Doctoral Dissertation

Characterization of the biosynthetic gene cluster for maklamicin, a spirotetronate-class antibiotic of the endophytic *Micromonospora* sp. NBRC 110955

植物内生放線菌由来スピロテトロン酸系抗生物質マクラマイシン生合成遺伝子群の機能解析

Ratama Daduang

September 2015

Division of Advanced Science and Biotechnology,

Graduate School of Engineering, Osaka University

Contents

Chapter 2 Characterization of the biosynthetic gene cluster for maklamicin, a spirotetronate-class antibiotic of the endophytic *Micromonospora* sp. NBRC 110955

Chapter 3 29-Deoxymaklamicin, a new maklamicin analogue produced by a genetically engineered strain *Micromonospora* sp. NBRC 110955

Chapter 1

General Introduction

1.1. Actinomycetes

Actinomycetes are a large group of phylogenetically related, filamentous, aerobic Gram-positive bacteria that show high GC-content in their genetic materials. The actinomycetes typically have two features. The first is a complex life cycle of morphological differentiation including the germination of spores, the elongation and branching of substrate mycelia, the formation of aerial mycelia, the septation of hyphae, and spore maturation (Chater and Losick 1997). The second feature is that actinomycetes are prolific sources of bioactive compound production. Among the bioactive compounds derived from microbial sources, actinomycetes produce over 10,000 compounds, representing the largest group (45%) of bioactive microbial metabolites (Lazzarini et al. 2001) (Table 1).

Table 1.1. Approximate numbers of bioactive microbial natural products (2002)

Source	Antibiotic	Other bioactive metabolites	Total bioactive metabolites (%)	Practically used
Actinomycetes	8,700	1,400	10,100 (45%)	100–120
Bacteria	2,900	900	3,800 (17%)	10–12
Fungi	4,900	3,700	8,600 (38%)	30–35
Total	16,500	6,000	22,500 (100%)	140–160

Of the known bioactive metabolites that are used directly in human and veterinary medicine and agriculture, most are derived from actinomycetes, and of the approximately 10,000 bioactive compounds produced by actinomycetes, 7,600 (74%) are produced by

Streptomyces. The sources of the other approximately 2,500 (26%) bioactive compounds are called rare actinomycetes or non-*Streptomyces* producers. In the group of rare actinomycetes, *Micromonospora*, *Actinomodura* and *Nocardia* are the most frequent producers; each genus produces several hundred bioactive compounds (Raja and Prabakarana 2011) (Table 1.2).

Table 1.2. Numbers of microbial metabolites produced by actinomycetes

Actinomycetales species	No.
Streptomyces	8,000
Micromonospora	740
Nocardia	357
Actinomadura	345
Streptoverticillium	258
Actinoplanes	248
Saccharopolyspora	131
Amycolatopsis	120
Streptosporangium	79
Mycobacterium	57
Other	665
Total	11,000

Rare actinomycetes produce the most diverse and most unique compounds, displaying excellent antibacterial activities and usually low toxicity. They produce some important compounds such as gentamicins, erythromycins, vancomycin and rifamycin. Among the rare actinomycetes, *Micromonospora* has been recognized as a significant microorganism that produces bioactive compounds second only to *Streptomyces* (Berdy 2005).

1.1.1. *Micromonospora*

The genus *Micromonospora* is Gram-positive, chemo-organotrophic and aerobic and has a high GC content like the rest of the actinomycetes. *Micromonospora* display a complex life cycle, differentiating into both substrate mycelia and spores, but they do not produce aerial mycelia. *Micromonospora* produce branched septate hyphae, and their colonies can be a variety of colors including white, orange, red and brown; sometimes the pigment may diffuse into the medium. When sporulation occurs, the single asexual spores of all *Micromonospora* species have spiny projections, and spores are formed on both short- and long-side branches (Vorbis 1992).

Micromonospora species are widely spread throughout nature, found in environments such as coastal sediments, marine sediment and plant rhizospears. Some species are known to form an intimate association with plants and are called endophytic *Micromonospora* (Hirsch and Valdés 2010).

Micromonospora species are prolific sources of various bioactive metabolites. Over 700 metabolites have been described from this genus (Table 1.2). They are well known for producing antibiotics, especially aminoglycosides, enediynes and oligosaccharides. The aminoglycosides, such as the gentamicins, are produced by *M. purpurea* and *M. echinospora*; sisomicin is produced from *M. inyonensis* and fortimicin from *M. olivoasterospora*. The macrolides compounds include rosamicin from *M. rosaria* and the mycinamicins, isolated from *M. griseorubida*. A family of macrolides closely related to lipiarmycin, the clostomicins, is produced by *M. echinospora*. Rifamycin (of the ansamycins family) is isolated from *M. halophytica*. The polysaccharide everninomycin is a typical product of *M. carbonacea* (Tiwari and Gupta 2012).

Micromonospora species have been reported to have the important roles in soil ecology such as biodegradation, bio-control and plant growth-promoting hormones (Hirsch and Valdés 2010). The versatile abilities of *Micromonospora* indicate that this genus is an important strain

for further study and development.

1.2. Secondary metabolites

"Natural products" are commonly defined as the chemical compounds isolated from various living things. These compounds may be derived from the primary or secondary metabolism of living organisms. The primary metabolites are normally found in all organisms. The secondary metabolites derived from microorganism and fungi are mostly low-molecular-weight (< 3,000 kDa) and comprise widely differing chemical compounds with unclear functions. The secondary metabolites studied to date display a variety of functions; some present as antibiotics with antimicrobial, antitumor and antiviral activities, and some exhibit activity as a growth regulator, modifying the replication and inhibition of the life cycle of prokaryotic or eukaryotic cells at the biochemical level (Berdy 2005).

Secondary metabolites are categorized into five main groups based on their core component, which provides the main structure of the compound (Turner and Aldridge 1984).

1. Carbohydrate from saccharide
2. Isoprenoid (or terpenoid) from isopentenyl diphosphate (IPP)
3. Aromatic amino acid from shikimic acid
4. Nonribosomal peptide from amino acid
5. Polyketide from malonyl coenzyme A

1.2.1. Carbohydrates

Carbohydrates are the most common components of plants, animals and microorganisms. The compounds in the carbohydrate family have a variety of functions. For example, the polysaccharide compound glycogen serves as a mammalian carbohydrate storage molecule (Paul 2009), and chitosan is an important industrial material used for water purification (Kumar et al. 2004).

Aminoglycosides form a valuable group of antibiotics and are notably recognizable as modified carbohydrate molecules. They have two or three uncommon sugars, mainly aminosugars, with glycoside linkages tethered to an aminocyclitol such as an amino-substitute cyclohexene system, which also has a carbohydrate origin (Magnet and Blanchard 2005). An example of an aminoglycoside is gentamicin (Fig. 1.1), which is the clinically most important of the aminoglycoside antibiotics, broadly used for the treatment of serious infections. Gentamicin has broad-spectrum activity against pathogenic enterobacteria (Unwin et al. 2004).

A second example is neomycin (Fig. 1.1), which shows activity against Gram-positive and Gram-negative bacteria. It is used for the oral treatment of intestinal infections and has applications in eye drops, ear drops and ointments (Huang et al. 2005).

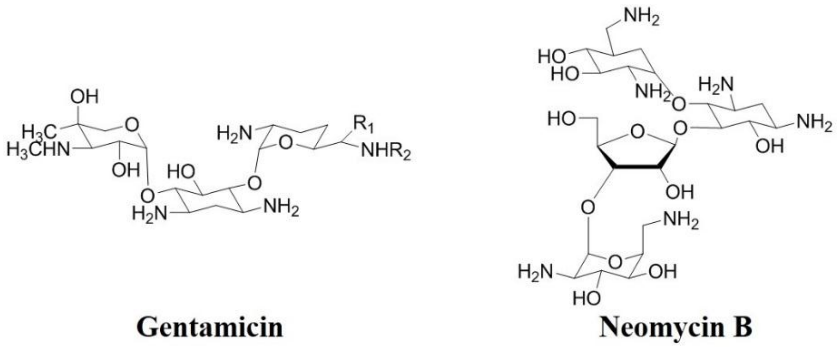

Fig. 1.1. The aminoglycoside antibiotics gentamicin and neomycin B.

1.2.2. Isoprenoids

Isoprenoids form a large and structurally diverse family of natural products derived from a C_5 isoprene unit joined in a head-to-tail fashion (Sacchettini and Poulter 1997). Isoprenoids and their derivatives play key roles in all aspects of life such as in reproductive hormones (steroids, gibberellins and abscisic acid), as components of the cell membrane (steroids), in respiration (quinones), as photosynthetic light-harvesting compounds (carotenoids), and in protein targeting and regulation (prenylation). In addition, particularly in plants, isoprenoids play diverse roles other than maintenance of the life cycle, including roles in protection,

communication, pollination attraction and plant dispersion and as a constituent of essential oils such as β-demascenone in rose, limonene in citrus and zingiberene in ginger (Chan and Keasling 2006). Typical examples of isoprenoids are shown in Figure 1.2.

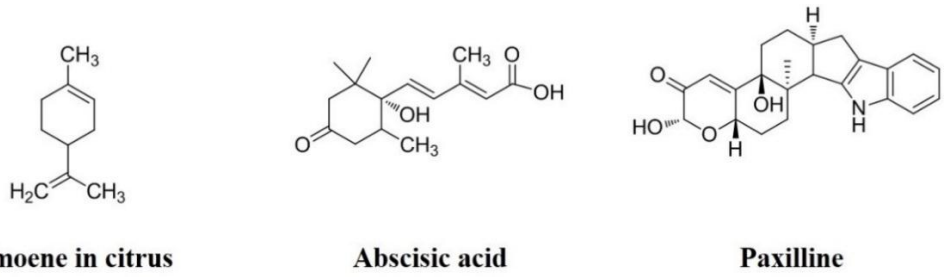

Fig. 1.2. Isoprenoids in nature: limoene, abscisic acid, and paxilline.

1.2.3. Aromatic amino acids

The aromatic amino acids such as L-phenylalanine, L-tyrosine and L-tryptophan are typically synthesized via the shikimate pathway (Paul 2009). A central intermediate in the pathway is shikimic acid (Fig 1.3), a compound isolated from plants. Shikimic acid is currently used as the raw material for the synthesis of the antiviral drug osetamivir (Tamiflu®) against avian influenza (Farina and Brown 2006). Phenylalanine and tyrosine are found in many natural products such as cinnamic acid, coumarins and flavonoids, along with the tryptophan precursor of a wide range of alkaloid structures (Knaggs 2003). The compounds in this group, such as chloramphenicol, display a broad spectrum of antibiotic activity and are synthesized via the shikimate pathway (He et al. 2001) as shown in Figure 1.3.

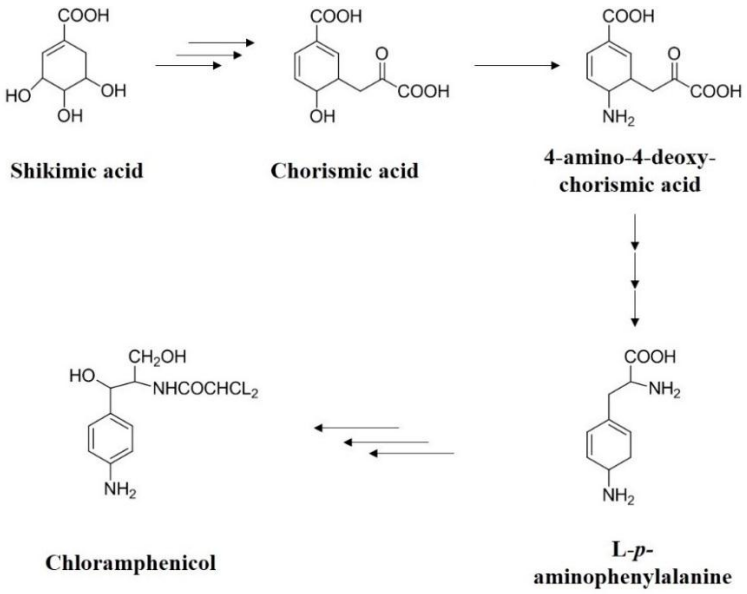

Fig. 1.3. Chloramphenicol synthesis via the shikimate pathway

1.2.4. Nonribosomal peptides

Nonribosomal peptides are a class of peptide secondary metabolites with pharmacological importance. They have a wide range of uses including applications as antibiotics, immunosuppressive agents, cytostatic agents and siderophores (Sieber and Marahiel 2005). The structures of some nonribosomal peptide compounds are shown in Figure 1.4.

Nonribosomal peptides contain a number of structural features that clearly separate them from ribosomal peptide compounds. For example, they contain non-proteinogenic amino acids such as ornithine in gramicidins, and hydroxyphenyl or dihydroxyphenyl-glycine in vancomycin (Schwarzer et al. 2003).

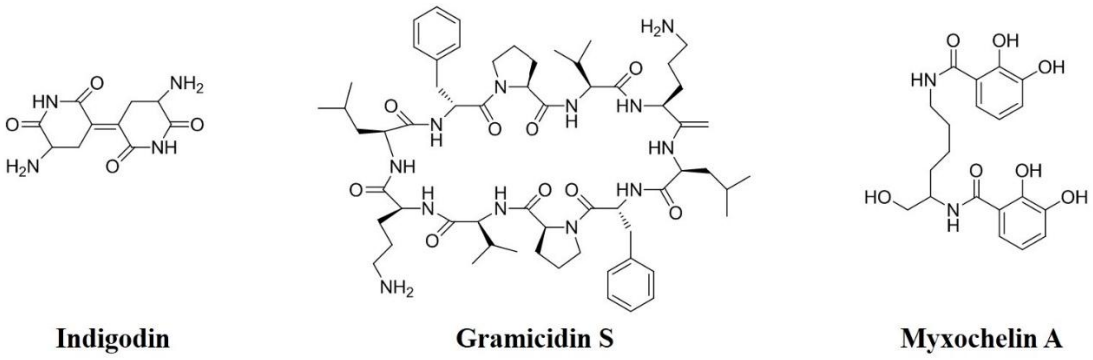

Fig. 1.4. Examples of nonribosomal peptide compounds.

Nonribosomal peptide compounds are synthesized through nonribosomal peptide synthetases (NRPSs). NRPSs are modularly organized multi-enzyme complexes, which display the extraordinary size and show the same time template and biosynthetic machinery (Schwarzer et al. 2003).

1.2.5. Polyketides

Polyketides are natural compounds containing alternating carbonyl and methylene group (β-polyketones)

Polyketides are an important group of secondary metabolites, display an incredible variety of functional and structural diversity with important activities including antimicrobial, anticancer, antifungal, antiparasitic and immunosuppressive activities (Staunton and Weissman 2001). Examples of polyketide compounds are shown in Figure 1.5.

Polyketides are synthesized through sequential reactions catalyzed by polyketide synthases (PKSs), which exert a series of enzymatic activities.

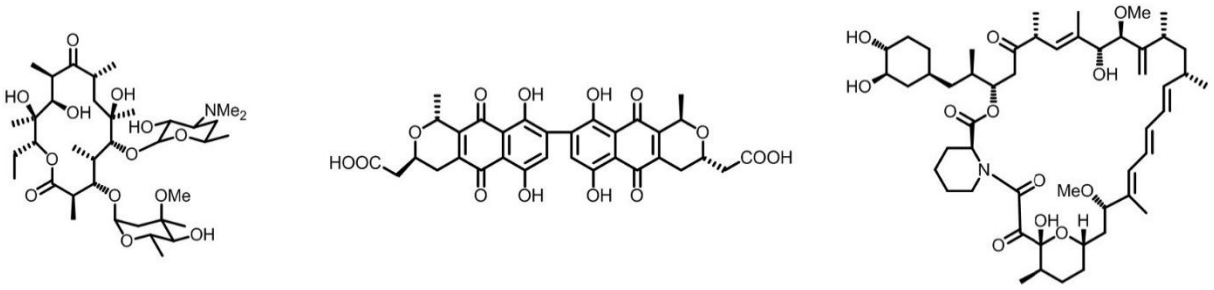

Fig. 1.5. Examples of polyketide compounds: erythromycin A, actinorhodin, and rapamycin.

1.2.5.1. Polyketide synthases

The process of synthesizing polyketide synthases (PKSs) is generally similar to fatty acid biosynthesis, in which the growing chain is constructed by a Claisen reaction and catalyzed by ketosynthase activity. In fatty acid biosynthesis, the reaction initially produces a β-ketoester,

and the ketone group is reduced after a condensation step and before the next round of chain extension. The overall reduction is achieved by a three-stage process: reduction to an alcohol by ketoreductase (KR), dehydration to the conjugated ester by dehydratase (DH), and reduction of the double bond by enoyl-reductase (ER) (Staunton and Weissman 2001). These reactions are illustrated in Figure 1.6.

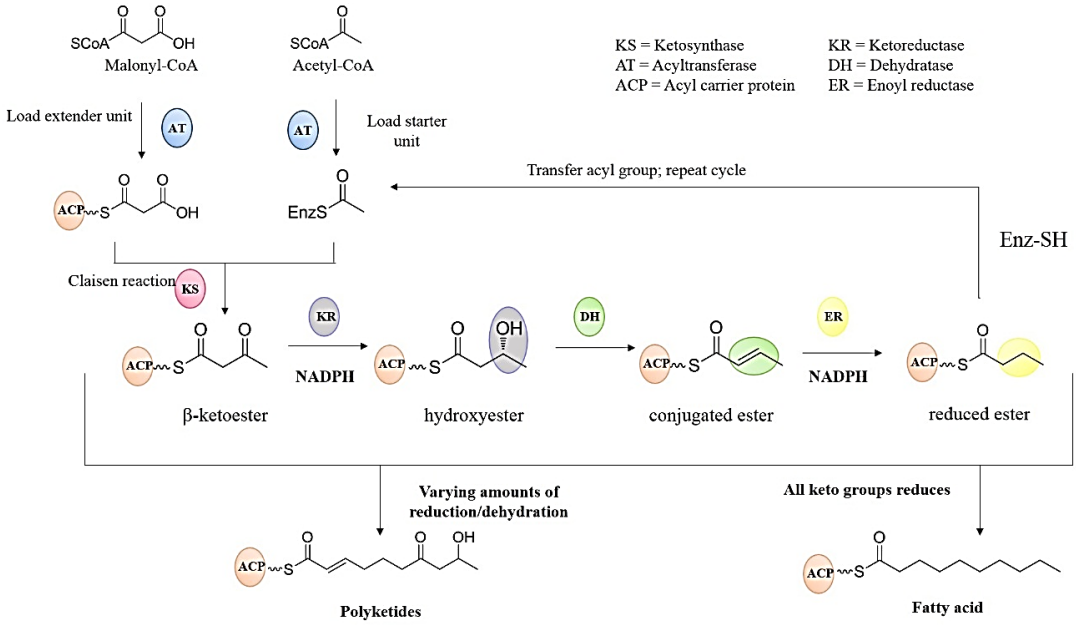

Fig. 1.6. Mechanisms underlying the biosynthesis of polyketide synthases.

In fatty acid biosynthesis, all keto groups will be reduced by the three-step process of reduction and dehydration as mentioned above. However, the biosynthesis of PKSs differs from fatty acid biosynthesis because of varying degrees of reduction or dehydration. The polyketide assembly line may contain a mixture of hydroxyl groups, carbonyl groups, double bonds and methylene groups. This means that the enzyme activities KR, DH and ER are not active during the specific extension cycles. The order in which the modifications occur or do not occur is closely controlled by the enzyme activities (Paul 2009). Finally, the condensation cycles continue until reach the proper chain length, then the action of thioesterase (TE) domain will take place and release the acyl chain from the ACP to yield premature polyketide compound

(Moss et al. 2004).

PKSs are classified into three groups according to their sequences, primary structure and catalytic mechanisms (Shen 2003), as follows.

(1) Type I PKSs: Very large multifunctional proteins with individual functional domains. Type I PKSs can either be modular or iteratively modular, usually when present in a bacterial or fungal system, respectively.

(2) Type II PKSs: Composed of a complex of individual monofunctional proteins; usually referring to the aromatic polyketide compounds.

(3) Type III PKSs: Homodimeric enzymes that essentially are iteratively condensing enzymes, type III PKSs are sometimes described as being involved in chalcone biosynthesis.

This dissertation will focus on modular type I PKSs.

1.2.5.2. Modular type I PKSs

Modular type I PKSs are large multifunctional polypeptides that are responsible for the biosynthesis of complex macrolides such as erythromycin and rapamycin. For typical modular PKSs, each module contains the essential domains ketosynthase (KS), acyltransferase (AT) and acyl carrier protein (ACP) for polyketide chain extension and the optional reductive domains ketoreductase (KR), dehydratase (DH) and enoyl reductase (ER) for reductively process β-keto ester. (Tian et al. 2015)

In modular type I PKSs, each successive chain extension cycle is catalyzed by various sets of domains located in different modules; one or more modules may be present on one multifunctional polypeptide, and each domain is used only once (Moss et al. 2004). An example is the biosynthesis of 6-deoxyerythronolide B (6-dEB), in which PKSs catalyze the decarboxylation condensation of six methyl malonyl-CoA extender units with a propionate starter unit derived from propionyl CoA. The 6-dEB synthase (DEBS) consists of three

multifunctional proteins (DEBS1, DEBS2 and DEBS3), and each gene harbors two extension modules. This linear mechanism appears to be common among modular PKSs in bacterial systems (Fig 1.7).

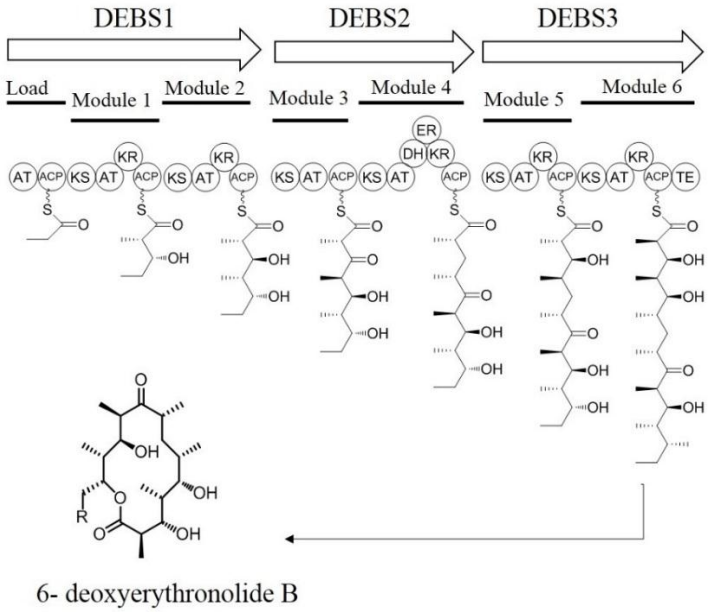

Fig. 1.7. The co-linear and nature of modular type I PKSs: The biosynthesis of 6-dEB by DEBS1, DEBS2 and DEBS3.

Modular type I PKS systems have been acknowledged as a potential system to be engineered and to generate novel analogues of known metabolites. Combinatorial biosynthesis techniques such as gene deletion or the gene modification of post-PKS tailoring in modular Type I PKSs are among the alternative ways to create new derivatives of polyketide compounds.

1.2.5.3. Post-PKSs tailoring step

After a PKS has been synthesized, post-PKS modification is an essential step to modify the polyketide framework. A modification such as the introduction of a hydroxy group or methyl group or glycosyl transfer can particularly change the therapeutic index of the parent polyketides, or alter some impact factors such as physio-properties, changing the solubility or the receptor binding ability of the modified parent compound (Rix et al. 2002). Figure 1.8

describes the post-PKS modification of erythromycin A.

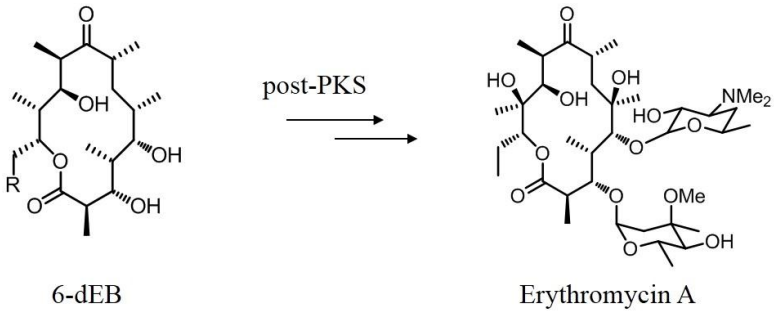

Fig. 1.8. Post-PKS modification of erythromycin A.

The post-PKS modification enzymes have play the important roles in natural products biosynthesis, among those enzymes, the cytochrome P450 monooxygenases (CYP450s) are well-known to be involved in regiospecific and stereospecific oxidation of polyketide compounds (Lamb et al. 2013). The studies on the modification of CYP450s have been attracted great attention for generation of novel polyketide derivatives.

For example, the study on *fscP* gene encoding CYP450 monooxygenase of candicidin biosynthesis by gene disruption on *Streptomyces* sp. strain FR-008 showed that Δ*fscP* mutant strain produced the new derivative of candicidin, decarboxylated candicidin (Fig. 1.9). However, the new derivative displayed lower antifungal activity than in candicidin (Chen et al. 2009).

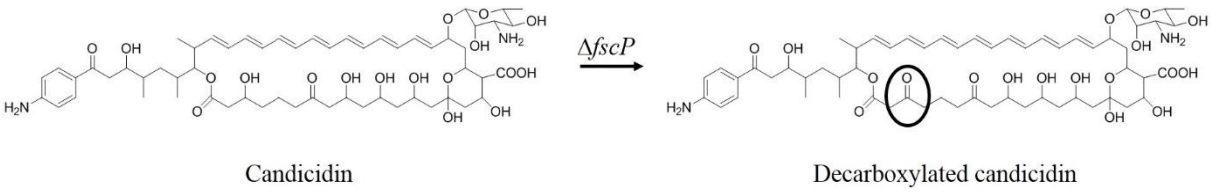

Fig. 1.9. The disruption of *fscP* gene in candicidin biosynthesis

1.2.5.6. Polyketide-class compounds

Several groups of compounds are categorized as polyketide-class compounds because their main structure is synthesized via a PKS pathway. The groups of compounds including macrolide antibiotics, polyene antibiotics, ansamycin antibiotics and the recently growing spirotetronate compound family are explained as follows.

Macrolide antibiotics are macrocyclic lactones typically composed of 12–16 ring-sized atoms, with some branching through methyl substituents. Two or more sugar units are attached through glycoside linkage, such as L-mycarose, D-mycinose and L-oleandrose. At least one sugar is an amino sugar; for example, D-desosamine, D-forosamine and D-mycaminose. The compounds in this group have a narrow spectrum of antibacterial activity especially against Gram-positive bacteria (Pal 2006). Examples of this group are erythromycin A (Fig. 1.5) and tylosin (Fig. 1.9).

Polyene antifungals are a group of macrocyclic lactones with very large (26–38)-member rings. They are categorized by the existence of a series of conjugated *E*-double bonds and are classified according to the presence of the longest conjugated chain. The polyene antifungals have no antibacterial activity, but they are promising as antifungal agents (Aparicio et al. 2004). The polyene compounds amphotericin B and nystatin are shown in Figure 1.9.

Ansamycin antibiotics are macrolides that have non-adjacent positions on an aromatic ring bridged by a long aliphatic chain. The aromatic portion may be replaced by naphlaene or naphthaquinone, or a benzene ring. The macrocycle in the ansamycins is closed by an amide rather than ester linkage. An example of this group is rifamycin (Fig 1.9), which is the only ansamycin currently used clinically (Floss and Yu 2005).

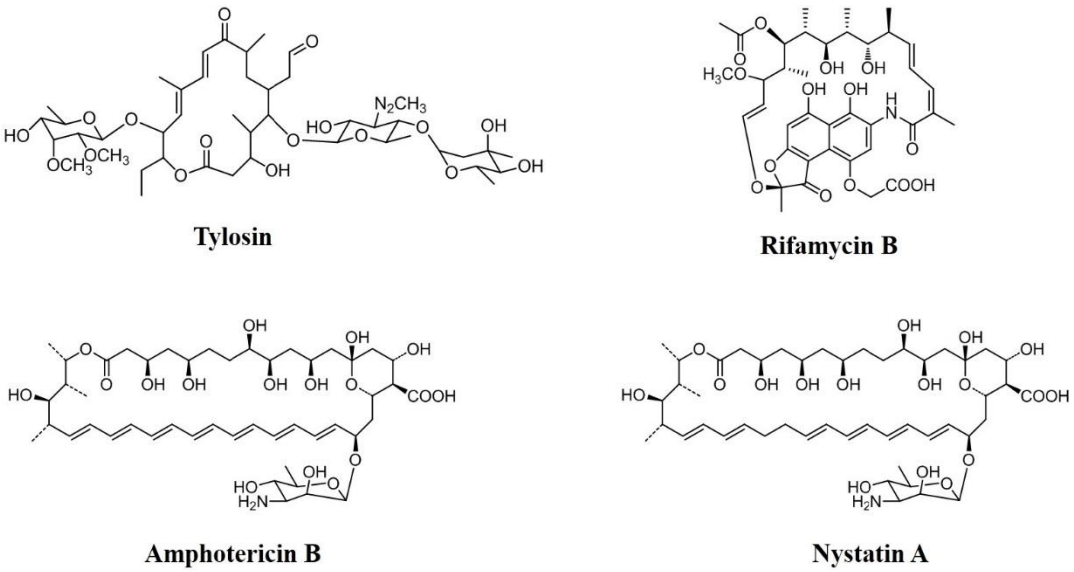

Fig. 1.9. Polyketide-class compounds.

1.3. Tetronate compounds

Tetronate compounds are a group of natural products that show great structural and functional diversity. The structure of this group consists of a linear fatty acid or polyketide backbone, tethered with a tetronic acid ring system (4-hydroxy-[*5H*] furan-2-one, Fig. 1.10). Several tetronate compounds have been reported, and the compounds can be classified according to distinct structural features such as linearity or the macrocyclization of the carbon backbone and the size of the central ring system (Vieweg et al. 2014). There are two types of tetronate compounds: linear tetronates and spirotetronates.

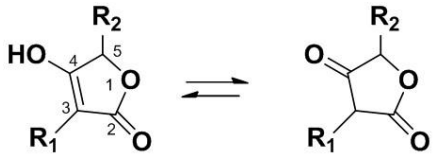

Fig. 1.10. Tetronic acid tautomer.

1.3.1. Linear tetronates

The linear tetronate compounds are characterized by the lack of macrocyclization of a linear carbon backbone attached to a tetronic acid moiety, although the occurrence of small-

and medium-sized rings within the molecule is accepted. Figure 1.11 shows examples of linear tetronates.

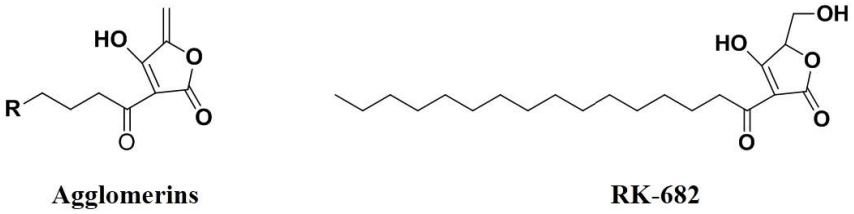

Fig. 1.11. The linear tetronates agglomerins and RK-682.

Agglomerin A and its derivatives B–D were isolated from *Pantoea agglomerans* PB-6024 (Shoji et al. 1989). The agglomerins consist of α-acyl-chains that are derived from primary fatty acid metabolism and a tetronic acid bearing an exocyclic double bond (Kanchanabanca et al. 2013). These compounds show activity against a range of anaerobic bacteria. Another linear α-acyl-chain tetronate compound is RK-682, which was isolated from *Streptomyces* sp. 88-682. This compound consists of a fatty acid tethered to a tetronic ring, which is decorated with a methyl-enehydroxy moiety instead of the exocyclic double bond that is present in agglomerins. RK-682 displays various activities and has been described as an inhibitor of the tyrosine phosphatases HIV-I protease and heparanase (Hamaguchi et al. 1995).

1.3.2. Spirotetronates

Spirotetronates are tetronate compounds in which tetronic acid *spiro*-links with cyclohexene ring sometimes conjugated with *trans*-decalin unit (Fig. 1.12). Several reports about spirotetronates support the hypothesis that all spirotetronate frameworks are synthesized according to a general mechanism involving polyketide assembly lines. It was suggested that the formation of a spirotetronate requires a conjugated pair of carbon-carbon double bonds in the tail region of the linear polyketide precursor plus a characteristic exocyclic double bond on the tetronic ring system. Spirotetronate compounds can be classified based on the size of the

central ring system and also the molecular weight, which is influenced mainly by the degree of glycosylation (Vieweg et al. 2014). Spirotetronates can be divided into three classes: small, medium and large spirotetronates.

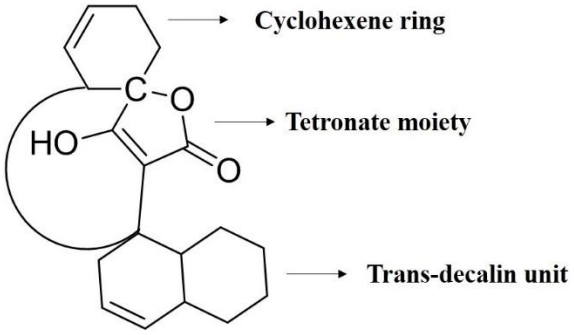

Fig. 1.12. The common structure of spirotetronates

1.3.2.1. Small spirotetronates

The small spirotetronates consist of macrocyclized tetronates with a polyketide backbone; they are low-molecular-weight and their central ring systems consist of 11 carbon atoms (Fig. 1.13). An example of a small spirotetronate is abyssomicin (Fig. 1.13). This compound has various derivatives that show anti-Gram-positive bacteria and anti-tuberculosis activities (Riedlingera et al. 2004).

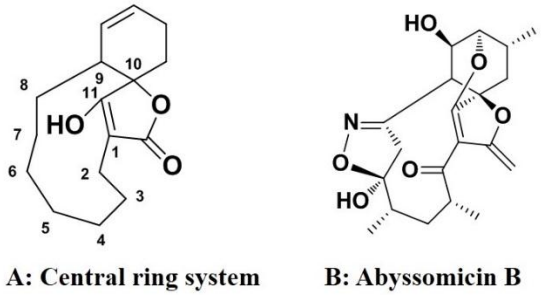

Fig. 1.13. A: central-ring system = C_{11}. B: Abyssomicin B, a small spirotetronate.

1.3.2.2. Medium spirotetronates

The family of medium-sized spirotetronates represents the largest group of tetronates. The compounds in this group are comprised of a polyketide scaffold with six carbon linkers

connecting a cyclohexene ring and a decalin unit, constructing a 13-membered ring. The scaffold was found to be a polyketide-derived carbon backbone. The spirotetronate moiety and the *trans*-decalin unit are thought to arise from a Diels-Alder reaction taking place after the biosynthesis of the linear precursor molecule. The compounds in this medium-sized family display a variety of glycosylation patterns, given the large number of derivatives (Vieweg et al. 2014). The best-studied spirotetronates in this family are chlorothricin, kijanimicin and tetrocarcin A (Fig. 1.14).

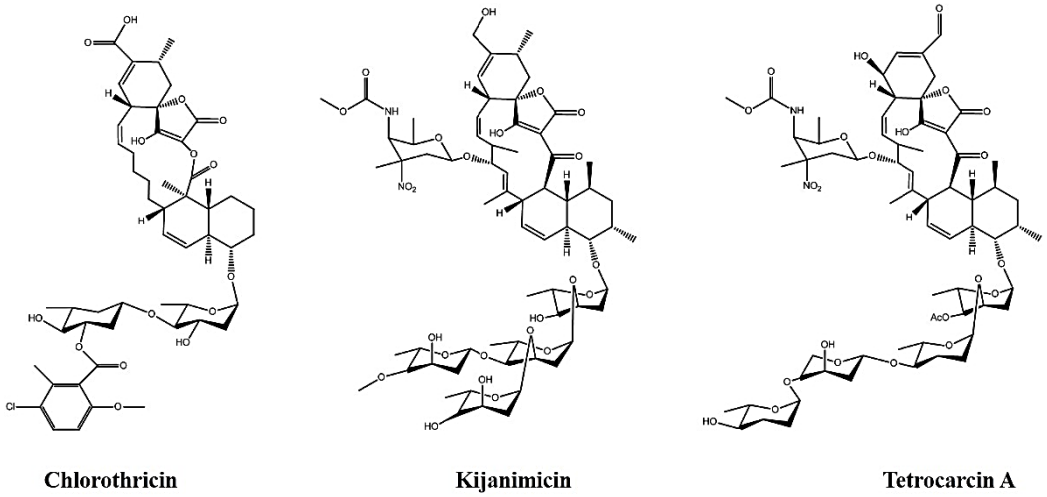

Fig. 1.14. The medium-sized spirotetronates chlorothricin,kijanimicin and tetrocarcin A.

Chlorothricin was isolated from *Streptomyces antibioticus* DSM 40725 and shows anti-Gram-positive and cholesterol biosynthesis inhibitor activities (Kawashima et al. 1991). The biosynthesis of chlorothricin has been elucidated since 2006, when it was confirmed that the assembly machinery involves a large polyketide synthase complex (Jia et al. 2006).

Kijanimicin was isolated from *Actinomadura kijaniata* SCC 1256 in 1981. It shows antibacterial activity, notably activity against *Propionibacterium acne* and *Bacillus subtilis*. This compound has an unusual nitrosugar called D-kijanose attached at the C-8 of the core structure and an L-digitose moiety attached at the decalin unit (Waitz et al. 1981). The biosynthetic gene cluster of this compound was isolated by Zhang *et al* and confirmed the

polyketide synthases are involved in construction of kijanimicin aglycone (Zhang et al. 2007).

A third medium-sized spirotetronate, tetrocarcin A, was isolated from a culture of *Micromonospora chalcea* NRRL 11289. It displays antitumor activity. Tetrocarcin A is closely related to kijanimicin, but the decalin unit of tetrocarcin A includes a tetrasaccharide comprised of L-digitose and L-amitose (Tomita et al. 1980). The biosynthetic gene cluster of tetrocarcin A reveals that the gene cluster responsible for the production of tetrocarcin A is mainly from polyketide synthases such as chlorothricin and kijanimicin (Fang et al. 2008).

1.3.2.3. Large spirotetronates

This group contains various large-sized spirotetronates in which the central-ring system is larger than the 13-membered ring. Quatromicins and versipelostatin are examples of this group (Fig. 1.15).

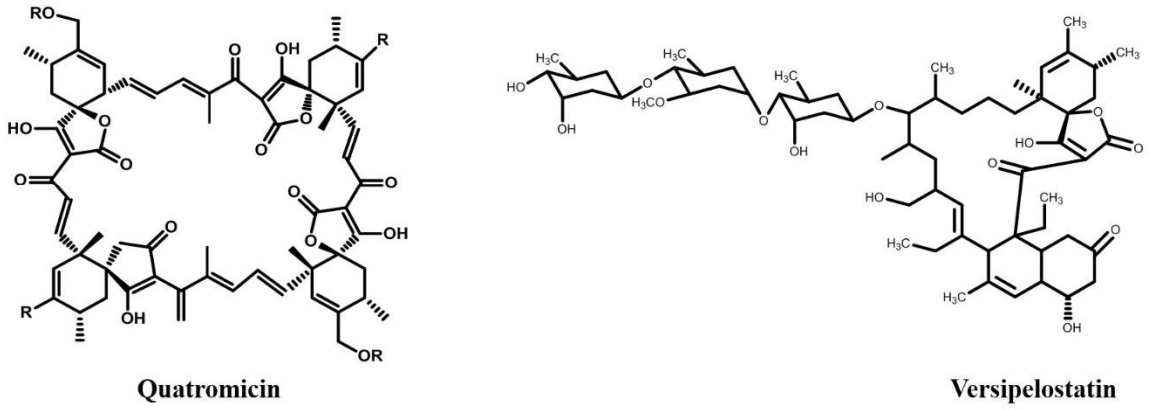

Fig. 1.15. The large spirotetronates quatromicin and versipelostatin.

Quatromicins were isolated from *Amycolatopsis orientalis*, and they display a unique structure harboring four spirotetronate moieties within one molecule. Quatromicins exhibit antiviral activity against herpes simplex virus (Kusumi et al. 1991).

With a 17-member ring, versipelostatin is the largest spirotetronate. It was first isolated from *Streptomyces versipellis* 4083-SVS6. It was found that versipelostatin downregurates of a

chaperon modulator (GRP78 chaperon protein) located in the endoplasmic reticulum (Park et al. 2002).

To date, more than 60 tetronates and spirotetronates have been identified, and they have been recognized as having potential antibiotic, anticancer and antiviral applications.

1.3.3. Maklamicin

Maklamicin, isolated from the endophytic *Micromonospora* sp. NBRC 110955, displays strong to moderate antibacterial activity against Gram-positive bacteria and also cancer cell cytotoxicity (Igarashi et al. 2011) (Fig. 1.16). Maklamicin belongs to the small-sized spirotetronate family, which shows two structural features that are distinct from those of other spirotetronates as described below.

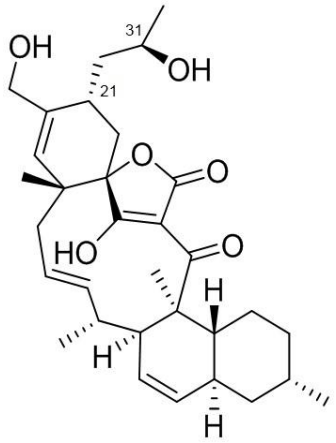

Fig. 1.16. The structure of maklamicin

1. The carbon chain linker of maklamicin is shortest in the spirotetronate family.

Maklamicin has a four-carbon chain connection between its trans-decalin unit and cyclohexene ring, forming an 11-member ring, whereas the carbon chain length between the two ring systems in other spirotetronates such as chlorothricin and versipelostatin is 6 or 10, respectively, constructing a 13- or 17- member ring.

2. The stereogenic center at C-31 at the substituent on the cyclohexene ring has never been reported.

Maklamicin contains a 2R-hydroxy-propyl group at C-21 as a substituent on the cyclohexene ring, creating a stereogenic center at C-31, whereas other spirotetronates have an achiral center substituent at the same position. Chlorothricin, compound AC6H, decatromicins and saccharocarcins have functional groups as a methyl, formyl ethyl and *n*-propyl groups, respectively (Fig. 1.17).

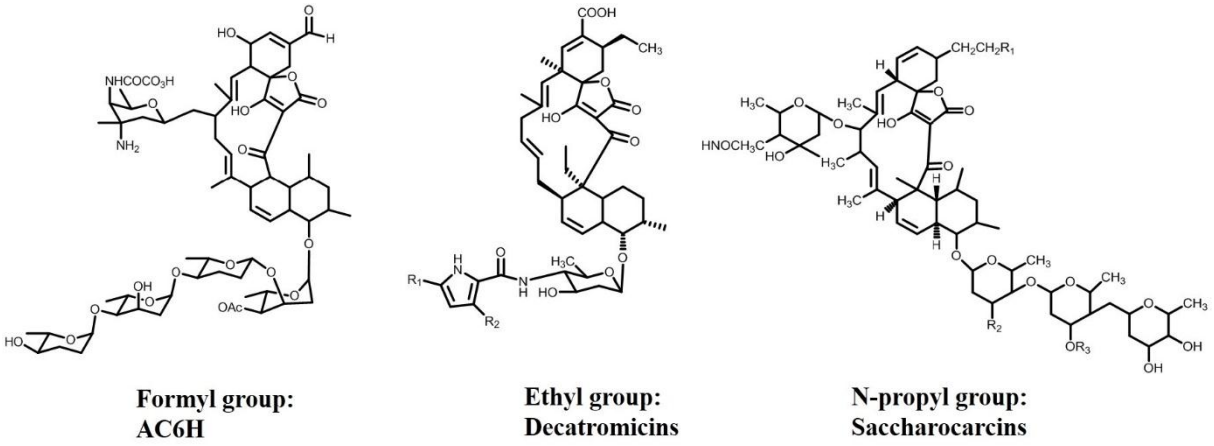

Fig. 1.17. Spirotetronates with an achiral substituent on the cyclohexene ring.

1.3.4. The biosynthesis of tetronates and spirotetronates

Several studies of the biosynthesis of tetronates and spirotetronates have been reported. The elucidation of chlorothricin, kijanimicin and tetrocarcin A biosynthetic gene clusters (Fang et al. 2008; Jia et al. 2006; Zhang et al. 2007) revealed that the main spirotetronate scaffold is synthesized via linear type I PKSs. Two Diels-Alder reactions were postulated to build up the cyclohexene ring attached to the tetronate moiety and *trans*-decalin system. A study of kijanimicin biosynthesis revealed a set of five genes called glycerate utilization operons that are suspected to be responsible for the incorporation of the C_3-unit from the glycolytic pathway for the formation of a tetronic acid moiety. However, there is no clear evidence to confirm the

function of the glycerate utilization operons.

The biosynthesis of the tetronate compound agglomerin, which harbors a tetronate moiety with an exocyclic double bond, was elucidated in 2013 (Kanchanabanca et al. 2013). It was the first biosynthesis model that clearly provided a reasonable explanation of the formation of a tetronic acid moiety. The function of glycerate utilization operons has been clarified, indicating that this gene cassette is highly conserved in the biosynthesis of all tetronates and spirotetronates.

1.3.4.1. Diels-Alder reactions

The Diels-Alder reaction is a main reaction in organic synthesis for the formation of carbocycles through [4+2]-cycloaddition reaction in which a diene reacts with a dienophile to yield a cyclohexene ring in a concerted manner (Fig. 1.18)(Kasahara et al. 2010). The Diels-Alder reaction is very useful in synthetic chemistry because of it has high region-stereoselectivity. Moreover, various kind of natural products biosyntheses such as polyketides, terpenoid or alkaloid have been suggested that Diels-Alder reactions are involved in their biosynthesis (Oikawa and Tokiwano 2004).

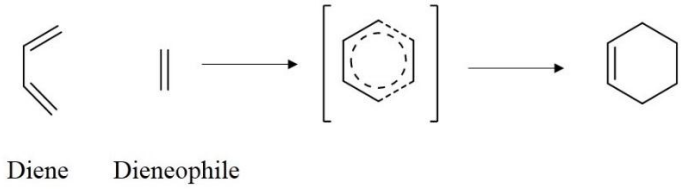

Fig. 1.18. The Diels-Alder reaction.

Although the Diels-Alder reactions have been reported to be involved in the biosynthesis of several natural products but the reports about the genes that encoding Diels-Alderase are insufficient. The first Diel-Alderase gene, solanapyrone synthase (SPS) was reported in 1995 on solanapyrone biosynthesis. Next, three additional Diels-Alderase, lovastatin nonakide synthase (LovB), macrophomate synthase (MPS) and spinosyn A (SpnF) biosynthesis were

reported and characterized their function (Oikawa and Tokiwano 2004).

The general step of [4+2] cycloaddition reaction involved in the biosynthesis pathway of four natural products was showed in figure 1.19

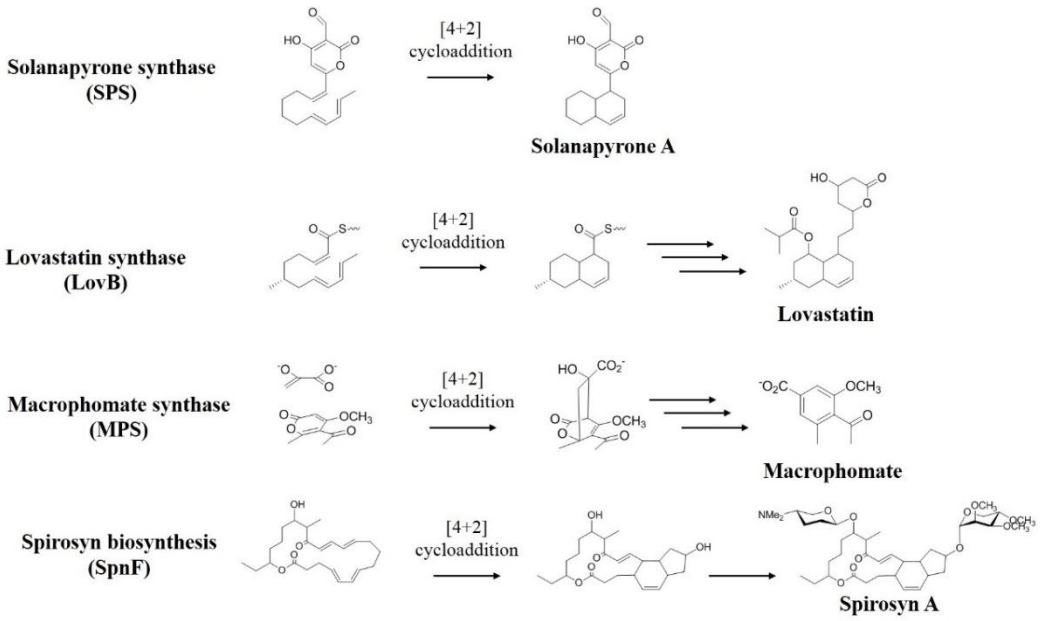

Fig. 1.19. Known [4+2] cycloaddition reactions

Since the first report of chlorothricin biosynthesis on 2006, several spirotetronates biosynthesis have been elucidated but no enzyme has been shown to facilitate Diels-Alder reactions (Vieweg et al. 2014). Until January 2015, Hashimoto and his colleagues have successfully identified and characterized one gene, *vstJ*, which was demonstrated to be involved in the [4+2]-cycloaddition reaction of the formation of cyclohexene ring on versipelostatin biosynthesis. (Hashimoto et al. 2015). Moreover, on March 2015, Tian and his group also reported about the genes involved in Diels-Alder reactions on pyrroindomycins, the spirotetramate antibiotic which its structure related to spirotetronates. In this study revealed that two genes, *pyrE3* and *pyrI4*, homologues of c*hlE3* and *chlL* in chlorothricin biosynthesis are involved in the formation of *trans*-decalin unit and cyclohexene ring, respectively (Fig. 1.20). The sequence of *pyrE3* and *pyrI4* does not share the sequence homology to each other (Tian et al. 2015).

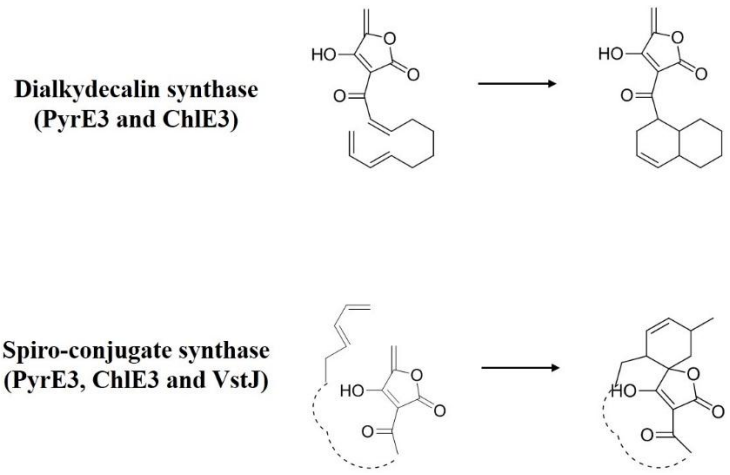

Fig. 1.20. The Diels-Alderase involved in decalin unit formation and cyclohexene ring formation of spirotetronate compounds

In general, the Diels- Alder reaction have been frequently used in the chemical synthesis of spirotetronate scaffold to assembly both decalin unit and cyclohexene ring system. Therefore, in the biosynthetic pathway, the decalin unit formation would undertake by an *endo*-selective Diels-Alder reaction whereas the cyclohexene ring formation would follow an *exo*-transition state. Moreover, all known type Diels-Alderase are phylogenetically difference, although the products share similar 6,6-bicyclic moiety (Tian et al. 2015).

Interestingly, the homologues of dialkyl decalin synthase and spiro-conjugate synthase were found to be conserved in the biosynthesis of several spirotetronates.

Based on the above-mentioned findings, a hypothesis regarding the biosynthesis of spirotetronates has been proposed in which the biosynthesis is based on the contribution of the following reactions. First, the assembly of linear type I polyketide compounds takes place. Second, the attachment of the tetronic acid moiety occurs, followed by a postulated Diels-Alder reaction to complete the macrocylization. Lastly, a tailoring modification such as hydroxylation or glycosylation modifies the core structure and may thus lead to the structural differentiation of spirotetronates (Vieweg et al. 2014)

1.4. Overview and objectives of this study

As described above, the spirotetronate polyketide antibiotic maklamicin has two structural features that are unique from those of other spirotetronates. This inspired me to investigate the detail mechanisms underlying the unique structure of maklamicin at the molecular level. The objective of my study was to obtain genetic information about the biosynthesis of maklamicin, which was isolated from the endophytic actinomycetes *Micromonospora* sp. NBRC 110955.

I began my investigation of maklamicin biosynthesis by conducting genome sequencing of *Micromonospora* sp. NBRC 110955, constructing the library, and performing a sequence analysis and gene disruption. As described in Chapter 2, the glycerate utilization operon involved in tetronate moiety formation was used as a probe for searching the genome database and for the screening of cosmid and Bacterial Artificial Chromosome (BAC) libraries. In addition, by chromosome walking and sequencing analyses, I identified the maklamicin gene cluster. Disruption of PKSs genes was performed to test the involvement of this gene cluster in maklamicin production. Lastly, I propose the pathway of maklamicin biosynthesis.

Chapter 3 describes how the function of genes involved in the post-PKS modification of maklamicin biosynthesis was characterized by gene disruption and an *in vivo* resting cell assay. Moreover, a new maklamicin analogue was obtained from the culture of a cytochrome P450 gene disruptant, and it displayed significant activities against Gram-positive bacteria. The general conclusions are given in Chapter 4.

Chapter 2

Characterization of the biosynthetic gene cluster for maklamicin, a spirotetronate-class antibiotic of the endophytic *Micromonospora* sp. NBRC 110955

2.1. Introduction

The spirotetronate-class antibiotics are a growing family of polyketide natural products with a wide range of biological activities, including anti-bacterial, anti-tumor, and anti-viral activities (Vieweg et al. 2014). The compounds in this family possess a characteristic tetronate moiety spiro-linked to a cyclohexene ring, sometimes conjugated with a *trans*-decalin moiety. To date, almost 60 related spirotetronate antibiotics have been reported from actinomycetes, and can be classified into three subgroups based on the size of the central ring system and their molecular weight, with the latter mainly reflecting the glycosylation pattern. The largest and most homogenous group of spirotetronate antibiotics is the family of medium-sized spirotetronates, which comprises a 13-membered carbon macrocycle and several sugar moieties. An intriguing point of this group is that the spirotetronate moiety is connected to a *trans*-decalin system either by a carbonyl unit (for kijanimicin or tetrocarcin A) or by a carboxylic ester (for chlorothricin) (Kawashima et al. 1991; Takeda et al. 1991; Waitz et al. 1981). Although the *trans*-decalin system is also found in versipelostatin A, one of the large-sized spirotetronates comprising a central ring system larger than a 13-membered macrocycle (Park et al. 2002), most of the large-sized spirotetronates have no such *trans*-decalin system. The most representative of the small-sized spirotetronates consisting of the central 11-membered carbon ring system are the abyssomicins, which lack the *trans*-decalin system in their frameworks (Riedlingera et al. 2004). Several studies have revealed that the main framework of

spirotetronate antibiotics is biosynthesized by type I polyketide synthase (PKS) machinery, which occasionally forms the *trans*-decalin system by a postulated Diels-Alder reaction (Fang et al. 2008; Jia et al. 2006; Zhang et al. 2007). Although the biosynthetic mechanisms of spirotetronates have been partially elucidated with respect to the formation of the carbon ring system as well as the *trans*-decalin system, the detailed mechanism causing the structural differences remains to be clarified.

Maklamicin, which is produced by the recently isolated endophytic *Micromonospora* sp. NBRC 110955, belongs to the family of small-sized spirotetronates and shows anti-microbial activity against Gram-positive bacteria, especially *Micrococcus luteus* (Igarashi et al. 2011). Unlike the small-sized spirotetronate abyssomicins, maklamicin includes a *trans*-decalin system that is connected to a spirotetronate moiety by both a carbonyl group and a four-carbon chain linker. The 4-carbon chain linker of maklamicin is the shortest in the spirotetronates family, whose members usually have a 6- or 10-carbon chain linker composing a 13- or 17-membered macrocyclic ring (Vieweg et al. 2014). Another unique structural feature of maklamicin is the presence of a 2*R*-hydroxy-propyl group at C-21. While other spirotetronates have an achiral substituent at the same position, only maklamicin contains a stereogenic center at the substituent on the cyclohexene ring. The structural novelty of maklamicin in the family of spirotetronate antibiotics has spurred us to elucidate the biosynthetic mechanism of maklamicin at the molecular level, which will contribute to the generation of new spirotetronates with potentially improved properties.

2.2. Materials and Methods

2.2.1. Bacterial strains, plasmids, and growth conditions

Micromonospora sp. NBRC 110955 isolated from the root of Maklam Phueak (*Abrus pulchellus* Wall. Ex Thwaites subsp. *pulchellus*) collected in Thailand. *Micromonospora* sp. NBRC 110955 was grown at 30^{o}C on ISP medium 2 (Becton, Dickinson and Company, Franklin

Lakes, NJ, USA). *Escherichia coli* DH5α was used for general DNA manipulation (Sambrook and Russell 2001), and the DNA methylation-deficient *E. coli* strain ET12567 containing pUZ8002 (Paget et al. 1999) was used for *E. coli*/*Micromonospora* conjugation. *E. coli* strains were grown in Luria-Bertani (LB) medium, with appropriate antibiotics when necessary. The plasmids used were SuperCos 1 (Agilent Technologies) and pKU503 (Komatsu et al. 2010) for construction of the cosmid and bacterial artificial chromosome (BAC) libraries for the *Micromonospora* sp. NBRC 110955 genome, respectively, and pIJ773 (Gust et al. 2003) for PCR amplification of the *oriT-aac(3)IV* cassette for gene disruption. *Micromonospora* sp. NBRC 110955 was cultivated in a test tube (15 by 160 mm) containing 5 ml of liquid ISP2 medium (0.2% yeast extract, 1% malt extract and 0.2% glucose, pH 7.2). The test tube was incubated on a reciprocal shaker (160 spm) at 30ºC for 5 days. An aliquot (700 μl) of the culture was then inoculated into 70 ml of liquid ISP2 medium in a 500 ml baffled flask on a rotary shaker (170 rpm), and mycelia were harvested after 36 h of cultivation at 30ºC. The mycelia were washed, resuspended in fresh liquid ISP2 medium, and stored at -80ºC until use as a seed culture. All the primers are listed in Table 2.1.

2.2.2. Genome sequencing and bioinformatics analyses

The seed culture of *Micromonospora* sp. NBRC 110955 was inoculated into 70 ml of liquid ISP2 medium in a 500 ml baffled flask, diluted to an OD_{600} of 0.05, and cultivated for 2 days. The mycelium was harvested by centrifuge and three grams of wet cell were used for Genomic DNA extraction according to the protocol from molecular cloning. Ten microgram of pure Genomic DNA was submitted to the Genome Analysis Centre, Norwich Research Park, UK, using 454 DNA pyrosequencing technology. One-quarter of a run of 454 sequencing yielded 104 Mb of sequence data which gave 14x coverage of the genome and were assembled using Newbler assembly software to give 373 contigs with a mean length of 18,317 bp. The assembled contig sequences and the Genome Matcher software (Ohtsubo et al. 2008) were used to analyze the potential maklamicin biosynthetic gene in the genome. Annotation of open

reading frames (ORFs) and gene functions was performed manually by using the FramePlot 4.0beta program (http://nocardia.nih.go.jp/fp4/), 2ndFind program (http://biosyn.nih.go.jp/2ndfind/), BLAST algorithm and PKS/NRPS analysis program (http://nrps.igs.umaryland.edu/nrps/). The nucleotide sequence data reported in this paper have been deposited in the DDBJ data bank under accession number LC021382.

Table 2.1. Oligonucleotides used in this study

Primer	**Sequence (5`-3`)**
For cosmid and BAC libraries screening	
makB1-Fw	GACAGCTGCGCCAAGCACCTGGAC
makB1-Re	CATTTTCGGCATGACCTGTTCGAG
makB5-Fw	ATCGTCAGCGTCAAGGAGCAGTGG
makB5-Re	CGGCGGGATGATCTCGTCACG
For construction of *makA1* disruptant	
makA1-Fw	ACTAGTGTGATCCGCATGAAGCATGACCACGACCCGGTCGTCCCGATTCCGGGGATCCG TCGACC
makA1-Re	TCTAGAGTCGCTCACCGCGGCCGGCAGGTCGCCGGCGCCCGCCCGTGTAGGCTGGAGCT GCTTC
makA1-cFw	TCAGGAACGGCTTTAACGACCGACAAG
makA1-cRe	AGATCGCGTCGACCTCCTCGGTGAGCA
For construction of *makA4* disruptant	
makA4-Fw	AAGTGGGTCACGGCGGATCTGCACAAGACCCGCCAGCGGATTCCGGGGATCCGTCGACC
makA4-Re	CTCCGGGGCGTGCGTACGCAGCCACTCCTGGCTGAGCGTTGTAGGCTGGAGCTGCTTC
makA4-cFw	GACGTGCGCGAGCGGCTGGACGGCGCCAG
makA4-cRe	GTGTCGGTCATGAGGAACTCCCTGTCGGTT

2.2.3. Construction and screening of cosmid and BAC libraries of genomic DNA

For construction of the cosmid library, 400 μg of genomic DNA of the *Micromonospora* sp. NBRC 110955 was partially digested with *Sau*3AI. The appropriated size of partially

digested DNA was checked by electrophoresis using 0.3% Agarose gel (Takara, Japan), the electrophoresis was ran for 24 hour with 20 volt to obtain the best DNA separation. Next, the target DNA size around 30-56 kb was excised from the gel then purified and dephosphorelated. Following, 2.5 μg of 30-56 kb DNA fragments were ligated into *Xba*I-*Bam*HI-digested of 1 μg dephosphorylated SuperCos 1. Southern blot analysis was performed to confirm the ligation between DNA fragment and SuperCos 1. Next, *E. coli* XL-1 Blue MR and Gigapack III plus packaging extract (Agilent Technologies) were used for the library construction according to the manufacturer's instructions. For construction of the BAC library, partially digested DNA fragments of 150 to 220 kb were purified, and cloned with a large *Bam*HI segment of pKU503 as described previously (Aroonsri et al. 2012; Komatsu et al. 2013). The cosmid library (1,100 colonies) and BAC library (2,700 colonies) were screened by PCR using primers designed from the 454 sequence data. The primer are listed in table 2.1.

2.2.4. Genetic disruption in the *Micromonospora* sp. NBRC 110955

Disruptants in which the internal region of the *makA1* and *makA4* genes were deleted were constructed by λRED-mediated PCR-targeted mutagenesis (Gust et al. 2003).

The approach of λRed-mediated PCR targeted mutagenesis of *Micromonospora* sp. NBRC 110955 is to replace a chromosomal sequence within *Micromonospora* sp. NBRC 110955 cosmid by apramycin marker that has been generated by PCR using primer with 39 nucleotide homology with gene to be deleted (Fig. 2.1). The primers was designed by 5' ends of primer is overlapping the regions of upstream and downstream of the *mak* coding region and 3' ends of primer designed to amplify the apramycin resistance *oriT-aac(3)IV* disruption cassette of pIJ773 (Fig 2.1) (Gust et al. 2003): makA1-Fw and makA1-Re for *makA1* disruption; makA4-Fw and makA4-Re for *makA4* disruption. The amplified DNA was introduced into the cosmid 1E1 for *makA1* or the cosmid 10E6 for *makA4* using λRED-mediated recombination with *E. coli* BW25113/pIJ790 (Gust et al. 2003).

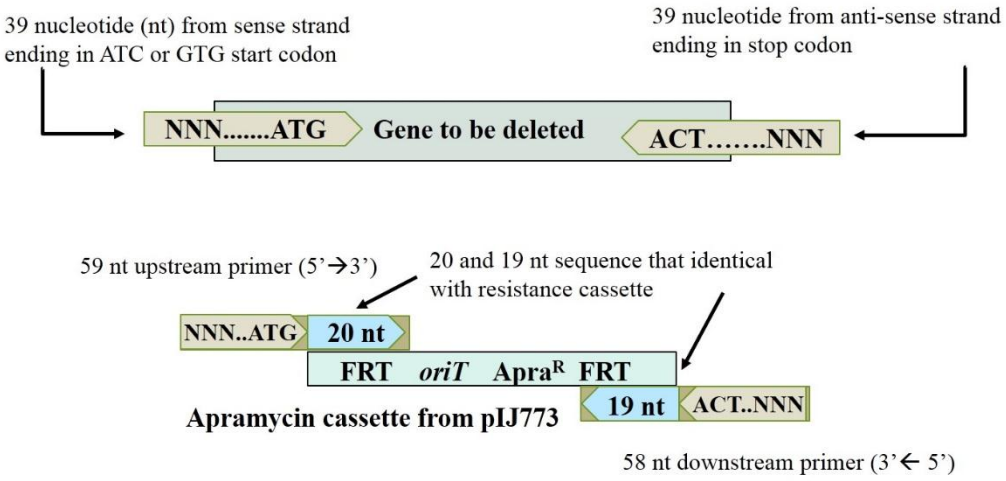

Fig 2.1. Designing PCR primers for making in-frame deletion. The 59 and 58 nucleotides (nt) primers were designed by 39 nt of primers (5') are overlapping with target regions upstream and downstream and 20 nt or 19 nt (3') primers are overlapping with apramycin cassette from pIJ773 plasmid.

The procedure of λRed-mediated PCR targeted mutagenesis was explained in Fig 2.2.

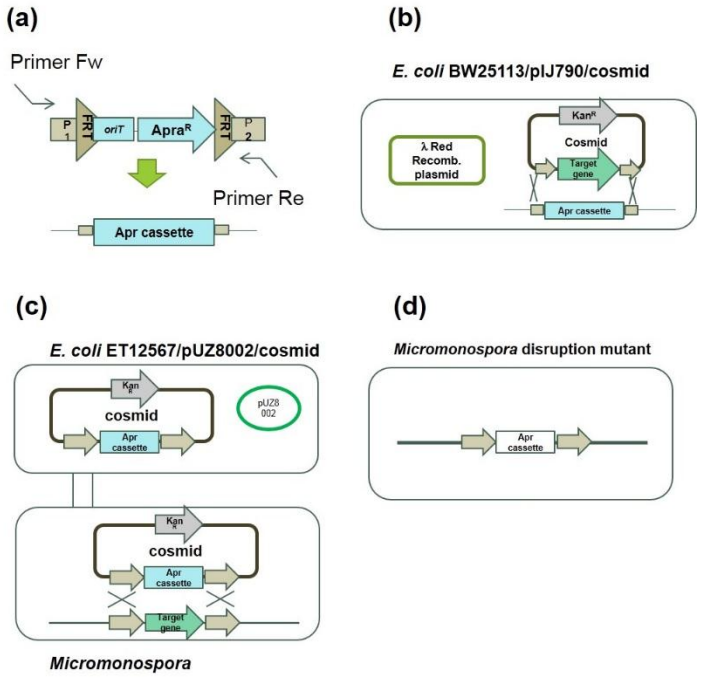

Fig. 2.2. The principle of λRed-mediated PCR mutagenesis in *Micromonospora* sp. NBRC 110955. **(a)** The amplification of apramycin (apr) cassette from pIJ773 by 59 and 58 nt primers according to **fig 2.1**. **(b)** The introduction of apramycin cassette to *E. coli* BW25113/pIJ790 (λ RED recombination plasmid) harboring *Micromonospora* NBRC 110955 cosmid to obtain the cosmid with the target region was replaced by

apramycin cassette (mutant cosmid). **(c)** The mutant cosmid was transferred to *E. coli* ET12567/pUZ8002 and conjugated with *Micromonospora* sp. NBRC 110955 **(d)** The target region in *Micromonospora* chromosome was replaced by apramycin cassette causing gene inactivation.

The disrupted cosmids were transferred via *E.coli* ET12567/pUZ8002 to the *Micromonospora* sp. NBRC 110955 by intergeneric conjugation. An LB culture of the donor *E. coli* ET12567/pUZ8002 harboring the disrupted cosmid was grown to an optical density at 600 nm (OD_{600}) of 0.4-0.6. The cells harvested were washed twice with LB and resuspended in a 0.1 volume of LB. The seed culture of the recipient *Micromonospora* sp. NBRC 110955 was inoculated into 70 ml of liquid ISP2 medium in a 500 ml baffled flask, diluted to an OD_{600} of 0.05, and cultivated for 2 days. The harvested mycelia were washed twice with Tryptone Soya Broth (TSB; Oxoid, Basingstoke, UK), resuspended in a 0.1 volume of TSB, and then mixed with the *E. coli* donor cells in a ratio of 1:1 (v/v). After incubation at 25^{o}C for 30 min, the mixture was spread on ISP medium 2 containing 30 mM $MgCl_2$. After incubation at 28^{o}C for 20 h, the plates were overlaid with water containing 1 mg of apramycin (Sigma, Japan) and 0.5 mg of nalidixic acid (Wako, Japan) and the incubation was continued at 30^{o}C. The genotype of candidates for disruption of the corresponding genes was confirmed by PCR analysis. The *Micromonospora* sp. NBRC 110955 *makA1* and *makA4* disruptants were abbreviated Δ*makA1* and Δ*makA4*, respectively.

2.2.5. Analysis of maklamicin production

The seed culture of *Micromonospora* sp. NBRC 110955 was cultivated in a 500 ml baffled flask containing 100 ml of V-22 medium (Igarashi et al. 2011). The baffled flask was incubated on a rotary shaker at 30^{o}C for 4 days. An aliquot (3 ml) of the culture was then inoculated into 100 ml of A11M medium (Igarashi et al. 2011) in a 500 ml baffled flask. After 6 days of cultivation, the culture broth was extracted with a half volume of *n*-butanol. After the organic layer was evaporated and dissolved with methanol, the crude extract was subjected to reversed-

phase HPLC on a CAPCELL-PAK C_{18} column (UG80; 5 μm; 4.6 by 250 mm; Shiseido, Tokyo, Japan) developed with a gradient system of acetonitrile (75% for 0-5 min; 75% to 95% for 5-25 min) containing 0.1% formic acid (flow rate, 1.2 ml/min; UV detection, 254 nm). Purified maklamicin was used as a standard for HPLC analysis.

2.3. Results

2.3.1. Cloning and identification of the maklamicin biosynthetic gene cluster

To identify the maklamicin biosynthetic gene cluster, the genome of the *Micromonospora* sp. NBRC 110955 was sequenced using 454 pyrosequencing technology. 104 Mb of sequence data were obtained, resulting in a 14-fold read coverage, and the reads were assembled into 373 contigs with an average length of 18,317 bp. The genome size is approximately 7.4 Mb. The chemical structure of maklamicin indicated that type I modular PKSs and tetronate-forming enzymes are involved in the biosynthesis of maklamicin. The study on biosynthesis of agglomerin A which is a tetronate antibiotic isolated from *Pantoea agglomerans*. Agglomerin A is not a spirotetronate antibiotic but has a tetronate moiety with an exocyclic double bond in the structure, revealed a set of proteins encoded by the “glycerate utilization operon” (*agg1* to *agg5*) for biosynthesis of the tetronate moiety (Fig. 2.3) (Kanchanabanca et al. 2013).

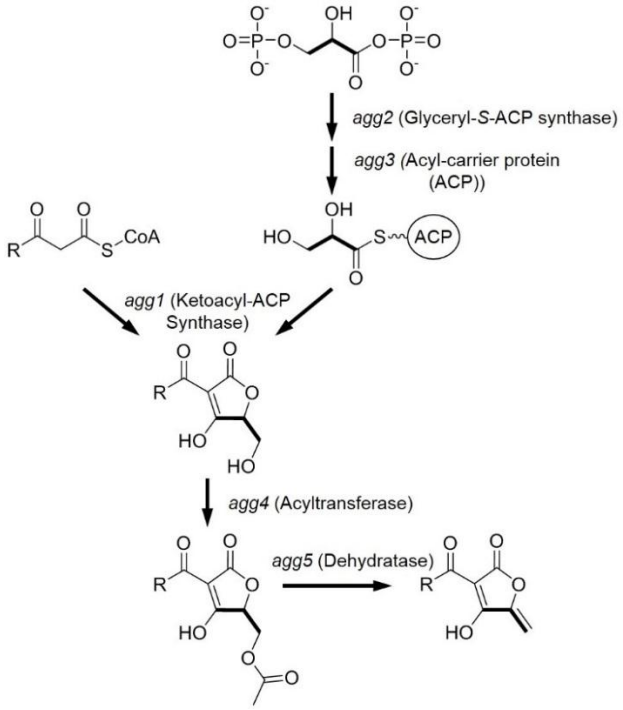

Fig. 2.3. Presumed steps for formation of the tetronate moiety. 1,3-Bisphosphoglycerate is activated by loading onto a discrete ACP by the action of a glyceryl-*S*-ACP synthase. Ketoacyl-ACP synthase fuses the ACP-coupled C_3-unit and a linear acyl-precursor together to form an initial ketoacyl-tetronate. In the next step, the premature tetronate moiety is acetylated by an acyltransferase, and then catalyzed by a dehydratase to generate an exocyclic methylene group of the mature tetronate moiety.

In addition, homologue genes of the glycerate utilization operon were found to be highly conserved in the biosynthetic gene cluster of spirotetronate antibiotics (Fang et al. 2008; Gottardi et al. 2011; Jia et al. 2006; Zhang et al. 2007) (Fig. 2.4), and closely associated with a particular PKS gene cluster. An *in silico* screening of the *Micromonospora* sp. NBRC 110955 contigs using gene clusters encoding the glycerate utilization operon identified a 15.7-kb contig containing a set of five genes encoding a ketoacyl-ACP synthase (*makB1* accession no. BAQ25501), a glyceryl-*S*-ACP synthase (*makB2* accession no. BAQ25502), an acyl-carrier protein (ACP) (*makB3* accession no. BAQ25503), an acyltransferase (makB4: accession no. BAQ25504), and a dehydratase (*makB5* accession no. BAQ25505). (Fig. 2.4).

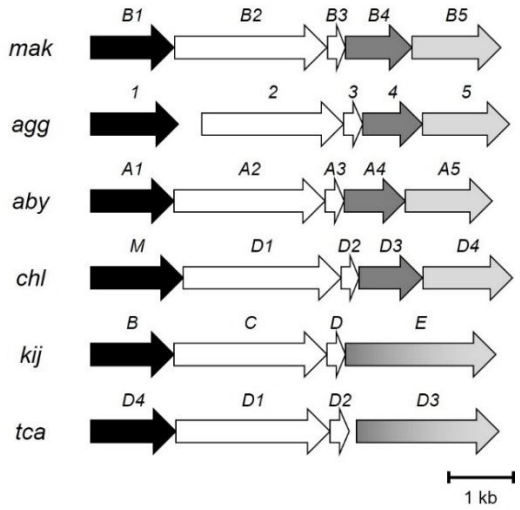

Fig. 2.4. Organization and comparison of the gene clusters for the tetronate ring formation and dienophile biosynthesis. *mak*, maklamicin; *agg*, agglomerin A; *aby*, abyssomicin; *chl*, chlorothricin; *kij*, kijanimicin; *tca*, tetrocarcin A. Genes are color-coded by the predicted function of their products: tetronate ring condensation (black), glycerate activation (white), and formation of an exocyclic double bond (dark gray and light gray).

The contig also contains the *makA5* gene, which encodes a type II thioesterase, homologs of which have been found in many other type I PKS systems and are thought to play an editing role in the biosynthesis of the polyketide (Heathcote et al. 2001), suggesting that these genes might belong to the family of maklamicin biosynthetic genes.

To ensure full coverage of the maklamicin biosynthetic gene cluster, a cosmid library of 1,100 clones and a BAC library of 2,700 clones were constructed and screened by PCR with probes of *makB1* and *makB5*. Further cosmids were identified by chromosome walking, and the gaps between each contig were bridged by PCR amplification and sequencing. In total, a region of about 152 kb of contiguous DNA was identified in this way (Fig. 2.5).

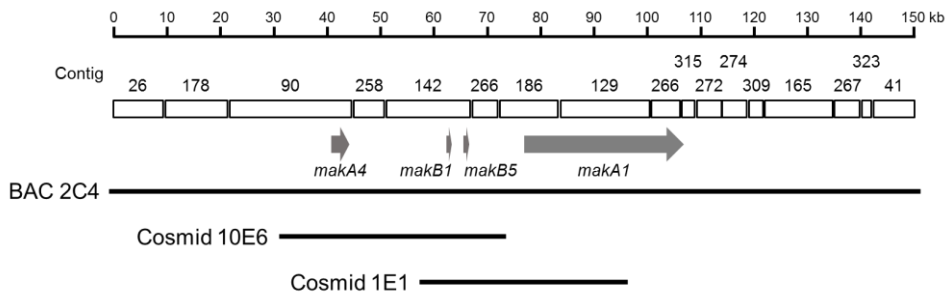

Fig. 2.5. A 152-kb DNA region from *Micromonospora* sp. NBRC 110955 represented by one BAC

clone and two cosmid clones. The arrows indicate *makA1*, *makA4*, *makB1* and *makB5* genes. The solid lines indicate the boundary of fragments from BAC2C4, cosmid 10E6, and cosmid 1E1, and the white boxes indicate the assembled contigs for the maklamicin biosynthetic gene cluster.

Annotation analysis of the sequence and comparison with genes in public databases revealed 46 ORFs, the genetic organization and proposed functions of which are shown in Fig. 2.6 and Table 2.3, respectively.

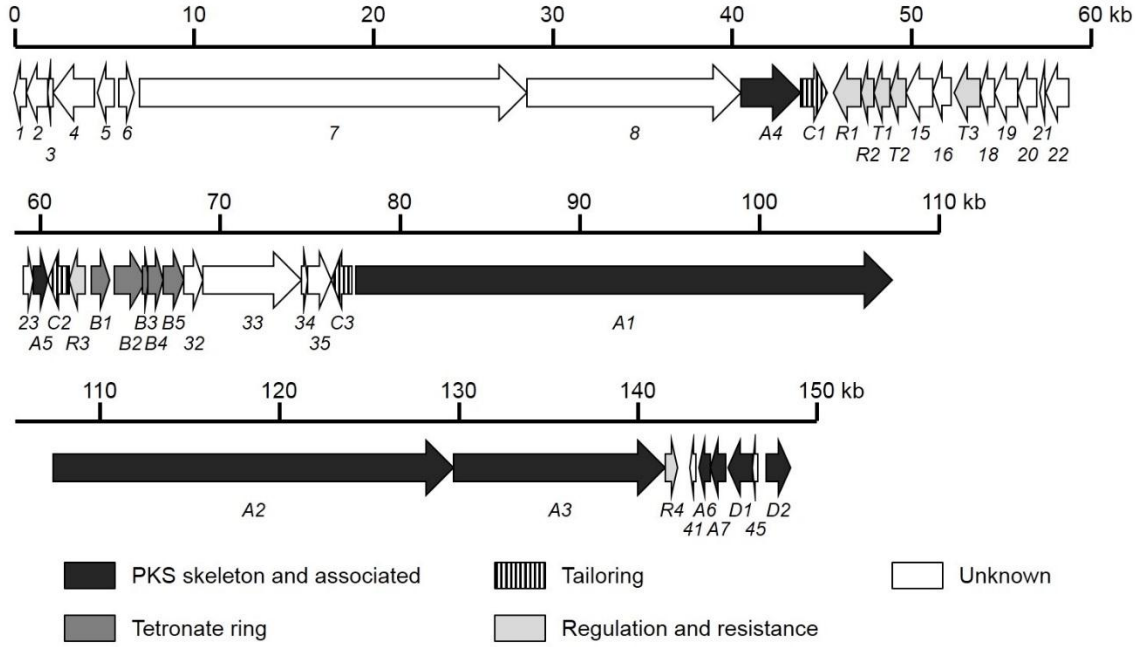

Fig. 2.6. Genetic organization of the maklamicin biosynthetic gene cluster. ORFs predicted to participate in maklamicin biosynthesis are shaded. The proposed functions of individual ORFs are indicated here and summarized in Table 2.3.

Six PKS genes, *makA1*, *makA2*, *makA3*, *makA4*, *orf7*, and *orf8*, were identified in the cluster, and are separated by the putative glycerate utilization operon (*makB1-makB5*). PKS modules encoded by the six PKS genes and the information of modules and domains of each PKS genes was explained on table 2.2.

Table 2.2 The PKS modules and domains from 6 PKS genes

Genes	Modules	Domains
makA1	Loading module (L)	KS^{Q}-AT-ACP
	M1	KS-AT-DH-KR-ACP
	M2	KS-AT-DH-KR-ACP
	M3	KS-AT-DH-KR-ACP
	M4	KS-AT-DH-KR-ACP
	M5	KS-AT-DH-KR-ACP
makA2	M6	KS-AT-DH-KR-ACP
	M7	KS-AT-DH-KR-ACP
	M8	KS-AT-DH-KR-ACP
	M9	KS-AT-DH-ER-KR-ACP
makA3	M10	KS-AT-DH-ER-KR-ACP
	M11	KS-AT-DH-KR-ACP
makA4	M12	KS-AT-ACP
orf7	M1	KS-AT-DH-KR-ACP
	M2	KS-AT-DH-KR-ACP
	M3	KS-AT-DH-KR-ACP
	M4	KS-AT-DH-KR-ACP
orf8	M1	KS-AT-DH-KR-ACP
	M2	KS-AT-DH-KR-ACP

From 19 modules of six PKS genes, only the first module of MakA1 contains a mutant ketosynthase^{Q} (KS^{Q}) domain that should function as a malonyl-thioester decarboxylase for polyketide chain initiation to yield an acetyl starter unit (Bisang et al. 1999) (Fig. 2.7).

```
                  *
MakA1_KSL    DAAQSSSL 164   ELHGTG 299   NVGHLEG 337
MakA1_KS1    DTACSSSL 177   EAHGTG 311   NLGHTQA 351
MakA1_KS2    DTGCSSSL 177   EAHGTG 311   NIGHTQA 351
MakA1_KS3    DTACSSSL 177   EAHGTG 311   NIGHTQA 351
MakA1_KS4    DTACSSSL 178   EAHGTG 312   NIGHTQA 350
MakA1_KS5    DTACSSSL 178   EAHGTG 312   NIGHTQA 350
MakA2_KS6    DTACSSSL 178   EAHGTG 312   NLGHTQA 351
MakA2_KS7    DTACSSSL 176   EAHGTG 310   NIGHTQA 348
MakA2_KS8    DTACSSSL 178   EAHGTG 312   NLGHTQA 351
MakA2_KS9    DTACSSSL 177   EAHGTG 311   NIGHTQA 349
MakA3_KS10   DTACSSSL 177   EAHGTG 311   NIGHTQA 351
MakA3_KS11   DTACSSSL 177   EAHGTG 311   NIGHTQA 351
MakA4_KS12   DTACSSSL 178   EAHGTG 312   NIGHTQA 352
Orf7_KS1     DTACSSSL 178   EAHGTG 312   NLGHTQA 351
Orf7_KS2     DTACSSSL 177   EAHGTG 311   NIGHTQA 349
Orf7_KS3     DTACSSSL 177   EAHGTG 311   NIGHTQA 349
Orf7_KS4     DTACSSSL 178   EAHGTG 312   NIGHTQA 350
Orf8_KS1     DTACSSSL 177   EAHGTG 311   NIGHTQA 351
Orf8_KS2     DTACSSSL 175   EAHGTG 309   NIGHTQA 347
```

Fig 2.7. Sequence alignment of the conserved motifs in KS domains from *mak* PKSs, Orf7, and Orf8. The conserved catalytic triad of C-H-H is highlighted in black, and the active site of KS domains is marked with an asterisk. The numbers indicate amino acid positions within each module.

Table 2.3. Deduced functions of ORFs in the maklamicin biosynthetic gene cluster.

Gene	Size[a]	Homolog[b] and origin	Identity/ similarity (%)	Proposed function
orf1	222	TcmI (EWM63272), *Micromonospora* sp. M42	78/86	Polyketide synthesis cyclase
orf2	407	Micau_3627 (YP_003836729), *Micromonospora aurantiaca* ATCC 27029	87/91	Cytochrome P450
orf3	106	MCBG_00403 (EWM63270), *Micromonospora* sp. M42	89/95	Antibiotic biosynthesis monooxygenase
orf4	759	Micau_3629 (YP_003836731), *Micromonospora aurantiaca* ATCC 27029	79/83	Monooxygenase
orf5	329	AMIS_50700 (YP_005464806), *Actinoplanes missouriensis* 431	79/86	dTDP-glucose 4,6-dehydratase
orf6	291	Micau_4024 (YP_003837123), *Micromonospora aurantiaca* ATCC 27029	72/83	Glucose-1-phosphate thymidylyltransferase
orf7	7211	FscC (YP_007749164), *S. albus* J1074	46/58	Type I polyketide synthase
orf8	3957	ChlA5 (AAZ77698), *S. antibioticus* DSM 40725	53/62	Type I polyketide synthase
makA4	1112	ChlA6 (AAZ77699), *S. antibioticus* DSM 40725	60/70	Type I polyketide synthase
makC1	502	ChlE3 (AAZ77700), *S. antibioticus* DSM 40725	54/64	FAD-dependent oxidoreductase
makR1	516	SCAT_p0196 (YP_004919488), *S. cattleya* NRRL 8057	53/62	Two-component system sensor kinase
makR2	242	SCAT_p0197 (YP_004919488), S. cattleya NRRL 8057	65/73	Two-component system response regulator
makT1	275	PyrJ4 (AFV71341), *S. rugosporus* NRRL 21084	44/60	ABC transporter permease
makT2	306	AMED_3162 (YP_003765355), *Amycolatopsis mediterranei* U32	67/75	Multidrug ABC transporter ATP-binding protein
15	461	Orf7 (CAC48373), *Amycolatopsis balhimycina* DSM 5908	64/78	Sodium:proton exchanger
16	345	ChlB3 (AAZ77676), *S. antibioticus* DSM 40725	62/75	3-Oxoacyl-ACP (acyl-carrier protein) synthase

Table 2.3. Continue

Gene	Size[a]	Homolog[b] and origin	Identity/ similarity (%)	Proposed function
makT3	495	ChlG (AAZ77686), *S. antibioticus* DSM 40725	51/68	Multidrug MFS (major facilitator superfamily) transporter
18	251	Ndas_2938 (YP_003680854), *Nocardiopsis dassonvillei* subsp. *dassonvillei* DSM 43111	50/61	Hypothetical protein
19	435	Ndas_2937 (YP_003680853), *Nocardiopsis dassonvillei* subsp. *dassonvillei* DSM 43111	54/66	Hypothetical protein
20	362	Ndas_2936 (YP_003680852), *Nocardiopsis dassonvillei* subsp. *dassonvillei* DSM 43111	68/78	ATPase AAA
21	78	Ndas_3539 (YP_003681446), *Nocardiopsis dassonvillei* subsp. *dassonvillei* DSM 43111	61/72	Hypothetical protein
22	402	ChlC6 (AAZ77691), *S. antibioticus* DSM 40725	49/62	Glycosyltransferase
23	185	ChlL (AAZ77701), *S. antibioticus* DSM 40725	43/61	Hypothetical protein
makA5	268	STSU_32540 (EIF88101), *S. tsukubaensis* NRRL18488	49/59	Type II thioesterase
makC2	396	ChlE2 (AAZ77695), *S. antibioticus* DSM 40725	57/66	Cytochrome P450
makR3	276	ChlF2 (AAZ77687), *S. antibioticus* DSM 40725	56/69	SARP family transcriptional regulator
makB1	342	ChlM (AAZ77702), *S. antibioticus* DSM 40725	67/78	Ketoacyl-ACP synthase
makB2	627	ChlD1 (AAZ77703), *S. antibioticus* DSM 40725	65/75	Glyceryl-S-ACP synthase
makB3	75	ChlD2 (AAZ77704), *S. antibioticus* DSM 40725	61/74	ACP
makB4	274	ChlD3 (AAZ77705), *S. antibioticus* DSM 40725	67/75	Acyltransferase

Table 2.3. Continue

Gene	Size[a]	Homolog[b] and origin	Identity/ similarity (%)	Proposed function
makB5	362	ChlD4 (AAZ77706), *S. antibioticus* DSM 40725	57/70	Dehydratase
32	346	ChlB3 (AAZ77676), *S. antibioticus* DSM 40725	66/78	3-Oxoacyl-ACP synthase
33	1801	ChlB1 (AAZ77673), *S. antibioticus* DSM 40725	55/85	Type I polyketide synthase
34	86	ChlB2 (AAZ77675), *S. antibioticus* DSM 40725	44/68	ACP
35	446	ChlB4 (AAZ77674), *S. antibioticus* DSM 40725	77/86	$FADH_2$-dependent halogenase
makC3	393	AORI_6618 (YP_008015604), *Amycolatopsis orientalis* HCCB10007	52/66	Cytochrome P450
makA1	9946	FscC (YP_007749164), *S. albus* J1074	50/61	Type I polyketide synthase
makA2	7431	FscC (YP_007749164), *S. albus* J1074	47/60	Type I polyketide synthase
makA3	3907	ChlA5 (AAZ77698), *S. antibioticus* DSM 40725	55/65	Type I polyketide synthase
makR4	210	AMIS_41060 (YP_005463842), *Actinoplanes missouriensis* 431	52/63	TetR family transcriptional regulator
41	99	Bra471DRAFT_05239 (EHR04437), *Bradyrhizobium* sp. WSM471	43/65	Hypothetical protein
makA6	239	SAV_1748 (NP_822924), *S. avermitilis* MA-4680	59/68	4'-Phosphopantetheinyl transferase
makA7	283	SCAB_84081 (YP_003493881), *S. scabiei* 87.22	72/80	Metallophosphoesterase
makD1	463	Micau_2481 (YP_003835596), *Micromonospora aurantiaca* ATCC 27029	66/73	Acyl-CoA synthetase
45	94	ML5_5878 (YP_004085486), *Micromonospora* sp. L5	61/79	Hypothetical protein
makD2	474	Strop_2764 (YP_001159584), *Salinispora tropica* CNB-440	68/78	Propionyl-CoA carboxylase

To confirm the involvement of *makA1* in the maklamicin biosynthesis, we generated a *makA1* disruptant (Δ*makA1*) in which a region encoding the first module of MakA1 was replaced with an *oriT-aac(3)IV* cassette by using λRED-mediated PCR-targeted mutagenesis (Fig. 2.8). The successful disruption of *makA1* gene was checked by PCR using primer pair makA1-cFw and makA1-cRe (Fig. 2.8). The *makA1* disruptant was confirmed the production of maklamicin by HPLC analysis. Based on HPLC result, the Δ*makA1* strain lost maklamicin production (Fig. 2.9), which clearly showed that *makA1* is responsible for the maklamicin biosynthesis and suggested that *makA2* and *makA3*, probably organized in a polycistronic operon with *makA1*, are also involved in the maklamicin biosynthesis.

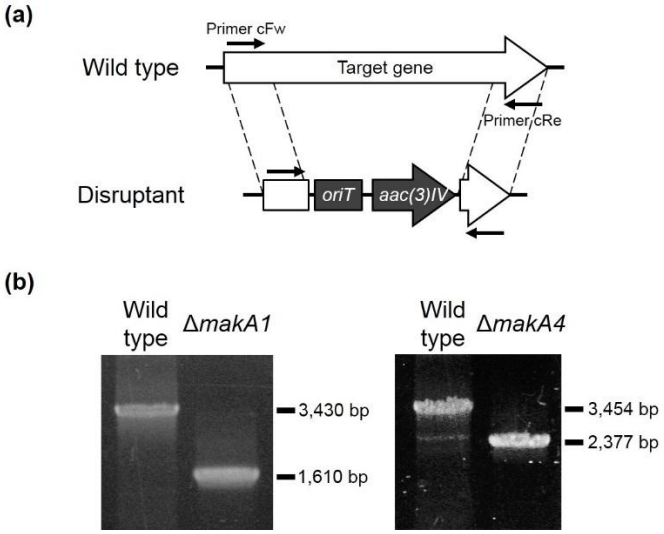

Fig. 2.8. Disruption of the *mak* PKS genes in the *Micromonospora* sp. NBRC 110955. **(a)** Schematic representation of the strategy for gene disruptions of *makA1* and *makA4*. The *oriT* sequence is the RK2 origin of transfer, and the *aac(3)IV* gene confers apramycin resistance. **(b)** PCR analysis to confirm gene-disruption of the *makA1* and *makA4* genes. Δ*makA1*, *makA1* disruptant; Δ*makA4*, *makA4* disruptant. With the primer pair makA1-cFw/makA1-cRe, an internal fragment (3,430 bp) of the intact *makA1* gene or a fragment (1,610 bp) containing the *oriT-aac(3)IV* cassette was amplified by PCR, respectively. With the primer pair makA4-cFw/makA4-cRe, an internal fragment (3,454 bp) of the intact *makA4* gene or a fragment (2,377 bp) containing the cassette was amplified by PCR, respectively.

Three PKS genes, *makA1*, *makA2*, *makA3*, encode the modular PKSs that consist of 11 active modules (including a loading module) with an inactive module (see below). Based on the structure of maklamicin, 12 PKS modules were predicted to be involved in the polyketide assembly process. Thus, through gene inactivation, we investigated the involvement of the *makA4* gene encoding a single PKS module (Fig. 2.8). Like the Δ*makA1* strain, the Δ*makA4* strain was unable to produce maklamicin (Fig. 2.9), indicating that the PKS module encoded by *makA4* is required for the maklamicin biosynthesis, and four PKS genes (*makA1-makA4*) are responsible for the polyketide assembly of maklamicin.

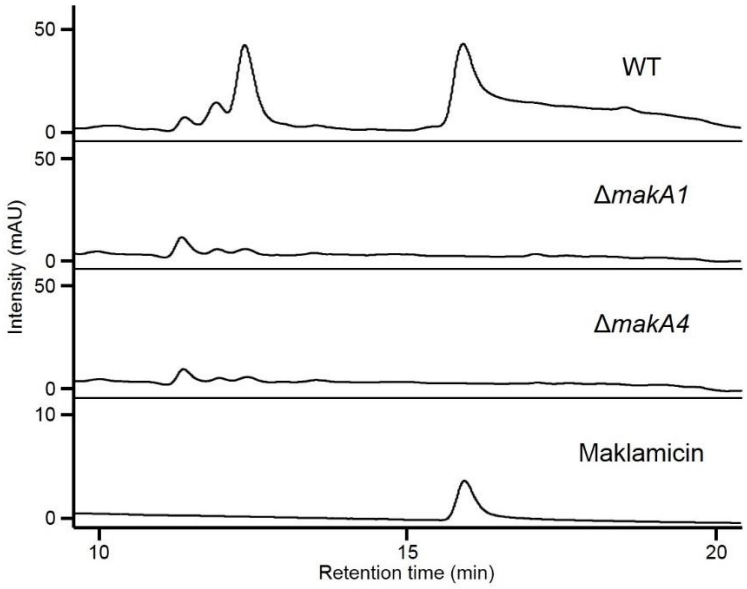

Fig. 2.9. Maklamicin production analyzed by the gene disruption of *makA1* and *makA4*. HPLC chromatograms of the *Micromonospora* sp. NBRC 110955 extracts are shown. WT, wild-type strain; Δ*makA1*, *makA1* disruptant; Δ*makA4*, *makA4* disruptant; maklamicin, an authentic standard of maklamicin. mAU, milliabsorbance units at 254 nm. Peaks corresponding to maklamicin are indicated by a heavy dot.

In contrast, disruption of the remaining PKS genes, *orf7* or *orf8*, located upstream of *makA4*, had no effect on the production of maklamicin (Fig. 2.10). These PKS genes appear to be involved in the production of another, as yet unidentified, polyketide compound.

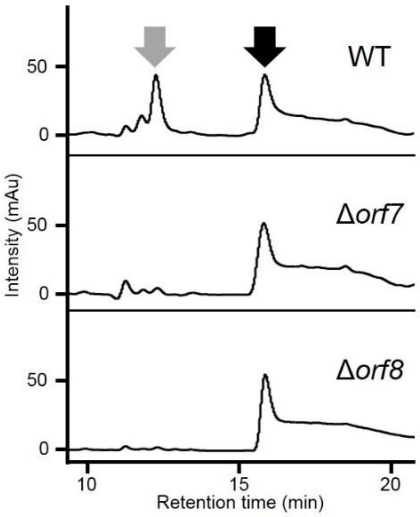

Fig. 2.10. Maklamicin production in the wild-type strain (WT), the *orf7* disruptant (Δ*orf7*) and the *orf8* disruptant (Δ*orf8*). HPLC chromatograms of *Micromonospora* sp. NBRC 110955 extracts are shown. mAU, milliabsorbance units at 254 nm. The peaks of maklamicin and an unidentified polyketide compound are indicated as a black arrow and a gray arrow, respectively.

2.3.2. Genes involved in the polyketide assembly of maklamicin

To predict the polyketide assembly process in the biosynthetic pathway of maklamicin, four PKSs (MakA1-MakA4) were analyzed *in silico*, and were found to contain 63 enzymatic domains organized into 13 PKS modules (Table 2.2).

Regards to figure 2.7, the first module of MakA1 contains a mutant KS^{Q} domain for decarboxylation of the ACP-bound malonate unit to form an acetyl group at the initiation of polyketide chain assembly, and thus functions as a loading module. The presence of the conserved active site (catalytic triad of Cys-His-His) in other KS domains (Fig. 2.7) indicates that they all function in the condensation steps (He et al. 2000)

Based on the sequence analysis of the acyltransferase (AT) domain within each module, the substrate specificities of the AT domains were predicted; the results showed that the AT domains of modules 2, 5, 7, 8, 10 and 12 as well as the loading module are specific for malonyl-CoA, whereas the AT domains of the remaining extender modules are specific for methylmalonyl-CoA (Fig. 2.11).

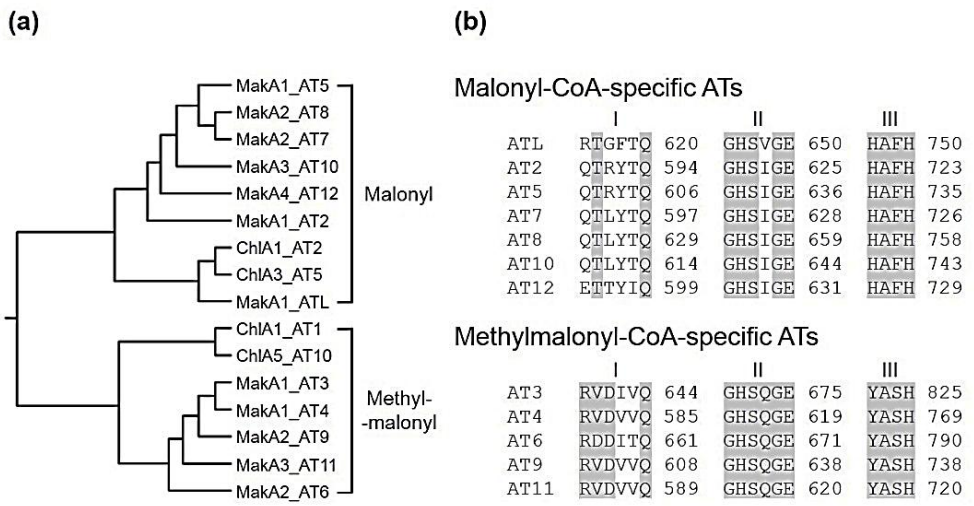

Fig. 2.11. Analysis of the AT domains from the maklamicin PKSs by phylogenetic tree and amino acid alignment. **(a)** Phylogenetic tree constructed by the unweighted pair group method with arithmetic mean. Multiple sequence alignment was conducted with the CLUSTALW program (http://www.genome.jp/tools/clustalw/). The corresponding domains are as follows ChlA1/ChlA3/ChlA5 for chlorothricin in *S. antibioticus* DSM 40725. **(b)** Sequence alignment of the conserved motifs in AT domains. Three dominant motifs, composed of 6, 6, and 4 amino acid residues, are assigned in each AT domain. The conserved amino acid residues are highlighted in gray. The numbers indicate amino acid positions within each module.

In contrast, an unusual AT domain was identified in module 1 of MakA1, in which an extra stretch of 62 amino acid residues including a polyalanine region (11 successive alanine residues) was found between the conserved motifs (Fig. 2.12), implying that this AT domain could be inactive, and an acetyl group on the loading module is directly transferred to a malonyl-*S*-ACP in module 2 by skipping module 1. These predicted substrates in the biosynthetic machinery for maklamicin are generally consistent with the structure of maklamicin. Regarding ACP domains, all the 13 ACP domains contain a signature motif around the conserved 4’-phosphopantetheine attachment site Ser residue (Fig. 2.13a) (Lai et al. 2006)

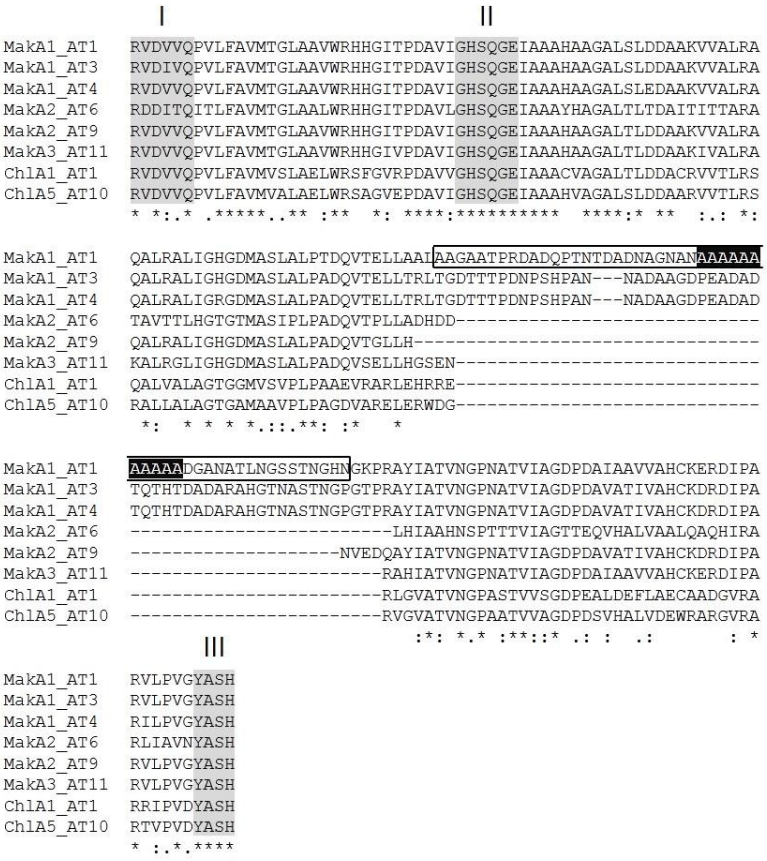

Fig. 2.12. Sequence alignment of the conserved motifs in AT domains from the *mak* PKSs and the chlorothricin PKSs. Multiple sequence alignment was conducted with the CLUSTALW program. Asterisks, identical or conserved residues in all sequences in the alignment; colons, conserved substitutions; single dots, semi-conserved substitutions. Three dominant motifs, composed of 6, 6, and 4 amino acid residues, are highlighted in gray. The extra stretch of 62 amino acid residues in the AT domain (module 1 of MakA1) is shown in a box with a polyalanine region (11 successive alanine residues) with black highlighting.

In addition, *makA6* and *makA7*, which are located downstream of the maklamicin PKS genes *makA1-makA3*, encode 4'-phosphopantetheinyl transferase and phosphoesterase, respectively. These two genes may be required for turnover of the 4'-phosphopantetheinyl group during the activation process of ACP domains (Murugan et al. 2010). Of the 11 predicted dehydratase (DH) domains found in the PKS modules, 2 DH domains (DH1 and DH2) contain a mutation in the conserved motif in which the active site His is replaced by other residues (Fig. 2.13b) (Wu et al. 2005), indicating that these DH domains should be inactive and the module 2 of MakA1 generates a hydroxy group by function of the ketoreductase (KR) domain (KR2).

Sequence analysis revealed that KR2 contains the stereochemistry signature "L(V)DD" motif for B-type KR domains to produce a hydroxy group of *R* configuration (Fig. 2.13c) (Caffrey 2003), which correlates with the stereochemistry of the C-31 hydroxy group in maklamicin. The two enoylreductase (ER) domains, ER9 in module 9 of MakA2 and ER10 in module 10 of MakA3, contain the conserved NADP(H)-binding site (Fig. 2.13d) (Cane 2010), and are likely responsible for the reduction of a double bond between C-8/C-9 and C-6/C-7, respectively.

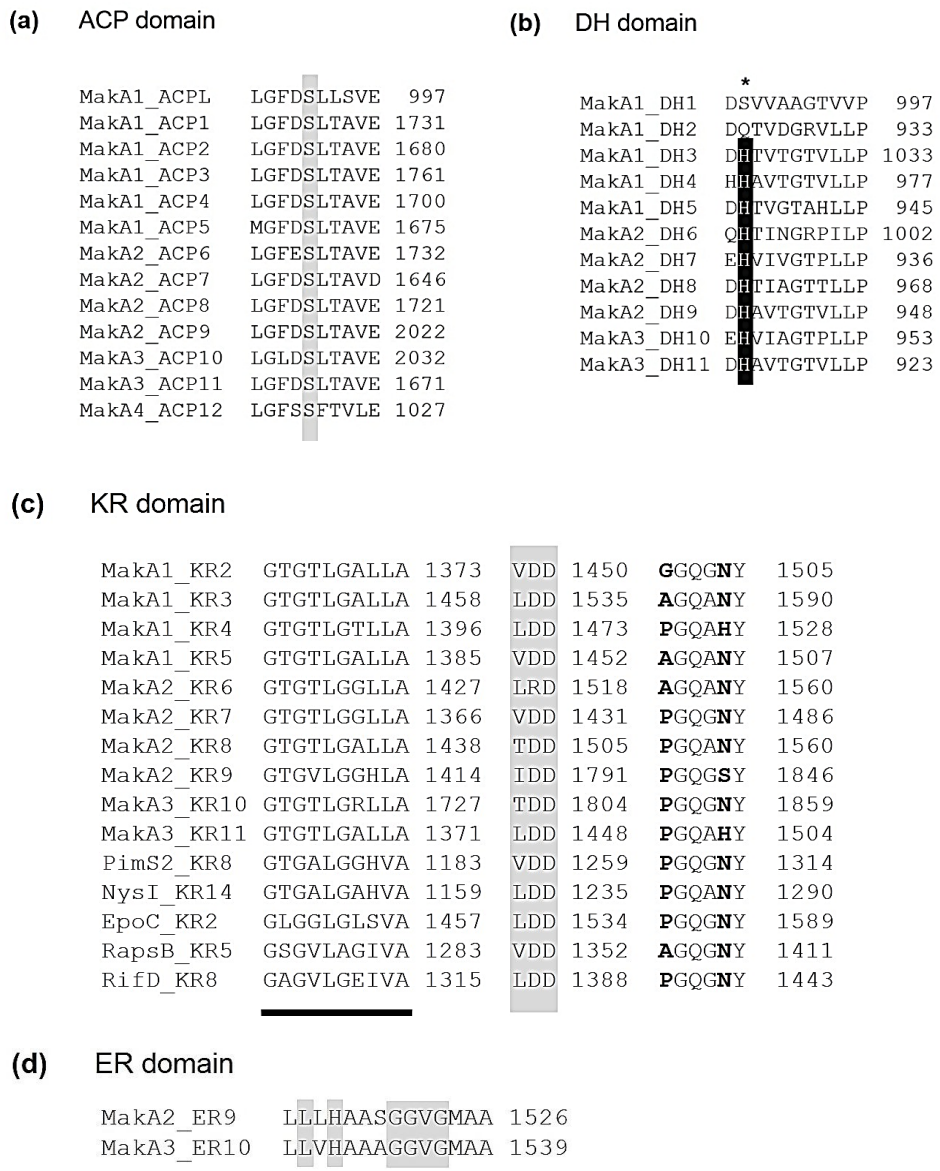

Fig. 2.13. Amino acid alignment of ACP, DH, KR and ER domains. The numbers indicate amino acid positions within each module. **(a)**: Sequence alignment of the conserved motif in ACP domains. The Ser residues as the phosphopantheteine binding site are highlighted in gray. **(b)** Sequence alignment of the

conserved motif in DH domains. An asterisk indicates the proposed active-site His in the DH domain, and the DH domains of module 1 and module 2 lack the active site His residue. **(c)** Sequence alignment of the conserved motifs in various B-type KR domains. The names are: PimS2, pimaricin; NysI, nystatin; EpoC, epothilone; RapsB, rapamycin; RifD, rifamycin. The conserved N-terminal NADP(H) binding motif is underlined (GxGxxGxxxA). The stereochemistry signature "LDD" motifs for B-type KR domains are highlighted in gray. The PN motifs are shown in bold type. **(d)** Sequence alignment of the conserved motif in ER domains. The proposed NADP(H)-binding site (LxHxxxGGVG) is highlighted in gray. The names and sequence accession numbers are shown in Appendix.

Consequently, for the biosynthesis of maklamicin, four PKSs (MakA1-MakA4) could catalyze the formation of the nascent linear polyketide intermediate through the successive decarboxylative condensations (Fig. 2.14). We postulated that the linear maklamicin intermediate undergoes an intramolecular Diels-Alder reaction by addition of the C-4/C-5 dienophile to the C-10/C-13 diene to yield the *trans*-decalin moiety. Further, we considered that an acyl-CoA synthetase (MakD1) and a propionyl-CoA carboxylase (MakD2) were responsible for the supply of precursors such as malonyl-CoA and methylmalonyl-CoA in the polyketide chain assembly, respectively.

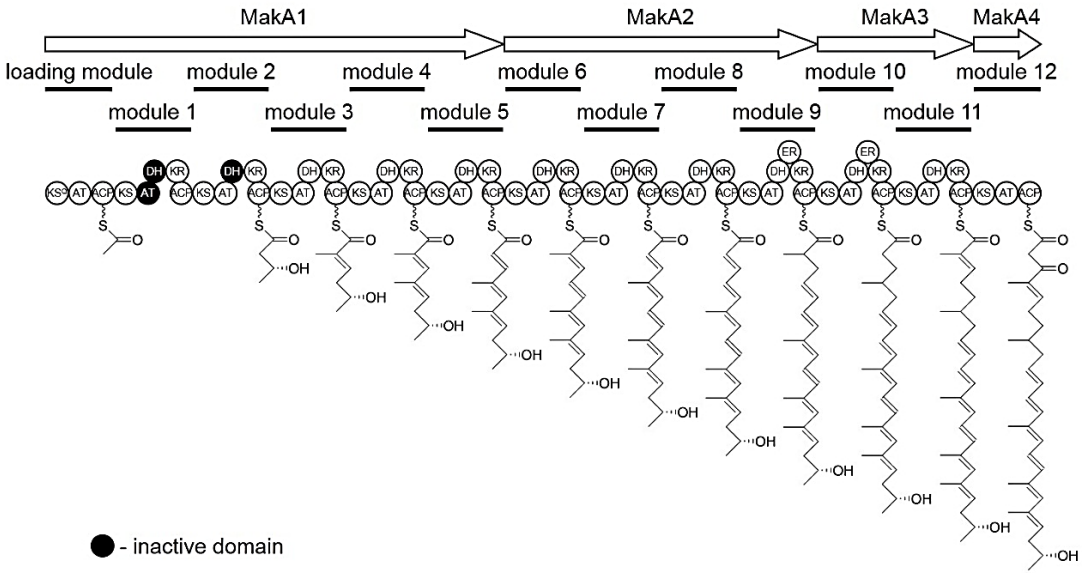

Fig. 2.14. Polyketide chain extension on the maklamicin PKSs encoded by *makA1*, *makA2*, *makA3* and *makA4*. The circles represent enzymatic domains in the PKS polypeptide; KS, ketosynthase; KS^{Q}, KS in which the active site Cys is replaced by Gln; AT, acyltransferase; DH, dehydratase; ER, enoylreductase; KR, ketoreductase; ACP, acyl carrier protein. The presumed inactive AT and DH domains of module 1 and module 2 are shaded in black.

2.3.3. Biosynthesis of the tetronate moiety of maklamicin

Five genes (*makB1-makB5*) are subclustered in the middle of the maklamicin biosynthetic gene cluster. Comparison with the glycerate utilization operon for the biosynthesis of other tetronate compounds suggested that these five genes are involved in biosynthesis of the tetronate moiety in maklamicin. In particular, both the sequence and organization of the *makB* genes were well coincident with those of *agg* genes (Fig. 2.4), the functions of which were recently characterized biochemically in the biosynthesis of agglomerin A (Kanchanabanca et al. 2013). MakB2, a putative glyceryl-*S*-ACP synthase showing both glyceryltransferase and phosphatase activities, activates 1,3-bisphosphoglycerate from the glycolytic pathway by loading onto the discrete ACP MakB3, yielding a glyceryl-*S*-ACP (Fig. 2.15). The putative ketoacyl-ACP synthase MakB1 catalyzes the condensation between the glyceryl-*S*-ACP and the polyketide intermediate to form the next maklamicin intermediate, which has the premature tetronate moiety with a *trans*-decalin moiety. Subsequently, the premature tetronate moiety could be acetylated by the putative acyltransferase MakB4, and then catalyzed by the putative dehydratase MakB5 to generate the exocyclic double bond of the mature tetronate moiety.

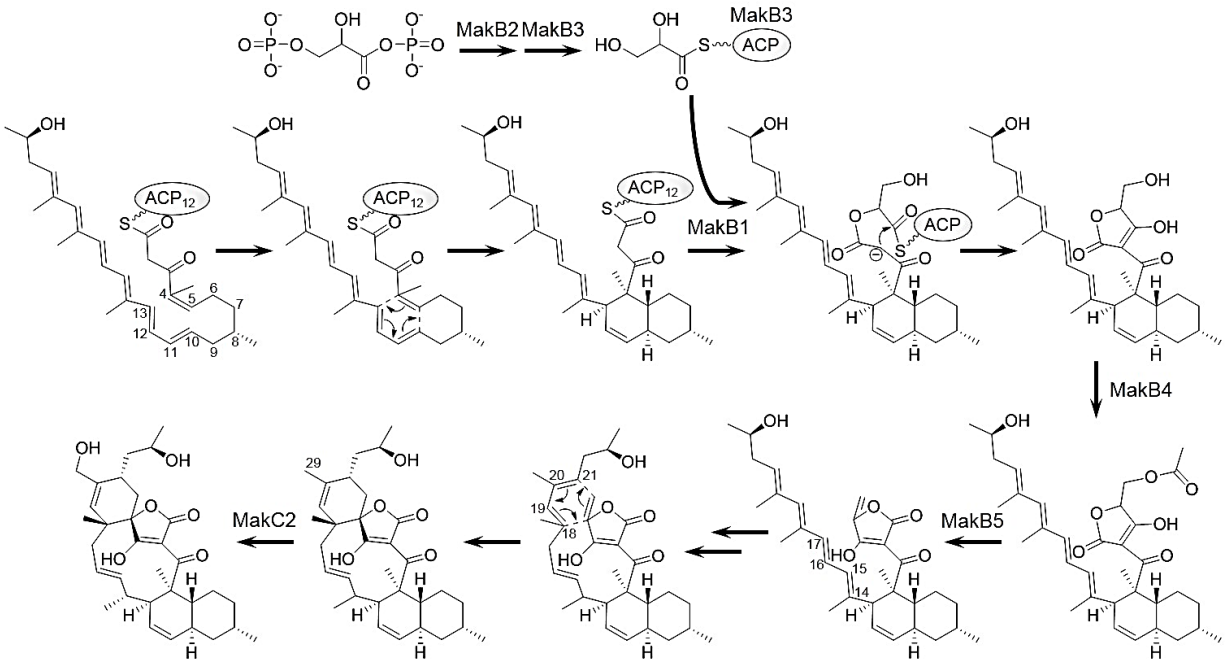

Fig. 2.15. Proposed pathway for maklamicin biosynthesis in the *Micromonospora* sp. NBRC 110955.

2.2.4. The tailoring steps of maklamicin biosynthesis

Prior to the generation of the cyclohexene moiety, the C-14/C-17 conjugated diene structure in the maklamicin intermediate would be reduced and converted to a single double bond between C-15 and C-16. Although the conversion of the diene structure seems to be indispensable for the maturation step in the maklamicin biosynthesis (see Discussion), the exact order of this double-bond transfer process remains to be unraveled. I postulated that the reduced intermediate is cyclized by the second Diels-Alder reaction between the exocyclic double bond of the tetronate moiety as a dienophile and a conjugated C-18/C-21 diene structure in the tail region to yield a premature maklamicin macrocycle showing a cyclohexene moiety. The gene products of both *makC2* and *makC3* strongly resembled cytochrome P450 monooxygenases, suggesting that either *makC2* or *makC3* or both might be associated with the post-modifications of maklamicin biosynthesis. Based on an experiment described in Chapter 3 indicated that MakC2 is required for the specific hydroxylation at C-29, which is probably the final step in the biosynthetic pathway of maklamicin.

2.2.5. Genes putatively involved in the regulation and the self-resistance of maklamicin biosynthesis

The *in silico* analysis of the *mak* gene cluster identified four genes coding for potential pathway-specific regulators in the control of the maklamicin biosynthesis. The proteins encoded by *makR1* and *makR2* are homologous to various sets of sensor kinases and cognate response regulators belonging to a bacterial two-component signal transduction system for detecting and responding to changes in the environment (Mascher et al. 2006). The deduced protein of *makR3* resembled transcriptional activators of the *Streptomyces* antibiotic regulatory protein (SARP) family that have been shown to positively control the production of antibiotics as cluster-situated regulators (Wietzorrek and Bibb 1997). This finding suggested the possibility that MakR3 positively regulates the production of maklamicin. A fourth regulatory gene,

makR4, was presumed to encode a transcriptional regulator of the TetR family (Ramos et al. 2005). Homologues of the *makR4* gene were found to be located inside the biosynthetic gene clusters of spirotetronate antibiotics possessing the *trans*-decalin moiety (*chlF1* for chlorothricin; *tcaR2* for tetrocarcin A; *kijA8* and *kijC5* for kijanimicin). Although the role of these putative regulators remains to be investigated, the high conservation of TetR-family regulatory genes implied that they might be common regulators for the production of those spirotetronate antibiotics, including maklamicin. MakT1 and MakT2 are homologous to members of ATP-binding-cassette (ABC) transport systems (Davidson and Chen 2004), and MakT3 is homologous to members of the multidrug MFS (major facilitator superfamily) transporters (Putman et al. 2000). Interestingly, in addition to regulators of the TetR family, the MFS transporter genes have also been identified in the biosynthetic gene clusters of chlorothricin, tetrocarcin A, and kijanimicin, suggesting that MakT3 might serve as the efflux pump for maklamicin as a self-resistance mechanism.

2.4. Discussion

The spirotetronate-class antibiotics have distinct chemical structures that include a tetronate moiety spiro-linked to a cyclohexene ring, sometimes conjugated with a *trans*-decalin moiety (Vieweg et al. 2014). In particular, the spirotetronates containing the *trans*-decalin moiety are characterized by a carbon chain length connecting the cyclohexene and the decalin units, which, together with the addition of several deoxy sugars and/or modification of other peripheral moieties, lead to the diversity of structures and biological activities, such as anti-microbial and anti-tumor activities. All representatives of the spirotetronates share these important structural characteristics, which supports the assumption that their main frameworks are biosynthesized by employing common polyketide assembly lines and glycerate utilization systems. In the present study, we successfully identified the biosynthetic gene cluster of maklamicin, one of the spirotetronates including the *trans*-decalin moiety, and proposed a

biosynthetic model composed of a polyketide assembly line, a glycerate utilization system and tailoring modifications. Furthermore, the identification of maklamicin biosynthetic genes enabled us to genetically compare the spirotetronate gene clusters to deduce common or unique biosynthetic pathways of spirotetronates.

Maklamicin has two structural features that are unique among the spirotetronates: a 2*R*-hydroxy-propyl group at C-21 and the shortest carbon chain length between the two ring systems. The 2*R*-hydroxy-propyl group of maklamicin is attached on the cyclohexene ring, whereas other spirotetronates have an achiral substituent at the same position, such as a methyl, a formyl, an ethyl, or an *n*-propyl group (Hegde et al. 1997; Momose et al. 1999; Park et al. 2007; Shimotohno et al. 1993). The attachment of a hydroxy group to the alkyl chain to form a peripheral moiety of a polyketide compound has been known to be catalyzed by specific enzymes in the post-PKS tailoring step (Rix et al. 2002). For example, the hydroxylation at C-29 in maklamicin, which appears to be the final step of the biosynthetic pathway, requires MakC2, a putative cytochrome P450 monooxygenase (see Chapter 3). However, the detailed analysis of maklamicin PKS modules demonstrated that the C-31 hydroxy group is formed on the nascent linear polyketide intermediate, by the cooperative function of both an inactive DH2 domain and a B-type KR2 domain. In the polyketide assembly lines of other representative spirotetronates (chlorothricin, kijanimicin, and tetrocarcin A), these initial processes have active DH domains that catalyze the generation of an enoyl group in the tail-region of the polyketide backbone (Fang et al. 2008; Jia et al. 2006; Zhang et al. 2007). Thus, the irregular PKS modules provide maklamicin with a unique stereogenic center on the substituent of the cyclohexene ring.

Maklamicin, which is classified as a small-sized spirotetronate, consists of a central ring system containing an 11-membered carbon macrocycle. With respect to the carbon chain between the cyclohexene and the decalin units, a four-carbon chain linker of maklamicin, in addition to the similar four-carbon chain linkers in nomimicin (Igarashi et al. 2012) and

PA46101 compounds (Matsumoto et al. 1989) , are the shortest ever such carbon chains reported in the family of spirotetronates. The ring size in the central ring system is dependent on the relative positional relationship between a conjugated pair of double bonds in the tail-region of the polyketide backbone and an exocyclic double bond at the C5-position of the tetronate moiety for undergoing Diels-Alder cycloaddition (Vieweg et al. 2014). The biosynthetic pathways of chlorothricin, kijanimicin, and tetrocarcin A have illustrated that the terminal diene structure is biosynthesized in the early steps of the pathway by two PKS modules (module 1 and module 2) containing two active DH domains. The DH domain in maklamicin PKS module 2, one of the corresponding modules, is rendered inactive by the replacement of the active site (Fig. 2.13b), which leads to macrocyclization by the second Diels-Alder reaction between the C-18/C-21 diene structure formed by modules 3 and 4 and the dienophile of the tetronate moiety to generate a cyclohexene ring. The maklamicin PKS assembly line shows the same number of PKS modules as observed in chlorothricin, kijanimicin, and tetrocarcin A, and their *trans*-decalin moieties are generated by the same polyketide assembling system via the first Diels-Alder reaction. Nonetheless, because the second Diels-Alder reaction in the maklamicin biosynthesis occurs with the alternative diene formed by different PKS modules, the carbon-chain linker in maklamicin is the shortest in the spirotetronates.

Another structural difference that distinguishes maklamicin from other spirotetronates is the presence of the C-15/C-16 double bond, which appears to be synthesized via some pathway other than the canonical PKS system. The corresponding double bond is also found in the structure of nomimicin, suggesting that a common mechanism underlies the biosynthesis of maklamicin and nomimicin. The conversion from a C-14/C-17 diene to the C-15/C-16 double bond would be a significant event for the macrocyclization between the C-18/C-21 diene and the exocyclic double bond of the tetronate moiety, because the diene structure presumably prefers to be located at a position proximal to the dienophile double bond. A PKS module encompassing the pair of a B-type KR domain and a DH domain typically generates polyketide

chain elongation intermediates with a *trans* (*E*) double bond (Li et al. 2009; Wu et al. 2005). Our finding that all maklamicin KR domains belonged to the B group (Fig. 2.13c) indicated that the maklamicin polyketide intermediate has *trans* double bonds that situate the C-18/C-21 diene distal to the dienophile (Fig. 2.15), when the C-14/C-17 diene is intact. The conversion of the C-14/C-17 diene to the C-15/C-16 double bond could provide the positional flexibility of the C-18/C-21 diene for easy access to the dienophile for performing the Diels-Alder reaction, but it remains to be determined which enzymes are responsible for the double bond transfer. A detailed genetic comparison among the gene clusters of maklamicin with nomimicin and spirotetronates lacking the C-15/C-16 double bond will elucidate the molecular mechanisms for the biosynthesis of the C-15/C-16 double bond.

The insights afforded by our identification and characterization of the maklamicin biosynthetic genes should open the way to a future comparative analysis with the biosynthetic genes of spirotetronates with or without the *trans*-decalin moiety to account for the structural commonalities and differences, and the production systems. Genes encoding a transcriptional regulator of the TetR family and an MFS transporter are well-conserved among the gene clusters of maklamicin, chlorothricin, kijanimicin, and tetrocarcin A, suggesting that these two genes, in addition to the biosynthetic genes for the polyketide backbone and the tetronate moiety, play a common role in the production of the spirotetronates. The recent studies reported intriguing enzymes for performing a Diels-Alder reaction, which demonstrated that VstJ catalyzes the stereoselective [4+2]-cycloaddition for the formation of spirotetronate structure in the versipelostatin biosynthesis (Hashimoto et al. 2015), and that ChlE3 is a dialkyldecalin synthase and ChlL is a spiro-conjugate synthase in the chlorothricin biosynthesis (Tian et al. 2015). These findings strongly suggests that Orf23, a homologue of VstJ and ChlL, catalyzes the formation of cyclohexene unit in the maklamicin biosynthesis and that MakC1, a ChlE3 homologue, catalyzes the formation of *trans*-decalin moiety. The genetic information of these conserved genes could be useful in genome mining and screening for the rapid identification of

actinobacteria producing new spirotetronates. Further understanding of the maklamicin biosynthetic machinery will enable us to apply combinatorial biosynthesis for the rational development of novel maklamicins and will facilitate expansion of the structural repertoire in the family of spirotetronate antibiotics.

2.5. Summary

In this chapter, maklamicin biosynthetic gene cluster were identified from *Micromonospora* sp. NBRC 110955. The gene cluster spans around 152 kb consists of 46 ORFs. The disruption of polyketide synthases genes, *makA1*and *makA4* resulted the complete loss of maklamicin production suggested the gene cluster is involved in maklamicin biosynthesis. Consequently, 24 genes can be assigned to have roles in the maklamicin biosynthesis including polyketide framework (*makA1-makA7* and *makD1-makD2*), spirotetronate (*makB1-makB5*) and peripheral moieties (*orf23, makC1-makC2*), self-resistance (*makT1-makT3*) and regulation (*makR1-makR4*) of maklamicin production. The proposed maklamicin biosynthesis indicated the inactivation of DH domain at initial module affecting the structure differentiation of maklamicin.

Chapter 3

29-Deoxymaklamicin, a new maklamicin analogue produced by a genetically engineered strain *Micromonospora* sp. NBRC 110955

3.1. Introduction

Polyketide natural products constitute an important group of secondary metabolites used in human/veterinary medicine and agriculture (Staunton and Weissman 2001). These products are typically biosynthesized in a step-by-step manner by several kinds of enzymes: polyketide synthases (PKSs) and tailoring enzymes for post-PKS modifications such as glycosylation, methylation, and hydroxylation (Olano et al. 2010). Among the tailoring enzymes, the cytochrome P450 monooxygenases are known to be involved in the regiospecific and stereospecific oxidation of polyketide compounds (Lamb et al. 2013). This enzymatic reaction is an important biosynthetic process of the polyketide compounds which renders them structurally diverse and provides them with desirable bioactivities.

Maklamicin is one of the spirotetronate-class antibiotics, a growing family of polyketide compounds with a wide range of biological activities, and shows anti-microbial activity against Gram-positive bacteria (Igarashi et al. 2011). Maklamicin consists of a polyketide scaffold with a tetronate moiety spiro-linked to a cyclohexene ring conjugated with a *trans*-decalin moiety. From chapter 2, I have recently identified the maklamicin biosynthetic (*mak*) gene cluster from the *Micromonospora* sp. NBRC 110955. This gene cluster includes four type I PKS genes (*makA1-makA4*) for the polyketide assembly and the glycerate utilization operon (*makB1-makB5*) for incorporation of the tetronate moiety, as well as putative genes involved in the regulation and self-resistance of this species. Furthermore, the homologues of VstJ which catalyses the stereoselective [4+2]-cycloaddition in the versipelostatin biosynthesis (Hashimoto

et al. 2015) and ChlE3 which is a diakyldecalin synthase and ChlL is a spirotetronate synthase in the chlorothricin biosynthesis (Tian et al. 2015) are present inside the *mak* gene cluster as an Orf23 (homologue of VstJ and ChlL) and MakC1 (homologue of ChlE3), suggesting that Orf23 and MakC1 are probably responsible for the formation of the spirotetronate structure. In addition to these genes, the *mak* cluster contains two more genes (*makC2* and *makC3*) encoding the cytochromes P450, which may participate in post-PKS modification in the maklamicin biosynthesis.

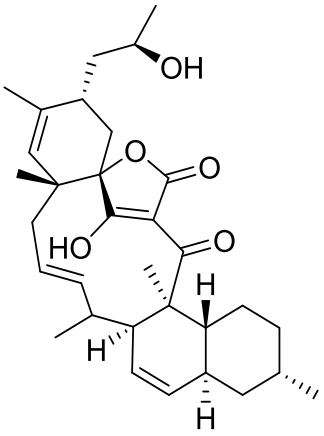

Fig 3.1. Structure of 29-deoxymaklamicin

3.2. Materials and methods

3.2.1. Bacterial strains, plasmids, and growth conditions

Micromonospora sp. NBRC 110955 (Igarashi et al. 2011) was grown at 30°C on ISP medium 2 (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). *Escherichia coli* DH5α was used for general DNA manipulation (Sambrook and Russell 2001), the DNA methylation-deficient *E. coli* strain ET12567 containing the RP4 derivative pUZ8002 (Paget et al. 1999) was used for *E. coli*/*Micromonospora* conjugation, and *E. coli* F^- *dcm* Δ(*srl-recA*)*306*::Tn*10* carrying pUB307-*aph*::Tn*7* was used for *E. coli*/*Streptomyces* conjugation. *E. coli* strains were grown in 2X yeast extract-tryptone medium, with appropriate antibiotics when necessary.

pIJ773 (Gust et al. 2003) was used to PCR amplify the *oriT-aac(3)IV* cassette for gene disruption, and pLT101, which contains the constitutive and strong promoter *ermEp** and a *tfd* terminator (Pulsawat et al. 2007), was used to construct a vector for genetic complementation. *Micromonospora* sp. NBRC 110955 was cultivated in a test tube (15 by 160 mm) containing 5 mL of liquid ISP2 medium (0.2% yeast extract, 1% malt extract and 0.2% glucose, pH 7.2). The test tube was incubated on a reciprocal shaker (160 spm) at 30°C for 5 days. An aliquot (700 μL) of the culture was then inoculated into 70 mL of liquid ISP2 medium in a 500 mL-baffled flask on a rotary shaker (170 rpm), and mycelia were harvested after 36 h of cultivation at 30°C. The mycelia were washed, resuspended in fresh liquid ISP2 medium, and stored at -80°C until use as a seed culture. All the primers are listed in table 3.1.

3.2.2. Genetic disruption in the *Micromonospora* sp. NBRC 110955

The *makC2* and *makC3* disruptants were constructed by a λRED-mediated PCR-targeted gene disruption protocol (Fig. 2.2, chapter 2) (Gust et al. 2003) using the following primer pairs: makC2-Fw and makC2-Re for *makC2* disruption; and makC3-Fw and makC3-Re for *makC3* disruption. The amplified DNA containing the *oriT-aac(3)IV* disruption cassette of pIJ773 was introduced into the cosmid 10E6 for *makC2* or the cosmid 1E1 (Fig 2.5, chapter 2) for *makC3* using λRED-mediated recombination with *E. coli* BW25113/pIJ790 (Gust et al. 2003). The disrupted cosmids were transferred via *E.coli* ET12567/pUZ8002 to the *Micromonospora* sp. NBRC 110955 by intergeneric conjugation. The genotype of candidates for disruption of the corresponding genes was confirmed by PCR analysis. The *Micromonospora* sp. NBRC 110955 *makC2* and *makC3* disruptants were abbreviated Δ*makC2* and Δ*makC3*, respectively.

3.2.3. Genetic complementation of the *makC2* disruptant

The apramycin resistance gene *aac(3)IV* of pLT101 was replaced by the hygromycin resistance gene *hyg* using the λRED-mediated recombination, yielding pLT129 as a vector for

complementation of the Δ*makC2* strain. The *makC2* gene was PCR-amplified by the primer pair makC2-comp-Fw/makC2-comp-Re and then cloned into the *Bam*HI site of pLT129 using a GeneArt Seamless Cloning and Assembly Kit (Life Technologies, CA, USA), resulting in pLT130. The plasmid pLT130 was introduced into the Δ*makC2* strain by intergeneric conjugation and integration. Integration of the plasmid was confirmed by hygromycin resistance gene (primers were showed in table 3.1) and PCR analysis.

3.2.4. Analysis of maklamicin production

The seed culture of *Micromonospora* sp. NBRC 110955 was cultivated in a 500 mL-baffled flask containing 100 mL of V-22 medium (Igarashi et al. 2011). The baffled flask was incubated on a rotary shaker at 30ºC for 4 days. An aliquot (3 mL) of the culture was then inoculated into 100 mL of A11M medium (Igarashi et al. 2011) in a 500 mL-baffled flask. After 6 days of cultivation, the culture broth was extracted with a half volume of 1-butanol. After the organic layer was evaporated and the residue dissolved with methanol, the crude extract was subjected to reversed-phase HPLC on a CAPCELL-PAK C_{18} column (UG80; 5 μm; 4.6 by 250 mm; Shiseido, Tokyo, Japan) developed with a gradient system of CH_3CN (75% for 0-5 min; 75% to 95% for 5-25 min) containing 0.1% HCO_2H (flow rate, 1.2 ml/min; UV detection, 254 nm). Purified maklamicin was used as a standard for HPLC analysis.

Table 3.1. Oligonucleotides used in this study

Primer	Sequence (5`-3`)
For construction of *makC2* disruptant	
makC2-Fw	GACTAGTCGGTCCACCGCGCCTGACCGCCCGTTCCGGCCAAGGGCTAATTCCGGGGATCCGTCGACC
makC2-Re	CGTCTAGACGTCGCGCGCTTCCGCTACCCCCTCGTAAGGAGGCTTCATGTGTAGGCTGGAGCTGCTTC
makC2-cFw	GGGTGAGGTGCTGGCCCAGGCACTGGT
makC2-cFw	TTCGACCCCCCGGAGGAGTTGGCATCGC
makC2-tFw	ACGACCTGCTGGTCTGCCACAGCTACA
makC2-tRe	TCTACCAGAACGCACGCGCCGTGCTCAGT
For construction of *makC3* disruptant	
makC3-Fw	GACTAGTCGAGATCCGGGCCGGGCTTCGGCGGTTGCGGGGTCGGTCAATTCCGGGGATCCGTCGACC
makC3-Re	CGTCTAGACGTGCCTCCCCTTTAGTCGGCGCGCCGACGATGGCGGGATGTGTAGGCTGGAGCTGCTTC
makC3-cFw	GCAGCGACTCCCAGCCGTTCATCAGGAT
makC3-cFw	ATGAGCAGCGACGTCCTGCACAGCAAC
makC3-tFw	ACGTACTGAAGATGCTCCAGGGCGACGT
makC3-tRe	CTCACTCGGCCTCGTACGGACGCCGT
For genetic complementation of the *makC2* disruptant	
makC2-comp-Fw	CGTGCCGGTTGGTAGGAGGCTTCATGACCGACCTC
makC2-comp-Re	CTTTAGATTCTAGAGCTACCAGCGCACCGGCAGGC
hyg-Fw	CTACGCGGAGCCTGCGGAACGAC
hyg-Re	GAGCAGCGCGGCCAGGATCTCGC
For transcriptional analysis of *makC2* in the recombinant *S. avermitilis*	
hrdB-Fw	ACTCGCTGTCACCGTCCTCAC
hrdB-Re	CGCGCCAAGAACCACCTCCT

3.2.5. Isolation and structure elucidation of a new maklamicin analogue

The seed culture of was Δ*makC2* strain was cultivated in a 500 ml baffled flask containing 100 ml of V-22 medium (Igarashi et al. 2011). The baffled flask was incubated on a rotary shaker at 30ºC for 4 days. An aliquot (3 ml) of the culture was then inoculated into 100 ml of A11M medium, (Igarashi et al. 2011) in a 500 ml baffled flask, in total 2 liters of culture. After 6 days of cultivation, 2 liters of culture broth was extracted with a half volume of 1-butanol, after which the 1-butanol layer was evaporated to dryness. The crude extract (1.56 g) was subjected to silica gel column chromatography with a step gradient of $CHCl_3$/MeOH (1:0, 20:1, 10:1, 4:1, 2:1, 1:1 and 0:1 v/v). Fractions 3 and 4 were combined and concentrated to give 358 mg of brown oil. Final purification was achieved by preparative C-18 HPLC using an XTerra RP18 column (5 μm; 10 by 150 mm; Waters, MA, USA) with 70% CH_3CN/0.1% HCO_2H at 3 mL/min and detection at 210 nm, to yield 5.3 mg of purified compound with a retention time of 38.5 min.

3.2.6. Chemical and analytical methods

The UV spectrum was recorded on a Hitachi U-3000 spectrophotometer. The IR spectrum was recorded on a FTIR-8400 S (Shimadzu, Kyoto, Japan). Optical rotation was measured on a JASCO P-1020 polarimeter. HRFABMS was measured on a JEOL JMS-700 spectrometer. NMR (^{1}H, 600 MHz; ^{13}C, 150 MHz) spectra were recorded on a Bruker UltraShield 600 Plus spectrometer and the ^{1}H and ^{13}C chemical shifts were referenced to the solvent signal (δ_H 7.26 and δ_C 77.0 in $CHCl_3$).

3.2.7. Biological assays

29-Deoxymaklamicin was tested in antimicrobial assays against *Bacillus cereus* NBRC15305, *Bacillus subtilis* PCI219, *Candida albicans* NBRC1594, *Enterococcus faecalis* NBRC100480, *Escherichia coli* NIH-JC2, *Micrococcus luteu*s ATCC9343, and *Staphylococcus*

aureus NBRC12732. Mueller Hinton broth (Difco, Detroit, MI, USA) was used for bacteria, and Yeast Nitrogen Base (Difco) supplemented with 2% glucose was used for *C. albicans*. Test microorganisms were inoculated into a 32-mL test tube containing 8 mL of the liquid medium. After incubation on a reciprocal shaker for 20 h at 30ºC, the cells were collected by centrifugation and the cell suspension (1.0 x 10^5 cells/mL) was prepared in saline. The liquid medium (135 μL), the cell suspension (15 μL), and the sample solution in DMSO (0.5 to 1 μL) were added into the wells of a 96-well culture plate and were mixed by gentle agitation. After incubation for 20 h at 37ºC (for *E. coli*) or 30ºC (for other bacteria and *C. albicans*), the absorbance at 650 nm was measured using a microplate reader. The concentrations of 29-deoxymaklamicin used for testing biological assay were 0.195, 0.391, 0.781, 1.56, 3.13, 6.25, 12.5, 25, 50 and 100 μg/ml respectively.

3.2.8. Resting cell conversion with *S. avermitilis* expressing *makC2*

The plasmid pLT130 was introduced into *S. avermitilis* SUKA22 (Komatsu et al. 2013) by intergeneric conjugation, followed by hygromycin selection and PCR analysis. Spores (1.0 x 10^8 CFU) of *S. avermitilis* strains were cultivated in a test tube (15 by 160 mm) containing 2 mL of APM medium (Kitani et al. 2009) at 28ºC for 2 days. An aliquot (700 μL) of the culture was inoculated into 70 mL of APM medium in a 500 mL-baffled flask, and mycelia were harvested after 2 days of cultivation. The 10 ml of cell culture was centrifuged and the mycelium was collected and washed twice with 50 mM potassium phosphate buffer (pH 7.2), and then the mycelia (1 gram wet-cell weight) was resuspended in 10 mL of 100 mM potassium phosphate buffer (pH 7.2) (Choi et al. 2012) supplemented with 0.5 mg of 29-deoxymaklamicin. The sample was incubated on a reciprocal shaker (185 spm) at 28ºC for 3 days, and extracted with a half volume of 1-butanol. The 1-butanol layer was concentrated to dryness under a vacuum, and the residue was dissolved in methanol and subjected to HPLC analysis by the same procedure as for the detection of maklamicin.

For gene expression analysis, 10 ml from 70 ml culture remained from resting cell conversion assay was used for RNAs extraction by an RNeasy Mini kit (QIAGEN Science, MD, USA) and treated with DNase I (Takara Bio, Shiga, Japan). The cDNA was synthesized using SuperScript III RNase H^- reverse transcriptase (Invitrogen, CA, USA) and random primers (Invitrogen) according to the manufacturer's instructions. The PCR amplification was performed by using GoTaq Green Master Mix (Promega KK, Tokyo, Japan) under the following conditions: 98ºC for 2 min, followed by 25 cycles of 98ºC for 30 s, 57ºC for 30 s, and 72ºC for 1 min. The primers used for the detection of the *makC2* transcript were makC2-cFw and makC2-cRe. The absence of DNA contamination was confirmed by RT-PCR without reverse transcriptase.

3.3. Results

3.3.1. Features of two putative cytochrome P450 genes, *makC2* and *makC3*

A homology search of the NCBI database demonstrated that MakC2 and MakC3 are significantly similar to the members of cytochrome P450, some of which are involved in the stereo- and regio-specific oxidation of secondary metabolites. More detailed analysis of the amino acid sequences indicated that MakC2 and MakC3 have a highly conserved threonine in the I helix, which has been suggested to play a critical function in oxygen activation in most cytochromes P450 (Yoshigae et al. 2013) (Fig. 3.2a). Similarly, both MakC2 and MakC3 possessed the well conserved K-helix ExxR motif (important to heme-binding and to stabilize protein conformation), and the heme-binding signature amino acid sequence FxxGxxxCxG, in which the cysteine residue serves as the fifth ligand to the heme iron (Werck-reichhart and Feyereisen 2000). These sequence characteristics suggested that both MakC2 and MakC3 can function as a cytochrome P450 monooxygenase. Moreover, phylogenetic analysis of MakC2 and MakC3 with other cytochromes P450 from actinomycetes indicated that MakC2 belongs to the CYP105 family and MakC3 to the CYP107 family (Fig 3.2b). The CYP105 and CYP107

families are the most abundant of the streptomycete CYP families, and are associated with antibiotic biosynthetic pathways and xenobiotic degradation (Lamb et al. 2003), suggesting that MakC2 and/or MakC3 may have functions in the biosynthesis of maklamicin, although their actual roles cannot be adequately predicted by sequence alignment.

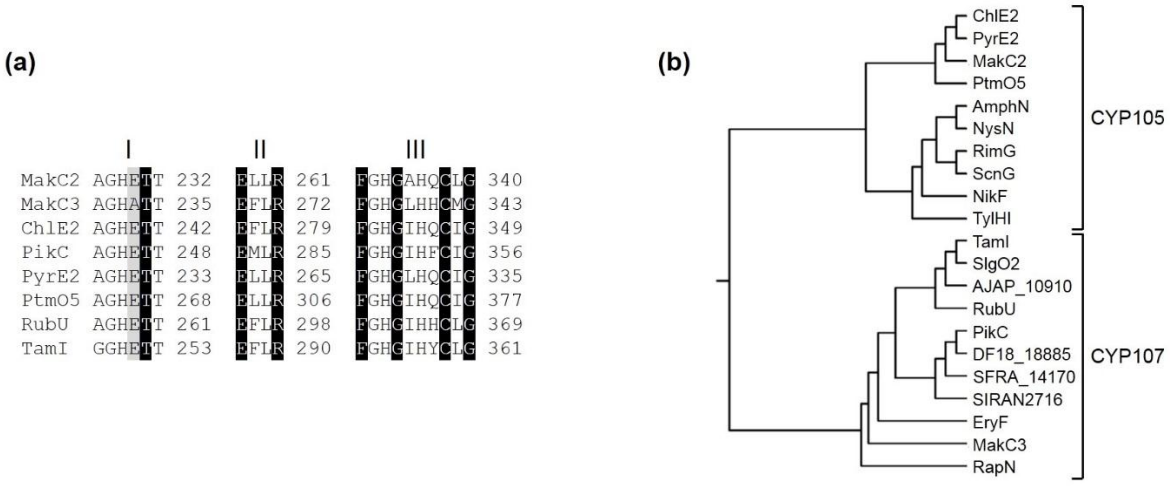

Fig. 3.2. Sequence analysis of MakC2 and MakC3 with cytochromes P450 from actinomycetes. **(a)** Sequence alignment of the conserved domains between MakC2, MakC3 and cytochromes P450 involved in the biosynthesis of polyketide compounds. I, amino acid residues involved in oxygen activation; II, ExxR motif in the K-helix; III, signature sequence (FxxGxxxCxG) for the cytochrome P450 heme binding domain. Active sites in each domain are highlighted in black and light grey. Numbers indicate amino acid positions within each sequence. **(b)** Phylogenetic tree of MakC2, MakC3 and several cytochromes P450 from actinomycetes. Multiple sequence alignment was conducted with the CLUSTALW program. Phylogenetic trees were constructed by the unweighted-pair group method with the arithmetic mean (http://www.genome.jp/tools/clustalw/). CYP105, cytochrome P450 (CYP) 105 family; CYP107, CYP107 family. The names and sequence accession numbers are shown in Appendix.

3.3.2. Effect of disruptions of *makC2* and *makC3* on maklamicin production

To investigate the roles of *makC2* and *makC3* in the biosynthesis of maklamicin, we generated both a *makC2* disruptant (Δ*makC2*) and a *makC3* disruptant (Δ*makC3*) by replacing the respective genes with an *oriT-aac(3)IV* cassette using λRED-mediated PCR-targeted mutagenesis (Fig. 3.3).

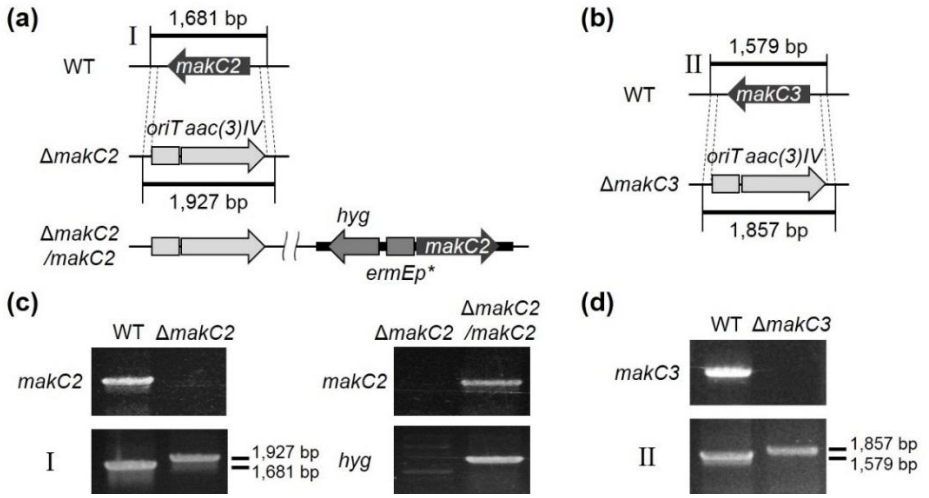

Fig. 3.3. Disruption of the *makC2* and *makC3* genes in *Micromonospora* sp. NBRC 110955 and genetic complementation of the *makC2* disruptant. (A and B) Schematic representation of the strategy for gene disruption of *makC2* **(a)** and *makC3* **(b)**. The *oriT* sequence is the RK2 origin of transfer. The *aac(3)IV* and *hyg* genes confer resistance for apramycin and hygromycin, respectively. **(c)** PCR analysis to confirm gene-disruption of *makC2* and its complementation. An internal fragment (1,001 bp) of the *makC2* gene was detected by using the primer pair makC2-cFw/makC2-cRe (upper panel). With the primer pair makC2-tFw/makC2-tRe, a fragment (I, 1,681 bp) containing an intact *makC2* gene or a fragment (1,927 bp) containing the *oriT-aac(3)IV* cassette was amplified with PCR (lower panel). The *hyg* (753 bp) gene was amplified using the primer pair hyg-Fw/hyg-Re. **(d)** PCR analysis to confirm the gene-disruption of *makC3*. An internal fragment (939 bp) of the *makC3* gene was detected by using the primer pair makC3-cFw/makC3-cRe (upper panel). With the primer pair makC3-tFw/makC3-tRe, a fragment (II, 1,579 bp) containing an intact *makC3* gene or a fragment (1,857 bp) containing the cassette was amplified with PCR (lower panel).

Maklamicin produced in the wild-type strain was readily detectable at the retention time of 15.7 min (Fig. 3.4b), whereas the Δ*makC2* strain completely lost the maklamicin production with concomitant appearance of a new compound at the retention time of 24.7 min (Fig. 3.4c). On the other hand, the disruption of *makC3* had no effect on the production of maklamicin (Fig. 3.4d), indicating that *makC3* is not involved in the biosynthesis of maklamicin. To confirm that the metabolic changes observed in the Δ*makC2* strain resulted solely from the *makC2* disruption, we reintroduced the entire *makC2* gene into the *makC2* disruptant (Fig.3.3). Because a narrow intergenic region (37 bp) between *makC2* and *makR3* has no plausible promoter sequences for the expression of *makC2*, *makC2* may form a bicistronic operon with *makR3*, the latter of which

encodes a putative regulator of the *Streptomyces* antibiotic regulatory protein (SARP) family (Wietzorrek and Bibb 1997). Thus, we constructed the plasmid pLT130, which harboured the *makC2* gene under the control of the strong constitutive *ermEp** promoter. Complementation of the *makC2* disruptant with pLT130 (Δ*makC2*/*makC2*) abolished peak at 24.7 minute and restored the production of maklamicin to the level in the wild-type strain (Fig. 3.4e). These results clearly indicated that *makC2*-encoded cytochrome P450 is essential for the biosynthesis of maklamicin.

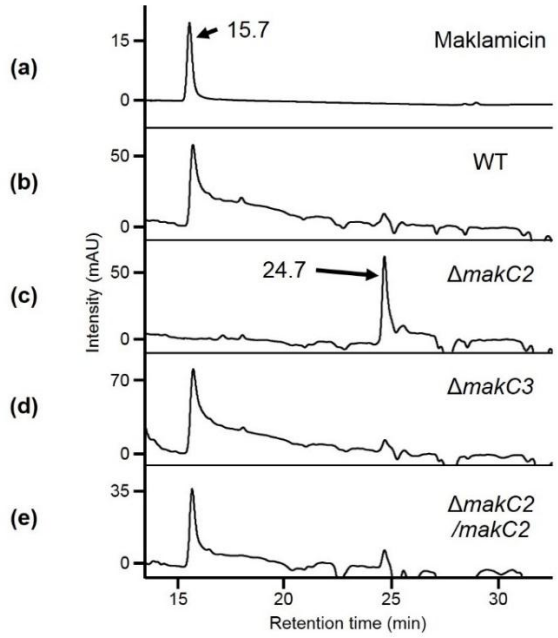

Fig. 3.4. HPLC analysis of metabolites produced by genetically modified strains of *Micromonospora* sp. NBRC 110955. **(a)** Maklamicin, an authentic standard of maklamicin **(b)** WT, wild-type strain **(c)** Δ*makC2*, *makC2* disruptant **(d)** Δ*makC3*, *makC3* disruptant **(e)** Δ*makC2/makC2*, *makC2*-complemented Δ*makC2* strain. mAU, milliabsorbance units at 254 nm. Maklamicin was detected at the retention time of 15.7 min.

3.3.3. Isolation and structure elucidation of compound 2 from the *makC2* disruptant

As described above, the accumulated compound at fig. 3.4c corresponding to peak at 24.7 in the Δ*makC2* strain may be an intermediate in the biosynthesis of maklamicin. Therefore, I purified the new compound from the Δ*makC2* strain and elucidated the structure. After purification, 5.3 mg of purified compound was obtained from 2 L of the culture broth. The molecular formula of compound (fig 3.4c) was deduced to be $C_{32}H_{44}O_5$ by the HRFABMS analysis (positive mode) {*m*/*z* 509.3273 $[M+H]^+$ (calculated exact mass for $C_{32}H_{45}O_5$: 509.6946)}, suggesting that this compound may be a maklamicin analogue lacking one oxygen atom. The UV spectrum of this compound detected in the HPLC/DAD analysis was also

identical to that of maklamicin. Subsequently, the structure of compound was established by the analyses of ^{1}H and ^{13}C NMR data, demonstrating that these NMR spectra were quite similar to those of maklamicin (Table 3.2). However, there were two important differences between the NMR data for this compound and maklamicin, one each involving C-20 and C-29. The chemical shift of the C-20 quaternary carbon of this compound was slightly upfield shifted to 133.2 ppm from that of 137.0 ppm for maklamicin. Notably, the functional group of C-29 was found to be a methyl (δ_C 21.9, δ_H 1.75), while maklamicin has a hydroxymethyl at the same position (δ_C 65.0, δ_H 3.99, 4.34). Together with the detection of key HMBC correlations for the primary frame of maklamicin, strong HMBC correlations were observed from the C-29 methyl protons to carbons at C-19, C-20 and C-21 (Fig. 3.5). Based on these findings, together with the data of ^{1}H-^{1}H COSY and HSQC correlation, I confirmed the structure of compound from fig 3.4c as 29-deoxymaklamicin (Fig. 3.1). (See Appendix for the definition of abbreviations).

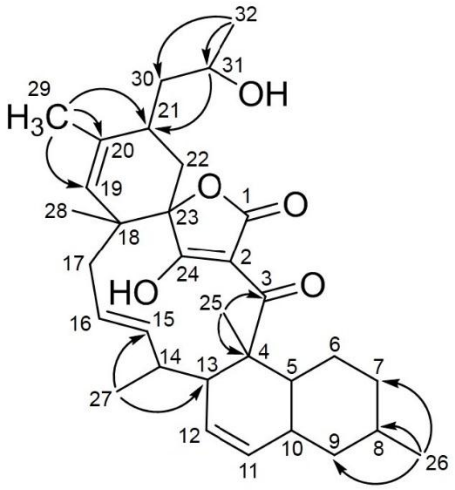

Fig. 3.5. Key HMBC correlations (H→C) of 29-deoxymaklamicin.

3.3.4. Biological activity of 29-deoxymaklamicin

To characterize the biological activity of 29-deoxymaklamicin as a new maklamicin analogue, I evaluated anti-microbial activity against 7 kinds of microorganisms previously tested (Table 3.3)

Table 3.2. NMR spectroscopic data of 29-deoxymaklamicin and maklamicin in $CDCl_3$

Position	29-Deoxymaklamicin			Maklamicin[b]	
	δ_C	δ_H mult (*J* in Hz)	HMBC[a]	δ_C	δ_H
1	167.0	-	-	167.0	-
2	107.3	-	-	107.2	-
3	201.8	-	-	201.9	-
4	50.6	-	-	50.6	-
5	42.2	1.48, dd (9.8, 10.7)	4, 7, 25	42.2	1.47
6	23.1	1.231, m	-	23.1	1.23
		2.02, m	-		2.00
7	32.73	1.575, m	26	32.71	1.57
		1.73, m	6, 8		1.73
8	27.7	2.08, m	-	27.7	2.08
9	39.4	1.41, m	5, 8, 10	39.5	1.40
		1.59, m	5		1.59
10	32.71	2.01, m	-	32.68	1.98
11	130.4	5.38, d (10.0)	10	130.38	5.38
12	125.0	5.45, ddd (10.0, 5.9, 2.4)	4, 10, 13	125.0	5.46
13	40.9	2.83, dd (5.34, 5.34)	4, 5, 11, 12, 14, 15, 25	41.0	2.83
14	39.8	1.85, m	4, 15, 16,	39.8	1.85
15	144.1	5.50, ddd (15.4, 9.6, 2.0)	5, 13	144.4	5.49
16	121.1	5.01, m (overlap)	14,17	120.7	5.01
17	42.3	1.91, br d (14.3)	15, 16, 18, 23	42.0	1.93
		2.38, dd (14.1, 11.6)	15, 16, 18, 28		2.37
18	39.2	-	-	39.1	-
19	129.7	5.02, s	18, 21, 23, 29	130.41	5.22
20	133.2	-	-	137.0	-
21	32.9	2.43, m (overlap)	-	28.6	2.74
22	29.6	1.76 (overlap)	21, 23, 30	31.2	1.72
		2.32, dd (14.5, 7.5)	21, 30		2.36
23	86.1	-	-	86.1	-
24	204.5	-	-	204.3	-
25	15.8	1.579, s	3, 4	15.8	1.57
26	18.9	1.04 d (7.2)	7, 8, 9	18.9	1.03
27	20.7	0.86, d (7.3)	13, 15	20.7	0.86
28	23.7	1.22, s	19, 23	23.4	1.25
29	21.9	1.75, s	19, 20, 21	65.0	3.99

Table 3.2. Continue

Position	29-Deoxymaklamicin			Maklamicin[b]	
	δ_C	δ_H mult (*J* in Hz)	HMBC[a]	δ_C	δ_H
30	41.3	1.65, dd (16.7, 10.9)	20, 21, 31, 32	41.6	1.67
		1.83, dd (14.3, 11.6)	20, 21, 22, 32		1.80
31	65.5	3.85, m	21	66.6	3.82
32	24.6	1.226, d (6.12)[c]	30, 31	24.6	1.21

[a] HMBC correlations are from proton(s) to the indicated carbon.

[b] Data from Igarashi *et al.* (4)

[c] *J*-coupling constant calculated from ^{1}H-NMR measured in 600 MHz using CD_3OH as a solvent.

29-Deoxymaklamicin showed an anti-Gram-positive bacteria spectrum comparable to that of maklamicin. Interestingly, the activity against *Staphylococcus aureus* NBRC12732 was 1.6 μg/mL as an MIC value, which was significantly lower than that of maklamicin (13 μg/mL). On the other hand, 29-deoxymaklamicin showed no activity against Gram-negative bacteria strain *E. coli* NIH-JC2 and yeast *C. albicans* NBRC1594, similar to maklamicin. These results suggested that the functional group at C-29 appears to be significant for exerting the activity against *Staphylococcus aureus*.

Table 3.3. Antimicrobial activity of 29-deoxymaklamicin and maklamicin

	MIC (μg ml^{-1})	
Microorganisms	29-Deoxymaklamicin	Maklamicin[a]
Micrococcus luteus ATCC9343	0.8	0.2
Staphylococcus aureus NBRC12732	1.6	13
Bacillus subtilis PCI219	3.1	1.7
Bacillus cereus NBRC15305	6.3	6.5
Enterococcus faecalis NBRC100480	6.3	13
Candida albicans NBRC1594	>100	50
Escherichia coli NIH-JC2	>100	>50

[a] Data from Igarashi *et al.*

3.3.5. Bioconversion of 29-deoxymaklamicin to maklamicin in recombinant *S. avermitilis* expressing *makC2*

According to the structure of 29-deoxymaklamicin, I assumed that *makC2* gene might function as the hydroxylase in maklamicin biosynthesis. Therefore, to confirm that MakC2 catalyses specific hydroxylation at C-29 *in vivo*, I performed a bioconversion study using *S. avermitilis* SUKA22 carrying the plasmid pLT130, which was used for genetic complementation of the *makC2* disruptant. The bioconversion using 29-deoxymaklamicin as the substrate with the recombinant *S. avermitilis* strain, in which heterologous expression of *makC2* was successfully achieved (Fig. 3.6), demonstrated that 29-deoxymaklamicin was converted to maklamicin (retention time of 16.3 min) (Fig. 3.7), while no such conversion was observed without 29-deoxymaklamicin. The UV-visible spectrum of the newly appeared peak exhibited the same maximum UV absorption as maklamicin (Fig. 3.8). In contrast, the parental *S. avermitilis* strain incubated with 29-deoxymaklamicin showed no conversion, indicating the *makC2*-dependent conversion to maklamicin. These results strongly indicated that MakC2 is the regio-specific hydroxylase converting 29-deoxymaklamicin into maklamicin *in vivo*, as a

last tailoring step of the maklamicin biosynthetic pathway.

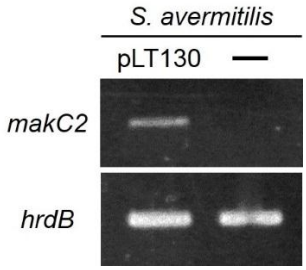

Fig. 3.6. Heterologous expression of *makC2* in *S. avermitilis* SUKA22. Transcription of the introduced *makC2* gene was measured by semiquantitative RT-PCR. The expression of *makC2* was examined in the *S. avermitilis* SUKA22 harbouring pLT130 (left lane) or no plasmid (right lane). The *hrdB*-like gene [*hrdB* encodes the major sigma factor in *Streptomyces coelicolor* A3(2)] was used as a control.

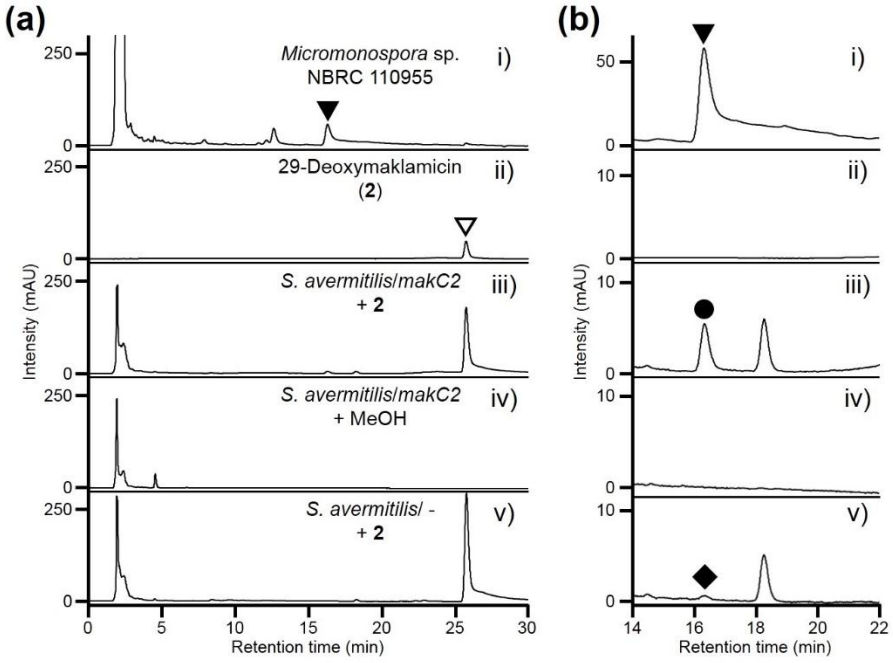

Fig. 3.7. Bioconversion of 29-deoxymaklamicin by recombinant *S. avermitilis* strains. **(a)** HPLC chromatograms of crude extracts in the resting cell reaction are shown. **(b)** The enlargement of HPLC chromatogram of **(a)** from 14 to 22 min. (i) Culture extract of *Micromonospora* sp. NBRC 110955; (ii) purified 29-deoxymaklamicin used as a standard; (iii) *S. avermitilis* harbouring *makC2* incubated with 29-deoxymaklamicin; (iv) *S. avermitilis* harbouring *makC2* incubated with MeOH; (iv) *S. avermitilis* incubated with 29-deoxymaklamicin. Authentic maklamicin and 29-deoxymaklamicin were detected at the retention times of 16.3 (filled inverted triangle) and 25.6 min (open inverted triangle), respectively.

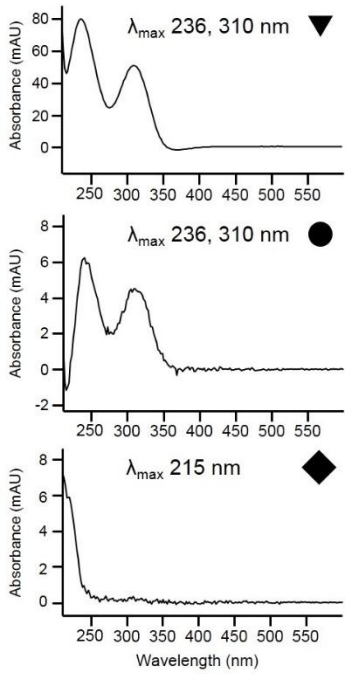

Fig. 3.8. The UV-visible spectrum of the peak appeared after incubation of *S. avermitilis* harboring *makC2* with 29- deoxymaklamicin. The symbols are identical to those indicated in Fig. 3.7; filled inverted triangle, peak at 16.3 min in panel i); filled circle, peak at 16.3 min in panel iii); filled diamond, peak at 16.3 min in panel v).

3.4. Discussion

The biosynthetic mechanism of the spirotetronate-class antibiotics has been shown to include a polyketide assembly line, a glycerate utilization system, a sugar modification, and a novel [4+2]-cycloaddition system for the spirotetronate skeleton (Hashimoto et al. 2015; Tian et al. 2015; Vieweg et al. 2014). However, there has been no report on systems for the modification of peripheral moieties on the main skeletons of these compounds. I recently identified the *mak* gene cluster and, based on this information, proposed a biosynthetic pathway for maklamicin. I have shown here that MakC2, a probable cytochrome P450 monooxygenase, catalyses a region-specific hydroxylation at the C-29 methyl group of 29-deoxymaklamicin via gene inactivation, structural elucidation of 29-deoxymaklamicin, and resting cell conversion. In contrast, MakC3, another cytochrome P450 in the *mak* cluster, is not involved in this biosynthetic process, and appears to be responsible for the production of another, as yet unidentified, previously proposed polyketide compound. The biosynthetic gene clusters of kijanimicin, versipelostatin, lobophorin, and chlorothricin (Hashimoto et al. 2015; Jia et al. 2006; Li et al. 2013; Zhang et al. 2007), all of which are spirotetronates containing the *trans*-

decalin moiety, are also found to have cytochrome P450 genes such as *kijA3*, *vstD*, *lobP1*, and *chlE2*, respectively. These genes have been proposed to be responsible for introducing a hydroxy group on each of their own core structures (*kijA3* for C-20 of kijanimicin, *vstD* for C-37 of versipelostatin, and *lobP1* for C-32 of lobophorin) or the oxidation of a methyl group to form a carboxy group (*chlE2* for C-20 of chlorothricin), without any functional characterization. Therefore, this work represents the first functional analysis of cytochrome P450 genes embedded in the biosynthetic gene cluster of spirotetronate antibiotics, and could provide information useful for deciphering tailoring steps in the biosynthesis of these spirotetronate antibiotics.

With respect to biological activity, 29-deoxymaklamicin showed an antimicrobial spectrum similar to that of maklamicin: both of them exhibited strong-to-modest anti-Gram-positive activities and no activity against Gram-negative bacteria and yeast. It is interesting that the lack of a C-29 hydroxy group in 29-deoxymaklamicin enhanced the activity to *Staphylococcus aureus*. Jia *et al.* (Jia et al. 2006) demonstrated that demethylsalicycloyl chlorothricin synthesized by a genetically engineered strain of the chlorothricin producer showed decreased biological activity in comparison to that of chlorothricin. However, little information is currently available on the degree to which the level of post-PKS modifications may affect the biological activity of spirotetronate antibiotics. Thus, 29-deoxymaklamicin and the cognate hydroxylase MakC2 would be useful for analysing the mode of action underlying the anti-Gram-positive activity of not only maklamicin, but also the spirotetronate antibiotics.

3.5. Summary

In this chapter, I characterized the function of cytochrome P450 encoded by *makC2* and *makC3* by gene inactivation. Gene deletion of *makC2* resulted in the complete loss maklamicin production and then created a new maklamicin analogue [29-deoxymaklamicin]. In contrast, gene deletion of makC3 did not affect the maklamicin production. 29-Deoxymaklamicin showed strong-to-modest anti-microbial activity against Gram-positive bacteria notably the activity against *Staphylococcus aureus*. Moreover, the bioconversion of 29-deoxymaklamicin into maklamicin by resting cell assay confirmed that MakC2 is the final-step hydroxylase in the formation of mature maklamicin.

Chapter 4

General conclusions

Actinomycetes are Gram-positive, filamentous and high-GC-content bacteria that display two typical features. The first is the complex life cycle of morphological differentiation, and the second is the ability to produce numerous types of secondary metabolites. Approximately two-thirds of the important antibiotics produced by actimomycetes are produced by the bacteria in the genus *Streptomyces.* However, non-*Streptomyces* actinomycetes (rare actinomycetes) are also promising sources of secondary metabolites and have attracted interest from the viewpoint of providing novel natural products.

A significant genus of the rare-actinomycetes group is *Micromonospora*. This genus displays a complex life cycle, differentiating into both substrate mycelia and spores, but no aerial mycelia are produced. *Micromonospora* species are prolific sources of various bioactive metabolites, second only to *Streptomyces*. The *Micromonospora* strains are well known for producing antibiotics, especially aminoglycosides, enediynes and oligosaccharides.

Secondary metabolites are compounds that are mostly low-molecular-weight but chemically diverse compounds. Secondary metabolites can be divided into several groups, and one of the major groups of secondary metabolites is polyketide compounds. Polyketides exhibit an incredible range of functional and structural diversity with significant activities including antibacterial, anticancer, antifungal, antiparasitic and immunosuppressive properties. Polyketides are produced by polyketide synthases (PKSs) by Claisen condensation of acyl-CoA precursors to generate β-ketoacyl, and then further modified by different reduction activities such as those of ketoreductase, dehydratase and enoly-reductase.

After the polyketide scaffold has been synthesized, post-PKS tailoring modification is an important step that changes the polyketide core structure. Modifications such as the introduction

of a hydroxyl group or methyl group or glycosyl transfer are essential for altering the biological activities of these groups of natural products.

Spirotetronates are polyketide-class compounds possessing an unusual structure composed of a tetronic acid moiety *spiro*-linked with a cyclohexene ring, sometimes conjugated to a *trans*-decalin unit. The compounds in this class exhibit a broad range of molecular targets and biological (e.g., antimicrobial, antitumor, antiviral and cholesterol biosynthesis-inhibiting) activities. To date, nearly 60 related compounds in the spirotetronate family have been reported, and their structural diversity was shown to be derived mostly from the glycosylation pattern and the length of the carbon chain connecting the decalin unit and cyclohexene ring.

Based on the size of its ring system and its molecular weight, maklamicin was classified as a small spirotetronate compound, consisting of an 11-member system. Among the nearly 60 known compounds, maklamicin has two structural features that are unique from those of other spirotetronates. (1) The four-carbon chain linker of maklamicin is the shortest in this family. (2) The presence of a 2*R*-hydroxy-propyl group at C-21, which creates the stereogenic center at C-31 on the cyclohexene ring, has not been reported. Based on the structural uniqueness of maklamicin in the family of spirotetronate antibiotics, I then attempted to elucidate the biosynthetic mechanism of maklamicin at the molecular level.

This thesis is divided into four chapters, organized as follows. The basic knowledge related to my study is given in Chapter 1, with a general introduction describing Actinomycetes, the genus *Micromonospora*, secondary metabolites, and the classification of compounds based on their core component, with a focus on polyketide compounds. The known information about polyketide compounds and polyketide synthases together with detailed findings regarding tetronate and spirotetronate compounds are summarized in this chapter.

In Chapter 2, I report the identification and characterization of the biosynthetic (*mak*) gene cluster in maklamicin. To increase our understanding of the structural uniqueness of

maklamicin, I sought to elucidate the maklamicin biosynthetic gene cluster through genome sequencing and library screening. The sequence analysis revealed that a plausible maklamicin cluster covers a 152-kb DNA region encoding 46 open reading frames (ORFs). Twenty-four genes can be assigned roles in the biosynthesis of maklamicin, including a polyketide backbone, spirotetronate and peripheral moieties, self-resistance, and the regulation of maklamicin production. Disruption of the PKS genes, resulting in a complete loss of maklamicin production, indicated that a type I modular PKS system is responsible for the biosynthesis of maklamicin. Moreover, the *mak* gene cluster contained a set of biosynthetic genes for the formation of a tetronate moiety, and these genes were found to be highly conserved in the gene clusters for spirotetronate. Subsequently, I propose a hypothesis regarding the biosynthesis of maklamicin based on the estimated gene cluster. In this proposed biosynthesis, the inactivation of the dehydratase (DH) domain at the initial module in the PKS assembly line causes the structural variation in maklamicin, which makes maklamicin unique from other spirotetronates.

Chapter 3 describes my investigation of the function of the genes involved in the post-PKS modification of maklamicin biosynthesis. One tailoring step in the biosynthesis is predicted to be post-PKS modification, which appears to be catalyzed by the putative cytochrome P450 monooxygenases MakC2 and/or MakC3. The gene disruption of *makC2* resulted in the complete loss of maklamicin production with a simultaneous accumulation of a new compound (29-deoxymaklamicin), whereas the gene disruption of *makC3* did not affect the maklamicin production. The structure of 29-deoxymaklamicin, which was obtained from a *makC2* disruption strain, shows the presence of a methyl group at C-29 instead of a hydroxy group in maklamicin. Moreover, the bioconversion of 29-deoxymaklamicin to maklamicin in recombinant *Streptomyces avermitilis* SUKA22 indicated that MakC2 is a post-PKS modification enzyme, responsible for hydroxylation in maklamicin biosynthesis. This information suggested that 29-deoxymaklamicin is an intermediate in the biosynthetic pathway

of maklamicin and would serve as the substrate of maklamicin. Additionally, 29-deoxymaklamicin displayed antibacterial activity against Gram-positive bacteria, with enhanced activity against *Staphylococcus aureus*. These results imply that altering the post-PKS tailoring pathway can be an alternative way to generate compounds with different biological activities.

In this study, the biosynthetic gene cluster of maklamicin, a spirotetronate-class polyketide antibiotic, was identified from the endophytic *Micromonospora* sp. NBRC 110955. Based on the maklamicin gene cluster, I was able to gain more insight into the biosynthesis of spirotetronate compounds and to increase our understanding of the detailed mechanisms underlying the structural changes in maklamicin. This understanding could be applied in other spirotetronates that feature the same structural variation. The information about the maklamicin gene cluster could contribute to the creation of new maklamicin analogues.

In addition, this study is the first report of the genetic inactivation of cytochrome P450 in the spirotetronate family, and I expect that the genetic engineering of this inactive cytochrome P450 will open the way to the creation of more spirotetronates with structural diversity and significantly improved properties. Lastly, my comparative analysis of the maklamicin gene cluster with those of other spirotetronates provides information about conserved genes that could be useful in genome mining and screening for the rapid identification of actinobacteria that produce new spirotetronates.

References

Aparicio JF, Mendes M V, Anton N, Recio E, Martin JF (2004) Polyene macrolide antibiotic biosynthesis. Curr Med Chem 11:1645–1656.

Aroonsri A, Kitani S, Hashimoto J, Kosone I, Izumikawa M, Komatsu M, Fujita N, Takahashi Y, Shin-ya K, Ikeda H, Nihira T (2012) Pleiotropic control of secondary metabolism and morphological development by KsbC, a butyrolactone autoregulator receptor homologue in *Kitasatospora setae*. Appl Environ Microbiol 78:8015–8024.

Berdy J (2005) Bioactive microbial metabolite. J Antibiot 58:1–26.

Bisang C, Long PF, Cortés J, Westcott J, Crosby J, Matharu a L, Cox RJ, Simpson TJ, Staunton J, Leadlay PF (1999) A chain initiation factor common to both modular and aromatic polyketide synthases. Nature 401:502–505.

Caffrey P (2003) Conserved amino acid residues correlating with ketoreductase stereospecificity in modular polyketide synthases. Chembiochem 4:649–662.

Cane DE (2010) Programming of erythromycin biosynthesis by a modular polyketide synthase. J Biol Chem 285:27517–27523.

Chan MCY, Keasling JD (2006) Production of isoprenoid pharmaceuticals by engineered microbe. Nat Chem Biol 2:674–681.

Chater KF, Losick R (1997) The mycelial life-style of *Streptomyces coelicolor* A3(2) and its relatives. In Bacteria as multicellular organisms. 3:149–182.

Chen S, Mao X, Shen Y, Zhou Y, Li J, Wang L, Tao X, Yang L, Wang Y, Zhou X, Deng Z, Wei D (2009) Tailoring the P450 monooxygenase gene for FR-008/candicidin biosynthesis. Appl Environ Microbiol 75:1778–81.

Choi K, Jung E, Jung D, An B, Pandey BP, Yun H, Sung C, Park H, Kim B (2012) Engineering of daidzein 3'-hydroxylase P450 enzyme into catalytically self-sufficient cytochrome P450. Microb Cell Fact 11:81–90.

Davidson AL, Chen J (2004) ATP-binding cassette transporters in bacteria. Annu Rev Biochem 73:241–268.

Fang J, Zhang Y, Huang L, Jia X, Zhang Q, Zhang X, Tang G, Liu W (2008) Cloning and characterization of the tetrocarcin A gene cluster from *Micromonospora chalcea* NRRL 11289 reveals a highly conserved strategy for tetronate biosynthesis in spirotetronate antibiotics. J Bacteriol 190:6014–6025.

Farina V, Brown JD (2006) Tamiflu: The supply problem. Angew Chemie - Int Ed 45:7330–7334.

Floss HG, Yu T-W (2005) Rifamycin-mode of action, resistance, and biosynthesis. Chem Rev 105:621–632.

Gottardi EM, Krawczyk JM, Suchodoletz H von, Schadt S, Mühlenweg A, Uguru GC, Pelzer S, Fiedler H-P, Bibb MJ, Stach JEM, Süssmuth RD (2011) Abyssomicin biosynthesis: formation of an unusual polyketide, antibiotic-feeding studies and genetic analysis. Chembiochem 12:1401–1410

Gust B, Challis GL, Fowler K, Kieser T, Chater KF (2003) PCR-targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. Proc Natl Acad Sci USA 100:1541–1546.

Hamaguchi T, Sudo T, Osada H (1995) RK-682, a potent inhibitor of tyrosine phosphatase, arrested the mammalian cell cycle progression at G1phase. FEBS Lett 372:54–58.

Hashimoto T, Hashimoto J, Teruya K, Hirano T, Shin-Ya K, Ikeda H, Liu H-W, Nishiyama M, Kuzuyama T (2015) Biosynthesis of versipelostatin: Identification of an enzyme-catalyzed [4+2]-cycloaddition required for macrocyclization of spirotetronate-containing polyketides. J Am Chem Soc 137:572–575.

He J, Magarvey NA, Piraee M, Vining LC (2001) The gene cluster for chloramphenicol biosynthesis in *Streptomyces venezuelae* ISP5230 includes novel shikimate pathway homologues and monomodular non-ribosomal peptide synthase gene. Microbiology 147:2817–2829.

He M, Varoglu M, Sherman DH (2000) Structural modeling and site-directed mutagenesis of the actinorhodin β-ketoacyl-acyl carrier protein synthase. J Bacteriol 182:2619–2623.

Heathcote M, Staunton J, Leadlay P (2001) Role of type II thioesterases: evidence for removal of short acyl chains produced by aberrant decarboxylation of chain extender units. Chem Biol 8:207–220.

Hegde VR, Patel MG, Das PR, Premanik B, Puar MS (1997) A family of novel macrocyclic lactones, the saccharocarcins produced by *Saccharothrix aerocolonigenes* subsp. *antibiotica.* II. Physico-chemical properties and structure determination. J Antibiot 50:126–134.

Hirsch AM, Valdés M (2010) *Micromonospora*: An important microbe for biomedicine and potentially for biocontrol and biofuels. Soil Biol Biochem 42:536–542.

Huang F, Haydock SF, Mironenko T, Spiteller D, Spencer JB (2005) The neomycin biosynthetic gene cluster of *Streotomyces fradiae* NCIMB 8233: characterisation of an aminotransferase involved in the formation of 2-deoxystreptamine. Org Biomol Chem 3:1410–1419.

Igarashi Y, Iida T, Oku N, Watanabe H, Furihata K, Miyanouchi K (2012) Nomimicin, a new spirotetronate-class polyketide from an actinomycete of the genus *Actinomadura*. J Antibiot 65:355–359.

Igarashi Y, Ogura H, Furihata K, Oku N, Indananda C, Thamchaipenet A (2011) Maklamicin, an antibacterial polyketide from an endophytic *Micromonospora* sp. J Nat Prod 74:670–674.

Jia XY, Tian ZH, Shao L, Qu XD, Zhao QF, Tang J, Tang GL, Liu W (2006) Genetic characterization of the chlorothricin gene cluster as a model for spirotetronate antibiotic biosynthesis. Chem Biol 13:575–585.

Kanchanabanca C, Tao W, Hong H, Liu Y, Hahn F, Samborskyy M, Deng Z, Sun Y, Leadlay PF (2013) Unusual acetylation-elimination in the formation of tetronate antibiotics. Angew Chem Int Ed Engl 52:5785–5788.

Kasahara K, Miyamoto T, Fujimoto T, Oguri H, Tokiwano T, Oikawa H, Ebizuka Y, Fujii I (2010) Solanapyrone synthase, a possible Diels-Alderase and iterative type I polyketide synthase encoded in a biosynthetic gene cluster from *Alternaria solani*. Chembiochem 11:1245–52.

Kawashima A, Nakamura Y, Ohta Y, Akama T, Yamagishi M, Kazunori H (1991) New cholresterol biosynthesis inhibitors MC-031 (*O*-demethylchlorothricin), -032 (*O*-demethylhydroxychlorothricin) -033 and -034. J Antibiot 45:207–212.

Kitani S, Ikeda H, Sakamoto T, Noguchi S, Nihira T (2009) Characterization of a regulatory gene, *aveR*, for the biosynthesis of avermectin in *Streptomyces avermitilis*. Appl Microbiol Biotechnol 82:1089–1096.

Knaggs AR (2003) The biosynthesis of shikimate metabolites. Nat Prod Rep 20:119–136.

Komatsu M, Komatsu K, Koiwai H, Yamada Y, Kozone I, Izumikawa M, Hashimoto J, Takagi M, Omura S, Shin-ya K, Cane DE, Ikeda H (2013) Engineered *Streptomyces avermitilis* host for heterologous expression of biosynthetic gene cluster for secondary metabolites. ACS Synth Biol 2:384–396.

Komatsu M, Uchiyama T, Omura S, Cane DE, Ikeda H (2010) Genome-minimized Streptomyces host for the heterologous expression of secondary metabolism. Proc Natl Acad Sci USA 107:2646–2651.

Kumar RMN V, Muzzarelli RAA, Sashiwa H, Domb AJ (2004) Chitosan chemistry and phamaceutical perspective. Chem Rev 104:6017–6084.

Kusumi T, Ichikawa A, Kakisawa H, Tsunakawa M, Konishi M, Oki T (1991) The structures of quartromicin A1, A2 and A3: novel macrocyclic antiviral antibiotics possessing four tetronic acid moieties. J Am Chem Soc 113:8947–8948.

Lai JR, Koglin A, Walsh CT (2006) Carrier protein structure and recognition in polyketide and nonribosomal peptide. Biochemistry 45:14869–14879.

Lamb DC, Ikeda H, Nelson DR, Ishikawa J, Skaug T, Jackson C, Omura S, Waterman MR, Kelly SL (2003) Cytochrome P450 complement (CYPome) of the avermectin-producer *Streptomyces avermitilis* and comparison to that of *Streptomyces coelicolor* A3(2). Biochem Biophys Res Commun 307:610–619.

Lamb DC, Waterman MR, Zhao B (2013) *Streptomyces* cytochromes P450: applications in drug metabolism. Expert Opin Drug Metab Toxicol 9:1279–1294.

Lazzarini A, Cavaletti L, Toppo G, Marinelli F (2001) Rare genera of actinomycetes as potential producers of new antibiotics. Antonie Van Leeuwenhoek 399–405.

Li C, Roege KE, Kelly WL (2009) Analysis of the indanomycin biosynthetic gene cluster from Streptomyces antibioticus NRRL 8167. Chembiochem 10:1064–1072.

Li S, Xiao J, Zhu Y, Zhang G, Yang C, Zhang H, Ma L, Zhang C (2013) Dissecting glycosylation steps in lobophorin biosynthesis implies an iterative glycosyltransferase. Org Lett 15:1374–1377.

Magnet S, Blanchard JS (2005) Molecular insights into aminoglycoside action and resistance molecular insights into aminoglycoside action and resistance. Chem Rev 105:477–498.

Mascher T, Helmann JD, Unden G (2006) Stimulus perception in bacterial signal-transducing histidine kinases. Microbiol Mol Biol Rev 70:910–938.

Matsumoto M, Kawamura Y, Yoshimura Y, Nakai H, Yoshida T, Shoji J (1989) Isolation, characterization and structures of PA-46101 A and B. J Antibiot 43:739–747.

Momose I, Hirosawa S, Nakamura H, Naganawa H, Iinuma H, Ikeda D, Takeuchi T (1999) Decatromicins A and B , new antibiotics produced by *Actinomadura* sp. MK73-NF4 II . Structure determination. J Antibiot 52:787–796.

Moss SJ, Martin CJ, Wilkinson B (2004) Loss of co-linearity by modular polyketide synthases: a mechanism for the evolution of chemical diversity. Nat Prod Rep 21:575–593.

Murugan E, Kong R, Sun H, Rao F, Liang Z-X (2010) Expression, purification and characterization of the acyl carrier protein phosphodiesterase from *Pseudomonas Aeruginosa*. Protein Expr Purif 71:132–138.

Ohtsubo Y, Ohtsubo WI, Nagata Y, Tsuda M (2008) GenomeMatcher: a graphical user interface for DNA sequence comparison. BMC Bioinformatics 9:376–384.

Oikawa H, Tokiwano T (2004) Enzymatic catalysis of the Diels-Alder reaction in the biosynthesis of natural products. Nat Prod Rep 21:321–352.

Olano C, Méndez C, Salas J a. (2010) Post-PKS tailoring steps in natural product-producing actinomycetes from the perspective of combinatorial biosynthesis. Nat Prod Rep 27:571.

Paget MSB, Chamberlin L, Atrih A, Foster SJ, Buttner MJ (1999) Evidence that the extracytoplasmic function sigma factor σ^E is required for normal cell wall structure *Streptomyces coelicolor* A3 (2). J Bacteriol 181:204–211.

Pal S (2006) A journey across the sequential development of macrolides and ketolides related to erythromycin. Tetrahedron 62:3171–3200.

Park HR, Chijiwa S, Furihata K, Hayakawa Y, Shin-Ya K (2007) Relative and absolute configuration of versipelostatin, a down-regulator of molecular chaperone GRP78 expression. Org Lett 9:1457–1460.

Park HR, Furihata K, Hayakawa Y, Shinya K (2002) Versipelostatin, a novel GRP78/Bip molecular chaperone down-regulator of microbial origin. Tetrahedron Lett 43:6941–6945.

Paul MD (2009) Medicinal Natural Products: A Biosynthetic Approach. 3rd edn. John Willey and Sons Ltd. United Kingdom.

Pulsawat N, Kitani S, Nihira T (2007) Characterization of biosynthetic gene cluster for the production of virginiamycin M, a streptogramin type A antibiotic, in *Streptomyces virginiae*. Gene 393:31–42.

Putman M, Veen HW van, Konings WN (2000) Molecular properties of bacterial multidrug transporters. Microbiol Mol Biol Rev 64:672–693.

Raja A, Prabakarana P (2011) Actinomycetes and drug- an overview. Am. J. Drug Discov. Dev. 2:75–85.

Ramos JL, Martines-Bueno M, Molina-Henares AJ, Teran W, Watanabe K, Zhang X, Gallegos MT, Brennan R, Tobes R (2005) The TetR family of transcriptional repressors. Microbiol Mol Biol Rev 69:326–356.

Riedlingera J, Reickea A, Zahnerb H, Krismerc B, Bulld AT, Maldonadoe LA, Warde AC, Goodfellow M, Bister B, Bischoff D, Sussmuth RD, Fiedler HP (2004) Abyssomicins, inhibitors of the para-aminobenzoic acid pathway produced by the marine *Verrucosispora* strain AB-18-032. J Antibiot 57:271–279.

Rix U, Fischer C, Remsing LL, Rohr J (2002) Modification of post-PKS tailoring steps through combinatorial biosynthesis. Nat Prod Rep 19:542–580.

Sacchettini JC, Poulter CD (1997) Creating isoprenoid discovery. Science (80-) 277:1788–1789.

Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual. 3rd edn. Cold spring Harbor Laboratory. Cold Spring Harbor.

Schwarzer D, Finking R, Marahiel M A (2003) Nonribosomal peptides: from genes to products. Nat Prod Rep 20:275–287.

Shen B (2003) Polyketide biosynthesis beyond the type I, II and III polyketide synthase paradigms. Curr Opin Chem Biol 7:285–295.

Shimotohno KW, Endo T, Furihata K (1993) Antibiotic AC6H, a new component of tetrocarcin group antibiotics. J Antibiot 46:682–684.

Shoji J, Sakazaki R, Hattori T, Mattsumoto K, Uotani N, Yoshida T (1989) Isolation and characterization of agglomerins A, B, C and D. J Antibiot 42:1729–1733.

Sieber S a, Marahiel M A (2005) Molecular mechanisms underlying nonribosomal peptide synthesis: approaches to new antibiotics. Chem Rev 105:715–738.

Staunton J, Weissman KJ (2001) Polyketide biosynthesis: a millennium review. Nat Prod Rep 18:380–416.

Takeda K, Kawanishi E, Nakamura H, Yoshii E (1991) Total synthesis of tetronolide, the aglycon of tetrocarcins. Tetrahedron Lett 32:4925–4928.

Tian Z, Sun P, Yan Y, Wu Z, Zheng Q, Zhou S, Zhang H, Yu F, Jia X, Chen D, Mándi A, Kurtán T, Liu W (2015) An enzymatic [4+2] cyclization cascade creates the pentacyclic core of pyrroindomycins. Nat Chem Biol 11:259–268.

Tiwari K, Gupta RK (2012) Rare actinomycetes: a potential storehouse for novel antibiotics. Crit Rev Biotechnol 32:108–132.

Tomita F, Tamaoki T, Shirahata K, Kasai M, Morimoto M, Ohkubo S, Mineura K, Ishii S (1980) Novel antitumor antibiotics, tetrocarcins. J Antibiot 45:668–670.

Turner WB, Aldridge DC (1984) Book Review:Fungal Metabolites II. Q Rev Biol.

Unwin J, Standage S, Alexander D, Hosted TJ, Horan AC, Wellington EMH (2004) Gene cluster in *Micromonospora echinospora* ATCC15835 for the biosynthesis of the gentamicin C complex. J Antibiot 57:436–445.

Vieweg L, Reichau S, Schobert R, Leadlay PF, Süssmuth RD (2014) Recent advances in the field of bioactive tetronates. Nat Prod Rep 31:1554–1584.

Vorbis G (1992) The genus Actinoplanes and related genera. Procaryotes A Handb Biol Bact Ecophysiol Isol identification, Appl 2:1029–1060.

Waitz J, Horan AC, Kalyanpur M, Lee BK, Loebenberg D, Marquez JA, Miller G, Patel MG (1981) Kijanimicin (Sch 25663), a novel antibiotic produced by *Actinomadura kijaniata* SCC 1256. Fermentation, isolation, characterization and biological properties. J Antibiot 34:1101–1106.

Werck-reichhart D, Feyereisen R (2000) Protein family review Cytochromes P450 : a success story. Adv Genome Biol 1:1–9.

Wietzorrek A, Bibb MJ (1997) A novel family of proteins that regulates antibiotic production in streptomycetes appears to contain an OmpR-like DNA-binding fold. Mol Microbiol 25:1181–1184.

Wu J, Zaleski TJ, Valenzano C, Khosla C, Cane DE (2005) Polyketide double bond biosynthesis. Mechanistic analysis of the dehydratase-containing module 2 of the picromycin/methymycin polyketide synthase. J Am Chem Soc 127:17393–17404.

Yoshigae Y, Kent UM, Hollenberg PF (2013) Role of the highly conserved threonine in cytochrome P450 2E1: Prevention of H_2O_2-induced inactivation during electron transfer. Biochemistry 52:4636–4647.

Zhang H, White-Phillip JA, Melançon CE, Kwon H, Yu W, Liu H (2007) Elucidation of the kijanimicin gene cluster: insights into the biosynthesis of spirotetronate antibiotics and nitrosugars. J Am Chem Soc 129:14670–14683.

Appendix 1

The protein names and sequence accession number

Protein name	Accession number	Source
ChlA1	AAZ77693	*Streptomyces antibioticus*
ChlA3	AAZ77696	*Streptomyces antibioticus*
ChlA5	AAZ77698	*Streptomyces antibioticus*
ChlE2	AAZ77695	*Streptomyces antibioticus*
PyrE2	AFV71331	*Streptomyces rugosporus* NRRL 21084
PtmO5	ACO31280	*Streptomyces platensis* MA7327
AmphN	AAK73509	*Streptomyces nodosus*
NysN	AAF71771	*Streptomyces noursei* ATCC 11455
RimG	AAR16519	*Streptomyces diastaticus* 108
ScnG	ADX66468	*Streptomyces chattanoogenesis* L10
NikF	CAB46536	*Streptomyces tandae* Tue901
TylHI	AAD12167	*Streptomyces fradiae*
TamI	ADC79647	*Streptomyces* sp. 307-9
SlgO2	CBA11565	*Streptomyces lydicus* NRRL 2433
AJAP_10910	AIG75072	*Amycolatopsis japonica*
RubU	AAM97370	*Streptomyces collinus* DSM2012
PikC	AAC68886	*Streptomyces venezuelae* ATCC15439
DF18_18885	KEF19452	*Streptomyces rimosus*
SFRA_14170	KDS87380	*Streptomyces fradiae* ATCC 19609
SIRAN2716	CDR05783	*Streptomyces iranensis*
EryF	AAA26496	*Saccharopolyspora erythraea* NRRL 2338
RapN	CAA60465	*Streptomyces rapamycinicus* NRRL 5491

Appendix 2

The table shows the definition of abbreviations from chapter 3

Abbreviations	Definition
HPLC-DAD	High-Performance Liquid Chromatography with Diode Array Detection
HRFABMS	High Resolution Fast Atom Bombardment Mass Spectrometry
NMR	Nuclear Magnetic Resonance
HMBC	Heteronuclear Multi-Bond Correlation
UV-Vis spectrum	Ultraviolet-Visible spectrum

List of publications

1. Daduang R, Kitani S, Hashimoto J, Thamchaipenet A, Igarashi Y, Shin-ya K, Ikeda H, Nihira T., (2015) Characterization of biosynthetic gene cluster for maklamicin, a spirotetronate-class antibiotic of the endophytic *Micromonospora* sp. NBRC 110955. Microbiol Res 180:30-39

2. Daduang R, Kitani S, Sudoh Y, Pait IGU, Thamchaipenet A, Ikeda H, Igarashi Y, Nihira T., (2015). 29 Deoxy-maklamicin, a new maklamicin analogue produced by genetically engineered strain of *Micromonospora* sp. NBRC 110955. J. Biosci. Bioeng doi: 10.1016/j.jbiosc.2015.04.004

Acknowledgements

First of all, I would like to express my gratitude deeply to my supervisor Professor Takuya Nihira who always encourages and teaches me his great insight for science. I am also would like to give my appreciation to Associated Professor Shigeru Kitani for his advices, and also teach me to do experiment. Likewise, I also do thank Assistant Professor Hiroshi Kinoshita for his suggests and comments.

I am also deeply grateful to my co-supervisors, Professor Eiichiro Fukisaki and Professor Hajime Watanabe, for their kind reviews and precious comments of this dissertation. I also would like to give special thanks to Professor Yasuhiro Igarashi (Toyama Prefectural University), Professor Haruo Ikeda (Kitasato University), Dr. Kazuo Shinya and Dr Junko Hashimoto (Biomedicinal Information Research Center) and Miss Yuri Sudoh (Tamagawa Universiry) for they excellent advices and helps, especially for experimental supports.

Moreover, I would like to express my thanks to Associated Professor Arinthip Thamchaipenet (Kasetsart University), for her advices, supports and helps. I would like to give a special thanks to all members in Nihira lab and all ICBiotech members for their corporation, especially, to Dr.Aiyada Aroonsiri and Dr. Suthitar Ogawa, my best senior ever, to listening to my ideas and give me good advices.

I am also grateful for the financial support provide by Japanese Government (MEXT).

At last, I would like to deeply express my gratefulness to my family, who always listen to my opinions, and encourage me, without your back up I can’t keep working.