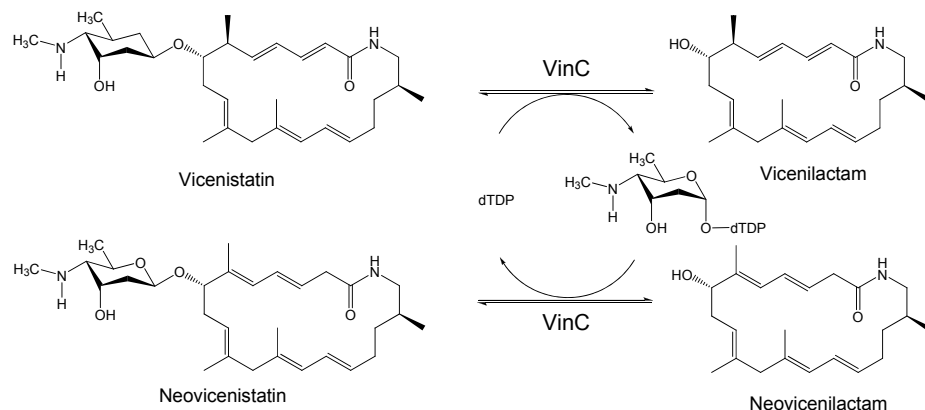

Figure 4-06. Preparation of NDP-deoxysugars by the reversible reactions catalyzed by glycosyltransferases (VinC from *S. halstedii* as an example*).*
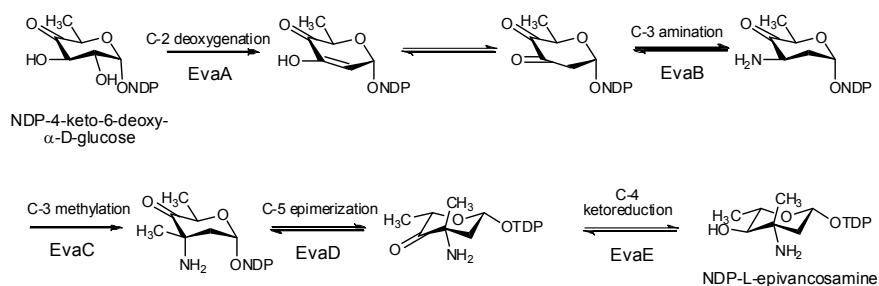
Figure 4-07. Preparation of NDP-deoxysugars using enzymatic reactions (NDP-L-epivancosamine as an example).

Professor Daniel Kahne from Harvard University kindly provided us with a structurally similar TDP-deoxysugar, TDP α,β-*epi*-L-vancosamine azide, from which we were able to prepare the NDP-amino sugar required in the enzymatic reaction for nitrososynthase ORF36. The NDP-amino sugar for ORF36, L-TDP-*epi*-vancosamine, was chemically prepared by the reduction of TDP α,β-*epi*-L-vancosamine azide using tris(2-carboxyethyl)phosphine (TCEP). HPLC-MS analysis showed that 95% of TDP α,β-epi-L-vancosamine azide could be reduced to L-TDP-*epi*-vancosamine for further characterization of ORF36 enzymatic activity. L-TDP-*epi*-vancosamine that we chemically prepared differs from the L-epivancosamine involved in everninomycin biosynthesis in containing an hydroxyl group instead of an OCH_3 group at C4 position (figure 4-08). However, everninomycin producer *M. africana* can also produce a structurally similar natural product, Sch 58773, which contains a nitro sugar with a hydroxyl group at C-4 position[43], indicating that the biosynthesis of nitro group is not affect by the presence of hydroxyl group or OCH3 group at C-4 position (figure 4-09).
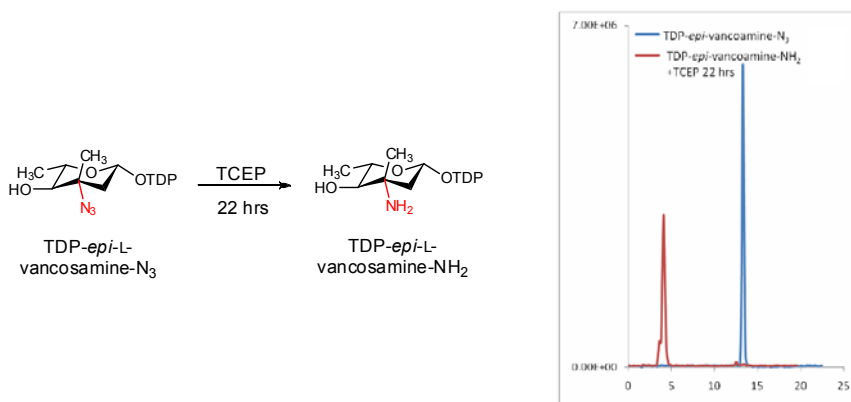
Figure 04-08. Reduction of TDP-*epi*-L-vancosamine-N$_3$ by TCEP to yield TDP-*epi*-L-vancosamine-NH$_2$.

Everninomycin R=CH$_3$
Sch 58773 R=H
Micromospora africana
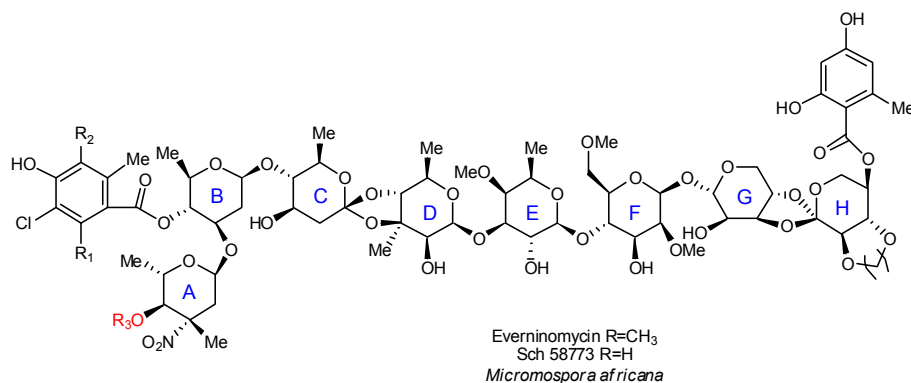
Figure 04-09. Everninomycin and Sch 58773 produced by *Micromonospora africana*.

Functional characterization of nitrososynthase ORF36

ORF36 from the everninomycin biosynthetic gene cluster in *M. africana* was proposed to be a flavin-dependent *N*-oxidase for the oxidation of an amino sugar to a nitroso sugar because of its high sequence similarity with other flavin-dependent oxidases such as DszC which could oxidize a sulfide to a sulfone and

IBAH which was responsible for the oxidation of an aliphatic amine to a aliphatic hydroxylamine. We speculated that ORF36 was also an *N*-oxidase which could oxidize an amino sugar to a nitrososugar and required the existence of reduced flavin for its activity.

To reconstitute the enzymatic activity of ORF36 *in vitro*, a flavin reductase was necessary to provide reduced flavin for ORF36 in the presence of excess NADPH. The *in vitro* reconstitution of nitrososynthase ORF36 required flavin, NADPH and flavin reductase. A commercially available flavin reductase from *Photobacterium fischeri* was directly utilized for flavin-dependent oxidase ORF36. The analysis of the enzymatic reaction catalyzed by ORF36 was carried out using HPLC-MS. HPLC-MS results showed that ORF36 was a nitrososynthase which was able to oxidize L-TDP-*epi*-vancosamine (m/z=544) to its corresponding nitroso product (m/z=560) through a hydroxylamine intermediate (m/z=558) (figure 4-10, 4-11).
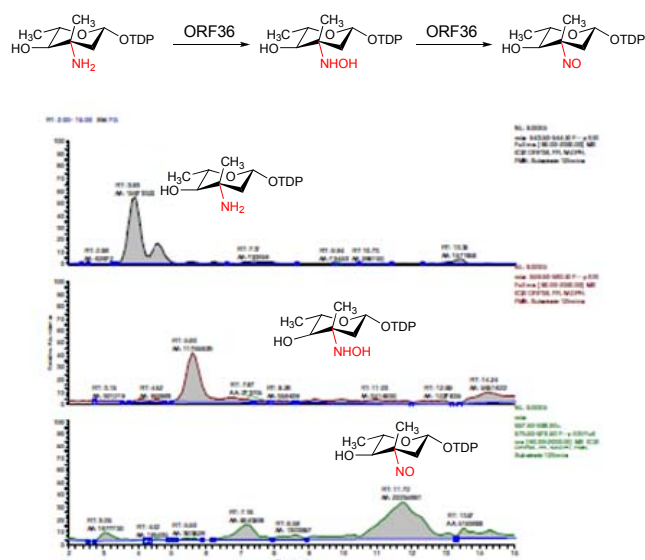
Figure 4-10. Oxidation reaction catalyzed by nitrososynthase ORF36 from everninomycin biosynthetic gene cluster in *M. africana*.

The oxidation reaction from a TDP-aminosugar to a TDP-nitrososugar has been demonstrated to be mediated by ORF36. This oxidation reaction is a flavin-dependent reaction in which a flavin reductase is required to provide reduced flavin for *N*-oxidase ORF36 in the presence of excess NADPH. A TDP-hydroxylaminosugar is proposed to be an intermediate in the ORF36 catalyzed oxidation reaction. The proposed mechanism for flavin-dependent N-oxidase is elucidated in figure 4-12.
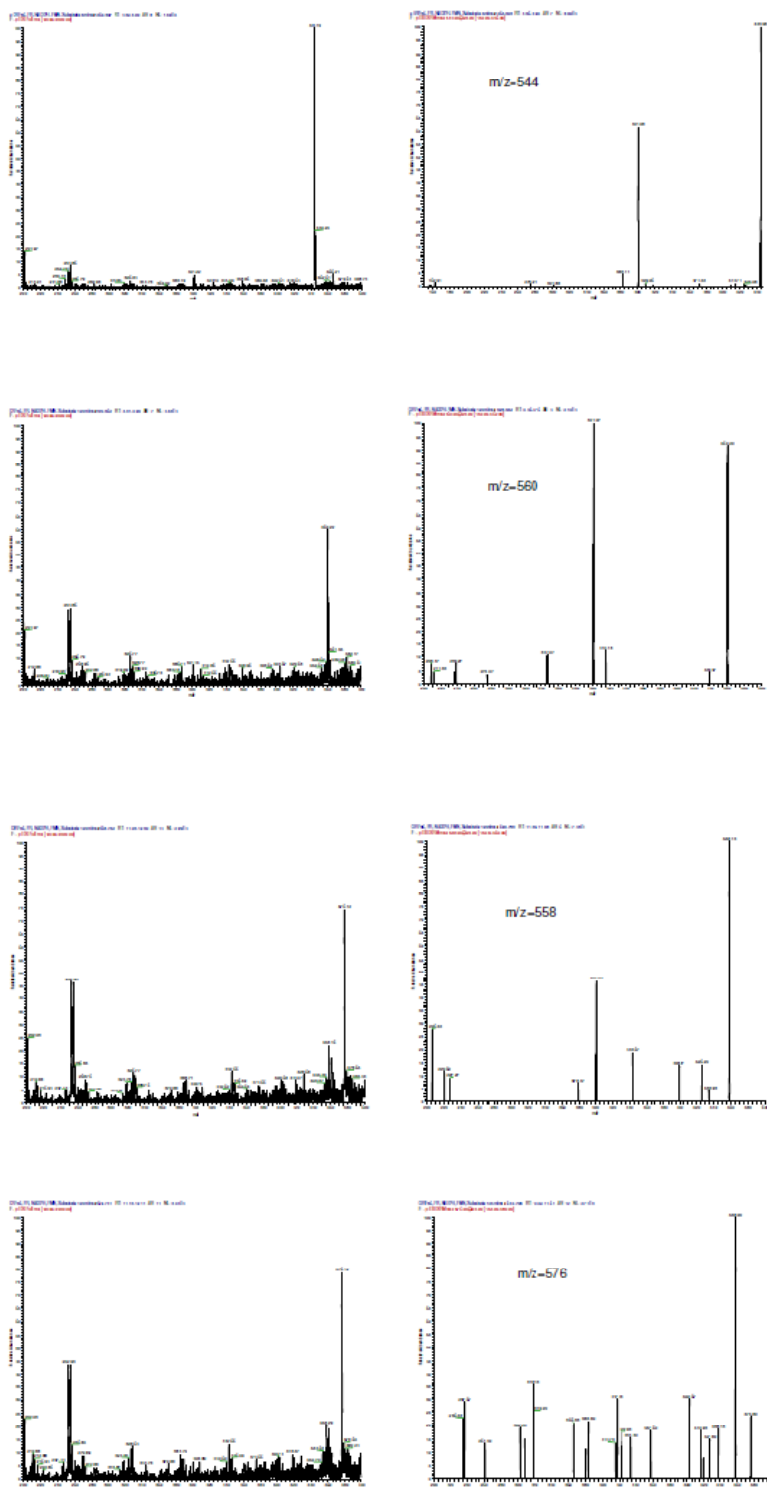
Figure 4-11. MS and Tandem MS of product ions for ORF36 catalyzed reactions.
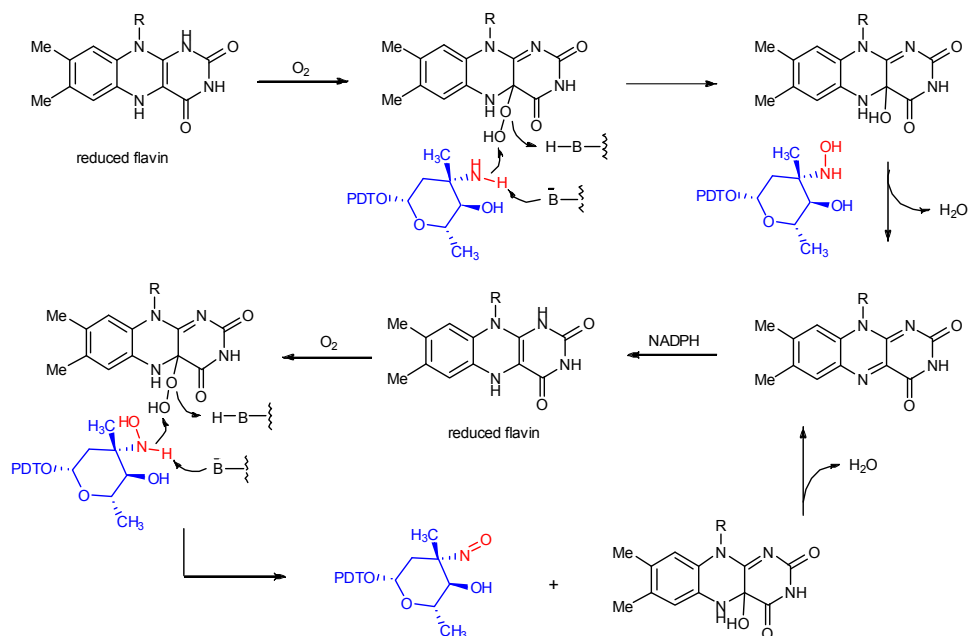
Figure 4-12. Proposed pathway for nitrososynthase ORF36 oxygenation reaction.

The function of ORF36 from the everninomycin biosynthetic gene cluster in *M. africana* has been demonstrated to be a nitrososynthase. In the case of rubradirin, which is another nitrosugar containing natural product produced by *S. rubradirius*, it has been proven that actually protorubradirin containing a nitrososugar is the direct product and oxidized to rubradirin by oxygen and UV light outside the cells via a chemical reaction. In our laboratory, another graduate student Ahmad Al-Mestarihi studied the function of RubN8, which is an ORF36 analog from rubradirin biosynthetic gene cluster. RubN8 was also confirmed to be an *N*-oxidase responsible for the oxidation of a TDP-aminosugar to a TDP-nitrososugar in rubradirin biosynthesis. In the process of studying the biochemical function of RubN8, Ahmad Al-Mestarihi also tried to identify whether

the TDP-nitrososugar could be converted to a TDP-nitrosugar by a chemical reaction. The RubN8 oxidation reaction system which contained the final product, a TDP-nitrososugar, was exposed to strong UV light (350 nm) and oxygen for two hours, but the compounds in this reaction system decomposed and no expected TDP-nitrosugar could be detected, possibly because those TDP-sugars were unstable or the reaction conditions were not suitable for the chemical oxidation from a TDP-nitrososugar to a TDP-nitrosugar. The biosynthetic mechanism for the oxidation from nitrososugar to nitrosugar needs further investigation.

Materials and Methods

Bacterial strains, Plasmids and Materials

All reagents were obtained from Sigma-Aldrich corporation and used without further purification unless otherwise noted. *E. Coli* TOP10 and BL21(DE3) competent cells were obtained from Invitrogen Inc. (Carlsbad, CA) and Novagen (Madison, WI), respectively. Restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs (Ipswich, MA). The pET28a expression vector was purchased from Novagen Inc. Taqplus DNA polymerase was purchased from Stratagene Inc. (La Jolla, CA). DNA primers were obtained from Operon Biotechnologies (Huntsville, AL). Solvents were reagent grade and were further dried when necessary. Analytical thin-layer chromatography was performed on glass plates precoated with silica gel (250 μm, Sorbent Technologies), with detection by UV and/or spraying with H_2SO_4 (50%). Flash chromatography was carried out on silica gel (60 Å, 32-63 μm), purchased from

Sorbent Technologies. Analytical HPLC of synthetic reaction mixtures was performed on a Hewlett-Packard 1100 series instrument using a Phenomenex Luna 5 μm C18 column (250 mm x 4.6 mm). Compounds bearing a thymidine chromophore were monitored at an absorbance of 270 nm. Synthetic reactions were monitored by HPLC using gradient A (a linear gradient from H2O/0.1% NH_4HCO_3 to 100% MeOH/0.1% NH_4HCO_3 over the course of 25 min). Preparative HPLC was performed on a Varian ProStar instrument using a Phenomenex Luna 10 μm C18 column (250 mm x 50 mm). NMR spectra were recorded on Varian Innova 400 or 500 MHz spectrometers. Mass spectra (ESI) for synthetic compounds were obtained using an Agilent Technologies LC/MSD instrument (Model #G1956B).

Preparation of Thymidine 5'-(3-amino-2,3,6-trideoxy-3-C-methyl-α,β-l-arabino-hexopyranosyl diphosphate), TDP α,β-epi-L-vancosamine azide.
The protected TDP *epi*-vancosamine (reference: Oberthur, M.; Leimkuhler, C.; Kahne, D., *Org Lett* **2004,** 6, (17), 2873-76) (38 mg, 60 μmol) was dissolved in MeOH/H2O/Et3N (2:2:1, 4 mL) and stirred at room temperature for 16 h. Following evaporation, the residue was redissolved in MeOH/H2O (2:1, 2 mL) and purified by reversed-phase HPLC. **2.25** (18 mg, 92%) was obtained as its ammonium salt, tR = 9.0 min, method A. 1H NMR (400 MHz, D2O): **2.25**β: δ = 7.62 (s, 1 H, 6-H), 6.24 – 6.21 (m, 1 H, 1'-H), 5.22 – 5.17 (dt, $J_{1,2b}$ = $J_{1,P}$ = 9.0, $J_{1,2a}$ = 2.1 Hz, 1 H, 1"-H), 4.47 (m, 1 H, 3'-H), 4.02 (m, 3 H, 4'-H, 5'-H2), 3.52–3.47 (m, 1 H, 5"-H), 3.16 (d, $J_{4,5}$ = 10 Hz, 1 H, 4"-H), 2.22 - 2.08 (m, 2 H, 2'-H2) 2.07 (d, ,$J_{2a,2b}$ = 12.6 Hz, 1 H, 2"-H$_a$), 1.71 (s, 3 H, thymidine CH3), 1.56-1.51 (dd, J1,2b = 9.0, $J_{2a,2b}$ = 12.6 Hz, 1 H, 2"-H$_b$), 1.25 (s, 3 H, 3"-CH3), 1.13 (d, $J_{5,6}$ = 6.4

Hz, 3 H, 6"-H$_3$); ^{31}P NMR (162 MHz, CD$_3$OD): δ = −10.61, −12.82. LRMS (ESI) for C$_{17}$H$_{27}$N$_5$O$_{13}$P$_2$ (570.12): 571 [M−1H]$^−$.

Cloning and overexpression of ORF36

The gene encoding ORF36 was amplified from genomic DNA of *Micromonospora carbonacea* var. *africana* (NRRL 15099) using the following primers: 5'GCACATATGGCGGCGGATCTTCGCGC3' and 5'TTGAAGCTTTTATTACGCCGAGGTCCGGGAGC 3' (*Nde*I and *Hind*III restriction enzyme sites underlined). PCR reactions were carried out using Taqplus DNA polymerase according to the manufacture's protocol. Subcloning of *orf36* into the *Nde*I/*Hind*III sites of pET28a yielded recombinant plasmid pET28-36N for expression as an *N*-terminal hexahistidine fusion protein with ORF36. Plasmid pET28-36N was transformed into *E. Coli* BL21(DE3) for heterologous expression of ORF36. Cultures of *E. Coli* BL21(DE3)/pET28-36N were grown at 37°C to an OD600 of 0.6, at which point the culture was induced with 0.1 mM isopropyl-beta-Dthiogalactopyranoside (IPTG) and grown an additional 6 hours at 28°C. Cells were harvested by centrifugation and stored at -80°C until needed. IPTG induced *E. Coli* BL21(DE3)/pET28-36N cells were resuspended in buffer A (20mM Imidazole, 0.5 M NaCl, 20mM Tris-HCl, pH 7.5) and lysed by sonication. The lysate was loaded onto a charged 5-ml Histrap crude column (Amersham Biosciences) and purified by FPLC at a flow rate of 5 ml/min. The column was washed with buffer A (20mM Imidazole, 0.5 M NaCl, 20 mM Tris-Cl, pH 7.5) and buffer B (500mM Imidazole, 0.5 M NaCl, 20 mM Tris-Cl, pH 7.5) using a step gradient. Fractions containing ORF36 were analyzed by SDS-page and buffer

was exchanged via a desalting column (HisTrap) using buffer C (20mM Tris-Cl, 1mM dithiothreitol and 5% glycerol, pH 7.5) and stored at -80 °C until assayed.

Preparation of L-TDP-epi-vancosamine

L-TDP-*epi*-vancosamine (4-*O*-desmethyl L-TDPevernosamine) was prepared by reduction of a synthetic azide congener **5** (0.5 mM) with 1 mM tris(2-carboxyethyl)phosphine (TCEP) in 20 mM Tris-HCl (pH 7.5) at 22°C for 24 hours. Amino sugar was stored in small aliquots at -80 °C until immediately prior to assays. HPLC/MS, performed for the reduction of the azide congener as described below for L-TDP-*epi*-vancosamine enzymatic reaction, indicated the extent of reaction was > 96%.

ORF36 enzymatic reactions

Solutions of flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide phosphate (NADPH) were made in 20 mM Tris-HCl pH 7.5 immediately before use. The concentration of substrate L-TDP-*epi*-vancosamine was determined by comparison of absorbance values of stock solutions to a TDP standard curve at 270 nm using a Nanodrop 1000 UV spectrometer (Thermo Fisher Inc). ORF36 were aliquoted in storage buffer [20 mM Tris.HCl, 5% glycerol, 1 mM DTT, pH 7.5], stored at -80 °C and thawed directly before use. Flavin reductase from *Photobacterium fischeri* (Roche Diagnostics GmbH, Mannheim, Germany) was stored in 40% glycerol, 1 mM EDTA, 0.1 mM DTT and 50 mM potassium phosphate, pH 7.0. The catalase and superoxide dismutase enzymes (Sigma Chemicals) were made in [20 mM Tris.HCl 40% glycerol, pH 7.5]. In a total volume of 50 µL, 250 µM of the substrate, L-TDP-*epi*-vancosamine,

was incubated with 30 μM FAD, 1 U/mL catalase, 1 U/mL superoxide dismutase, 0.001 mg/mL flavin reductase, and 0.4 – 2.0 mM NADPH. The reaction was initiated by the addition of ORF36. The time course of the reaction was followed by HPLC/MS at 30°C.

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